METHODS IN MOLECULAR BIOLOGY™

Volume 295

Immunochemical Protocols

Third Edition

Edited by

Robert Burns

Immunochemical Protocols

METHODS IN MOLECULAR BIOLOGY™

John M. Walker, SERIES EDITOR

309 **RNA Silencing:** *Methods and Protocols,* edited by *Gordon Carmichael, 2005*

308 **Therapeutic Proteins:** *Methods and Protocols,* edited by *C. Mark Smales and David C. James, 2005*

307 **Phosphodiesterase Methods and Protocols,** edited by *Claire Lugnier, 2005*

306 **Receptor Binding Techniques:** *Second Edition,* edited by *Anthony P. Davenport, 2005*

305 **Protein–Ligand Interactions:** *Methods and Protocols,* edited by *G. Ulrich Nienhaus, 2005*

304 **Human Retrovirus Protocols:** *Virology and Molecular Biology,* edited by *Tuofu Zhu, 2005*

303 **NanoBiotechnology Protocols,** edited by *Sandra J. Rosenthal and David W. Wright, 2005*

302 **Handbook of ELISPOT: Methods and Protocols,** edited by *Alexander E. Kalyuzhny, 2005*

301 **Ubiquitin–Proteasome Protocols,** edited by *Cam Patterson and Douglas M. Cyr, 2005*

300 **Protein Nanotechnology:** *Protocols, Instrumentation, and Applications,* edited by *Tuan Vo-Dinh, 2005*

299 **Amyloid Proteins:** *Methods and Protocols,* edited by *Einar M. Sigurdsson, 2005*

298 **Peptide Synthesis and Application,** edited by *John Howl, 2005*

297 **Forensic DNA Typing Protocols,** edited by *Angel Carracedo, 2005*

296 **Cell Cycle Protocols,** edited by *Tim Humphrey and Gavin Brooks, 2005*

295 **Immunochemical Protocols,** *Third Edition,* edited by *Robert Burns, 2005*

294 **Cell Migration:** *Developmental Methods and Protocols,* edited by *Jun-Lin Guan, 2005*

293 **Laser Capture Microdissection:** *Methods and Protocols,* edited by *Graeme I. Murray and Stephanie Curran, 2005*

292 **DNA Viruses:** *Methods and Protocols,* edited by *Paul M. Lieberman, 2005*

291 **Molecular Toxicology Protocols**, edited by *Phouthone Keohavong and Stephen G. Grant, 2005*

290 **Basic Cell Culture**, *Third Edition,* edited by *Cheryl D. Helgason and Cindy Miller, 2005*

289 **Epidermal Cells**, *Methods and Applications,* edited by *Kursad Turksen, 2004*

288 **Oligonucleotide Synthesis**, *Methods and Applications,* edited by *Piet Herdewijn, 2004*

287 **Epigenetics Protocols**, edited by *Trygve O. Tollefsbol, 2004*

286 **Transgenic Plants:** *Methods and Protocols,* edited by *Leandro Peña, 2004*

285 **Cell Cycle Control and Dysregulation Protocols:** *Cyclins, Cyclin-Dependent Kinases, and Other Factors,* edited by *Antonio Giordano and Gaetano Romano, 2004*

284 **Signal Transduction Protocols,** *Second Edition,* edited by *Robert C. Dickson and Michael D. Mendenhall, 2004*

283 **Bioconjugation Protocols,** edited by *Christof M. Niemeyer, 2004*

282 **Apoptosis Methods and Protocols,** edited by *Hugh J. M. Brady, 2004*

281 **Checkpoint Controls and Cancer, Volume 2:** *Activation and Regulation Protocols,* edited by *Axel H. Schönthal, 2004*

280 **Checkpoint Controls and Cancer, Volume 1:** *Reviews and Model Systems,* edited by *Axel H. Schönthal, 2004*

279 **Nitric Oxide Protocols,** *Second Edition,* edited by *Aviv Hassid, 2004*

278 **Protein NMR Techniques,** *Second Edition,* edited by *A. Kristina Downing, 2004*

277 **Trinucleotide Repeat Protocols,** edited by *Yoshinori Kohwi, 2004*

276 **Capillary Electrophoresis of Proteins and Peptides,** edited by *Mark A. Strege and Avinash L. Lagu, 2004*

275 **Chemoinformatics,** edited by *Jürgen Bajorath, 2004*

274 **Photosynthesis Research Protocols,** edited by *Robert Carpentier, 2004*

273 **Platelets and Megakaryocytes, Volume 2:** *Perspectives and Techniques,* edited by *Jonathan M. Gibbins and Martyn P. Mahaut-Smith, 2004*

272 **Platelets and Megakaryocytes, Volume 1:** *Functional Assays,* edited by *Jonathan M. Gibbins and Martyn P. Mahaut-Smith, 2004*

271 **B Cell Protocols,** edited by *Hua Gu and Klaus Rajewsky, 2004*

270 **Parasite Genomics Protocols,** edited by *Sara E. Melville, 2004*

269 **Vaccina Virus and Poxvirology:** *Methods and Protocols,*edited by *Stuart N. Isaacs, 2004*

268 **Public Health Microbiology:** *Methods and Protocols,* edited by *John F. T. Spencer and Alicia L. Ragout de Spencer, 2004*

267 **Recombinant Gene Expression:** *Reviews and Protocols, Second Edition,* edited by *Paulina Balbas and Argelia Johnson, 2004*

266 **Genomics, Proteomics, and Clinical Bacteriology:** *Methods and Reviews,* edited by *Neil Woodford and Alan Johnson, 2004*

265 **RNA Interference, Editing, and Modification:** *Methods and Protocols,* edited by *Jonatha M. Gott, 2004*

264 **Protein Arrays:** *Methods and Protocols,* edited by *Eric Fung, 2004*

METHODS IN MOLECULAR BIOLOGY™

Immunochemical Protocols

Third Edition

Edited by

Robert Burns

Antibody Unit, Scottish Agricultural Science Agency, Edinburgh, Scotland

HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American Standards Institute)

Permanence of Paper for Printed Library Materials.

Cover illustration: Immunonosorbent electron migrograph (ISEM) of potato latent virus. Cover art courtesy of R. Burns, SASA, Edinburgh, Scotland.

Production Editor: C. Tirpak
Cover design by Patricia F. Cleary

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com; or visit our Website: www.humanapress.com

eISBN: 1-59259-873-0

ISSN 1064-3745

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

Immunochemical protocols. – 3rd ed. / edited by Robert Burns.
p. ; cm. -- (Methods in molecular biology ; 295)
Includes bibliographical references and index.
ISBN 1-58829-274-6 (alk. paper)
1. Immunochemistry--Laboratory manuals. I. Burns, Robert, 1959-.
II. Series: Methods in molecular biology (Clifton,N.J.) ; v. 295.
[DNLM: 1. Antibodies--isolation & purification--Laboratory Manuals. 2. Immunochemistry--methods--Laboratory Manuals. W1 ME9616J v.279 2004 / QW 25 I334 2005]
QR183.6.I43 2005
616.07'9--dc22

2004009405

Preface

Immunochemical techniques have been in use for many years with early examples of bacterial strain typing dating back to the 1940s. The basis for the science is the exquisite elegance of the mammalian immune system with its ability to recognize foreign proteins and to manufacture antibody molecules that strongly bind to the substances that elicited them. Not only are potentially harmful pathogens and toxins recognized by the immune system, but the system can be persuaded to manufacture antibodies to an astonishing array of substances.

In the early days of this science, all antibodies for investigative work were produced by immunizing mammals with the substance of interest, followed by regular donor bleeds that yielded antisera. Serum produced in this way yields heterogenic populations of antibody molecules recognizing different epitopes on the target protein, which may be adequate for its intended purposes, but can also cause problems of crossreactivity. In 1975, Kohler and Milstein reported that spleen cells from immune donor animals could be immortalized, cloned from single cells, and grown in continuous culture. This original work described the method for the production of monoclonal antibodies.

The development of techniques based on antibodies has increased across the years and the routine use of them is now commonplace in many kinds of diagnostic and other investigative work. Workers new to the science may find the array of techniques and reagents bewildering, and this third edition of *Immunochemical Protocols* seeks to provide both the basic methods for producing and using antibodies along with some advanced protocols that will prove especially valuable to the more experienced worker. It provides full details of methods for antigen selection and preparation, antibody production, reagent manufacture, as well as protocols covering many of the areas where immunochemical techniques are used. Each protocol has been written by an investigator who has precise, practical knowledge and hands-on experience of the individual technique. Chapters contain detailed background to the use of the method, step-by-step instructions and a Notes section based on the authors' practical experience.

Immunochemical Protocols, Third Edition should provide workers with limited experience of antibody technology a broad array of protocols immediately of use in the laboratory. Experienced workers will find details of

alternative methods to those being currently used by them, and should also find new techniques that they may wish to use for their investigative work.

I would like to thank Dr. Gerry Saddler and the members of Diagnostics, and Molecular Biology at SASA for their encouragement and support throughout this project. A special thanks goes to Dr. Alex Reid of SASA for his help in dealing with the graphics and some of the more unusual file formats.

Robert Burns

Contents

Contributors

ROBERT BURNS • *Antibody Unit, Scottish Agricultural Science Agency, Edinburgh, Scotland*

JEFFREY J. CHALMERS • *Department of Chemical Engineering, The Ohio State University, Columbus, OH*

STEFAN DÜBEL • *Professor of Biotechnology, Institute of Biochemistry and Biotechnology, Carolo-Wilhelmina Technical University, Braunschweig, Germany*

GARY ENTRICAN • *Head of Immunology, Moredun Research Institute, Bush Loan, Edinburgh, Scotland*

DAVID C. HANCOCK • *Signal Transduction Laboratory, Cancer Research UK, London Research Institute Laboratories, London, UK*

ROSARIA P. HAUGLAND • *Molecular Probes Inc., Eugene, OR*

JAMES D. HIRSCH • *Molecular Probes Inc., Eugene, OR*

DAVID HUGHES • *Nuffield Department of Surgery, John Radcliffe Hospital, Oxford, UK*

MICHAEL HUST • *Department of Biotechnology, Institute of Biochemistry and Biotechnology, Carolo-Wilhelmina Technical University, Braunschweig, Germany*

WILLIAM JORDAN • *Department of Immunology, University of Medicine and Dentistry of New Jersey, Newark, NJ*

GRAHAM D. KEMP • *Centre for Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland*

OSCAR R. LARA • *Department of Chemical Engineering, The Ohio State University, Columbus, OH*

SABRINA LIBERATORI • *Department of Molecular Biology, University of Siena, Siena, Italy*

BARBARA MAGI • *Department of Molecular Biology, University of Siena, Siena, Italy*

GLENN E. MORRIS • *Biochemistry Group, North East Wales Institute, Wrexham, UK*

VINCENT MULHOLLAND • *Diagnostics and Molecular Biology, Scottish Agricultural Science Agency, Edinburgh, Scotland*

NICOLA J. O'REILLY • *Cancer Research UK, London Research Institute Laboratories, London, UK*

WEILIN L. SHELVER • *Department of Agricultural Chemical Research, USDA-ARS Biosciences Research Laboratory, Fargo, ND*

G. Brian Wisdom • *School of Biology and Biochemistry, The Queen's University, Belfast, Northern Ireland, UK*
Daphne M. Wright • *Plant Health Group, Central Science Laboratory, York, UK*
Gareth Young • *Immunology Group, Moredun Research Institute, Bush Loan, Edinburgh, Scotland*
Maciej Zborowski • *Department of Biomedical Engineering, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH*

1

Immunization Strategies for Antibody Production

Robert Burns

Summary

A range of immunization techniques can be used for the successful production of antibodies. The choice of method used is dependent on the nature of the antigen and the type of antibody needed by the user.

Key Words: Immunization; antibody; antigen; immunogen; immunity; epitope; monoclonal; polyclonal; immunoglobulin.

1. Introduction

Vertebrate immune systems are capable of producing antibodies to a greater or lesser degree in response to the presence of a foreign protein within the tissues of the animal. The presence of the foreign protein initiates a sequence of events, mediated by the cells of the immune system that lead to the release of antibody molecules in blood and some body secretions.

Antibodies produced by vertebrate immune systems bind strongly to the protein that elicited their formation, and it is this unique ability that is harnessed in all branches of immunochemistry.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

Vertebrates have evolved this immunological strategy to help them combat pathogens of viral, bacterial, and fungal origin; however, almost all foreign substances, regardless of source, can induce antibody responses. The immunological response in mammals is particularly well developed, and it is this group of vertebrates that are normally used for antibody production.

There are two main approaches for antibody production in vertebrates, each having strengths and weaknesses depending on intended application. Before discussing these two approaches, it would be beneficial to describe some background immunology.

Mammalian embryos are extremely tolerant of foreign proteins while still *in utero*, and all substances within the developing organism are accepted as "self." This is essential during development to ensure that immune responses are not raised to proteins and peptides produced during this time. Any immunological response to developmental proteins, hormones and growth factors would have disastrous results.

Shortly before or immediately after birth, the neonatal immune system matures and learns to differentiate between "self" and "non-self" ***(1)***. The immature immune system contains millions of cells within the bone marrow capable of producing antibodies (B-lymphocytes). This cell population is in effect a "starter pack," containing cells that will be capable of responding to a huge number of target proteins. These neonatal lymphocytes are produced by random reassortment of the antibody genes and because of this many of them will recognize and respond to proteins within the developing individual. A process of clonal deletion takes place and any lymphocytes, which recognize proteins within the developing organism, are killed. As the young mammal matures, it is incapable of mounting an immunological response to "self" antigens as a result of the process of clonal deletion.

Remaining B-lymphocytes in the bone marrow have the potential to respond to an enormous number of foreign substances (antigens). Once exposed to antigens, the cells, which have the best fit antibody to the target, undergo clonal expansion to increase the cell numbers and affinity maturation to increase the specificity (fit) of the antibody molecules produced.

The ability of the mammalian immune system to respond to foreign substances is based on the molecular shape of antigen fragments produced as a result of digestion by cells called macrophages. The antigen fragments produced by this process are generally about the size that one antibody-binding site can physically adhere to. These fragments are known as epitopes, and although there will be many on any target substance, a single antibody will only be able to recognize and bind to one of them.

The response to any antigen will involve the recruitment of many B-lymphocytes, each making antibodies to an individual epitope on the target molecule. These lymphocytes, which have responded to the epitopes on the antigen, undergo clonal expansion so that many descendant B-lymphocytes are produced from each of the original cells. This process gives rise to a population of cells descended from single progenitors (clones) each with their own specific antibody to epitopes on the antigen. These clones of cells are resident in lymphoid tissue and are particularly concentrated in the marrow, spleen, and gut-associated lymphoid tissue. The resulting pool of antibody molecules produced by these cells is known as polyclonal antibody, because it is derived from multiple clones, each with unique specificity to single epitopes.

Monoclonal antibodies are produced as a result of immortalizing and expanding the individual antibody secreting cells artificially in tissue culture ***(2)***. Cells grown in this way all have identical epitope specificity and because they are derived from single clones, their product is known as monoclonal antibody. Cells that secrete monoclonal antibodies are known as hybridomas and are typically derived by fusion of two cell types. B-lymphocytes, which have the capacity to make antibody, are obtained from a donor spleen and are physically fused to a tumor cell line, which is immortal. The resulting hybridom as are immortal and produce antibody into the synthetic medium in which they are growing.

Exposure to antigens can be by a variety of routes but ultimately the cellular changes leading to B-lymphocyte activation are blood borne. Natural immunization takes place as a consequence of infection through respiratory, digestive, urogenital and skin surfaces. Medical immunization to prevent infection by pathogens is normally

conducted by intramuscular injection, although other routes, such as oral dosing for poliomyelitis and intradermal injection for tuberculosis, may be used. The main feature, which characterizes immunization, is the presentation of antigens to the cells of the immune system, which induces B-lymphocyte priming. These primed B-cells will undergo clonal expansion and will secrete antibody until the antigen has been destroyed. As soon as the antigen has been removed, the B-cell lineage making antibodies to it will become quiescent and will form a stable population within the tissues of the immune system (memory cells). If the antigen is encountered again by the organism these quiescent B-cells can undergo rapid clonal expansion and can mount an antibody response much faster than during the primary challenge. Each time that the immune B-cells are exposed to the antigen, the affinity (fit) of the antibody produced will be improved and the number of quiescent B-cells after each challenge increases. Each challenge also increases the amount of antibody produced in the blood and after three or four immunizations the individual reaches a status of hyperimmunity. This is characterized by high levels of circulating specific antibody typically in the range of 10–20 mg/mL of serum. Hyperimmunity is rarely ever seen as a result of natural immunization but is commonly used for the in vivo production of polyclonal antibody. Risks are associated with hyperimmunity as further exposure to the antigen can lead to an overwhelming immune response known as anaphylaxis, which can be rapidly fatal. Paradoxically, repeated exposure to the antigen can lead to immunological tolerance, where the B-cells making the antibody are destroyed by the immune system leaving the individual unable to mount an immune response to the antigen.

As previously stated, immunization is a phenomenon mediated by cells of the immune system and normally is the result of a blood-borne challenge by antigen. The route of introduction can be very important in determining how well the individual will respond to an antigen.

It is extremely important when immunizing animals for antibody work to choose the correct approach for the type of antigen to be

used. This chapter describes a number of immunization routes for polyclonal antibody production, monoclonal antibody cell donors and also one method for inducing selective immune tolerance as a preparative method.

Polyclonal and monoclonal antibodies should be seen as complimentary in their use. Each has strengths and weaknesses and the choice of which to use should be carefully evaluated before embarking on antibody production. In general, polyclonal antibodies have a much broader specificity because the antiserum pool comprises many species of antibody molecule each with different target epitopes on the antigen. This lack of specificity can be advantageous in situations where variation in the target substance is known and polyclonal antibodies may provide a more robust test. Monoclonal antibodies are derived from clonal cell lines and their specificity is directed to a single epitope on the antigen. The highly specific nature of the monoclonal antibody allows the development of assays where two very closely related substances can be differentiated from each other. Examples of these highly specific tests are found in virus testing for strain differentiation and in clinical assays where levels of a synthetic hormone may be detected in spite of the presence of its naturally occurring counterpart.

1.1. Legislation

There are strict regulations governing the welfare of laboratory animals used for antibody production in most countries. Before deciding on a particular approach for antibody production it is important that the appropriate authorities are contacted with a project proposal to ensure that the methods to be used are permissible. Local ethical review committees may also have input into project design to ensure that numbers of animals used are appropriate and that other diagnostic alternatives have been investigated. Legislation and ethical review typically covers animal species, numbers to be used, immunization route, bleeding regimes, and welfare issues, such as project duration.

2. Materials

1. Balb-c mice are the preferred laboratory strain used as cell donors in monoclonal antibody work (*see* **Note 1**).
2. New Zealand rabbits are the preferred laboratory strain used for polyclonal antiserum production (*see* **Note 2**).
3. Suitable adjuvant for addition to antigen (*see* **Note 3**).
4. Animal house facilities licensed for the specific required procedures under animal welfare legislation.
5. Parenteral anesthetic agents as prescribed by veterinary surgeon (*see* **Note 4**; Hypnorm + Hypnovel).
6. Diethyl ether.

3. Methods

3.1. Immunizing Rabbits for Polyclonal Antiserum Production

1. Mix 0.5 mL of 1 mg/mL solution of antigen with 0.5 mL of appropriate adjuvant.
2. Inject into muscle of hind leg or subcutaneously into neck scruff.
3. Repeat on d 14 and 44.
4. Test bleed (1–2 mL) on d 54 and assess for antibody activity (i.e., ELISA).
5. Bleed from marginal ear vein on d 60 and then every 28 d until antibody titre drops.
6. Give boost dose and either commence bleeding regime 10 d later or perform terminal exsanguination under anesthesia.

3.2. Immunizing Mice for Monoclonal Antibody Production

1. Mix 0.15 mL of 0.5 mg/mL solution of antigen with 0.15 appropriate adjuvant and mix (volumes based on group of three mice).
2. Inject 0.1 mL of adjuvant/antigen mixture per dose intraperitoneally (IP).
3. Repeat on d 14 and 44.
4. Obtain test bleeds by tail tip amputation under light anesthesia on d 54 (*see* **Note 5**) and assess for antibody titre. Mice should be marked by ear punching or tattooing to allow subsequent identification.
5. Rest the mice for a period of 60 d or more to allow the B-cells to become quiescent.

6. Inject (boost) the best responding mouse with an IP injection of 0.05 mL antigen (0.5 mg/mL solution) without adjuvant.
7. Kill the mouse 3 d later by cervical dislocation and remove its spleen aseptically for cell harvesting.

3.3. Modified Immunization Protocol for Anamnestic Antigens

When it is known from other work that the antigen is highly glycosylated or comes from a source known to be rich in polysaccharides (bacterial cell walls) it is highly unlikely that the animal will ever produce a classic full immune response. Usually these antigens do not produce quiescent B-cells after the rest period and each immunization is seen as a primary challenge. Substances that invoke this incomplete response are known as anamnestic antigens, and immunoglobulins produced to them are always class M. There is no point in carrying out a full immunization protocol over several months, and a shortened one is recommended.

1. Mix 0.15 mL of 0.5 mg/mL solution of antigen with 0.15 appropriate adjuvant and mix (volumes based on group of three mice).
2. Inject 0.1 mL of adjuvant/antigen mixture per dose IP. Repeat on d 14.
3. Take a test bleed by tail tip amputation under light anesthesia on d 21 and assess for circulating antibody. All antibody produced will be IgM and so assessment method (ELISA) must take this into account. Mark the mice by ear punch or tattoo for subsequent identification.
4. Give the mouse exhibiting the highest titre of circulating antibody an IP injection of 0.1 mL (0.5 mg/mL solution) of antigen on d 23.
5. Harvest the spleen and perform the cell fusion on d 30.

3.4. Induction of Immune Tolerance in Neonatal Mice (3,4)

As previously mentioned fetal and newborn mammals have immature immune systems that do not yet have the capacity to differentiate self and nonself. This lack of maturity can be harnessed to our advantage when working with antigens, which are naturally found along with closely related (cross-reacting) substances. This technique does not guarantee success but can swing the odds in favor

of the researcher producing hybridomas with the desired specificity. Situations where this methodology is used include work on viruses, bacteria, and fungi, where there may be many shared epitopes between the organism of interest and closely related species. The cross-reacting antigen used for the technique is a whole preparation of the closely related species. This technique causes the suppression of an immune response to shared epitopes, which may swamp the immune response and favors specific immunity to epitopes found only on the species of interest.

1. Obtain a "time mated" female Balb/c mouse 10 d into the pregnancy and maintain in standard cage used for mouse breeding.
2. Observe until litter are born.
3. Inject the neonates daily on d 1–5 after birth with 0.05 mL 0.5 mg/mL of crossreacting antigen into the neck scruff. (The neonatal immune systems are maturing at this time and because the cross-reacting antigen is present they will adopt these proteins as "self" and lose the ability to mount an immune response to them.)
4. Immunize three of the animals with specific antigen of interest when they are 6 wk old using the standard monoclonal immunization protocol provided previously.

3.5. Intrasplenic Immunization

Intrasplenic immunization is used for the production of hybridomas in situations where only very small quantities of the antigen are available. Typically, it lends itself extremely well to producing antibodies to proteins that have been purified by electrophoresis and subsequent blotting onto nitrocellulose. Antibodies produced by this route are always immunoglobulin class M as only one immunization is used. This method is covered by Animal Procedures legislation in most countries as it is an invasive surgical procedure and welfare issues must be addressed.

The antigen must be in a highly aggregated or immobilized form. This technique is frequently used to produce antibodies to proteins separated by electrophoresis. The gel is stained in the normal manner and the proteins blotted over onto nitrocellulose. The band containing the protein of interest is then excised from the blot and used

as the antigen. The presence of protein stains and other reagents makes little difference and as the in vivo part of the protocol is very short there are no long-term animal welfare issues.

1. Induce and maintain anesthesia using a mixture of fentanyl/fluanisone with midazolam (Hypnorm/Hypnovel; *see* **Note 4**). The dosage for these agents is 0.25 mL of each active ingredient plus 0.5 mL of water given IP at a rate of 0.1 mL per mouse.
2. Shave the hair along the midscapular line above the position of the spleen and make a 1-cm incision made through the skin. Cut through the muscle wall to expose the spleen, and then deliver it through the opening complete with its pedicle.
3. Introduce the antigen into a pocket made in the spleen through the capsule and return the organ to the body cavity.
4. Close the muscle layer with three or four sutures and then close the skin likewise.
5. Keep the mice warm and close to a source of drinking water until they recover from the anesthetic, which takes approx 1–2 h. Generally, the mice suffer no side effects and will be feeding, grooming and showing no signs of discomfort soon after recovery from anesthesia.
6. Use the mice as donors for cell fusion 7 d after the intrasplenic immunization.

3.6. In Vitro Immunization

This technique is performed in tissue culture and works well when only small quantities of antigen (1–2 µg) are available. It also allows the production of antibodies to substances that are toxic in whole animals. There is no immunological processing of antigens so only soluble, simple antigens such as peptides can be used for this approach. A source of interleukins-4 and -5 is required for the method to work and the easiest way of obtaining them is from thymocyte-conditioned medium.

3.6.1. Thymocyte-Conditioned Medium

1. Kill two 6-wk-old Balb-c mice and remove their thymus glands aseptically.

2. Homogenize the tissue to produce single cells and resuspend in 10 mL of RPMI 1640 medium containing 15% fetal bovine serum.
3. Incubate the medium at 37°C/ 5% CO_2 for 24 h (*see* **Note 6**) and then harvest the supernatant by centrifugation (700*g*). Store the conditioned medium at –20°C until required.

3.6.2. Immunization

1. Kill a non-immunized balb-c mouse and remove its spleen aseptically.
2. Homogenize the spleen to produce single cells, and then resuspend them in 10 mL of the thymocyte-conditioned medium.
3. Add 1–2 µg of antigen to the cell suspensions and incubate at 37°C/ 5% CO_2 for 72 h (*see* **Note 7**).
4. Harvest the spleen cell by centrifugation (500*g*) and use immediately for a cell fusion.

4. Notes

1. Balb-c mice are an inbred strain ideally suited to monoclonal antibody work. Females are usually used because they do not fight when housed together in project groups of three to five individuals. This mouse is also known as the "barber" strain because the dominant female will remove the whiskers from the others in the group.
2. New Zealand rabbits are normally used for serum production; they are easily handled and adapt well to individual cages or group floor pens. This strain has half-lop ears, which make blood collection from marginal veins a fairly straightforward procedure.
3. Most antigens require an adjuvant to increase their immunogenicity and a number of formulations can be used. Regulations on their use should be consulted prior to embarking on a course of immunizations. For many years Freund's complete and incomplete adjuvant were the formulation of choice for all immunization work. In recent years welfare issues have been raised over the use of these adjuvants and a number of alternatives based on water-soluble bacterial cell wall components have become available.
4. Parenteral anesthetic agents are preferable to gaseous ones as the size of the mouse creates problems when using standard anesthetic machines.

5. Tail tip amputation is normally used to obtain test bleeds from mice, and this is normally carried out under light anesthesia induced with diethyl ether.
6. It is extremely important that the medium is harvested after 24 h of incubation. Longer incubation periods may induce the formation of suppressing cytokines, which will block the desired cell stimulation.
7. It is important that the thymocytes are not disturbed for the full 72-h incubation and that they are rapidly harvested and fused after this period of time.

References

1. Roitt, I., Brostoff, J., and Male. D. (1996) Cell cooperation in the antibody response, in *Immunology*. Mosby, London, UK, pp. 13–16.
2. Kennet, R. H., Denis, K. A., Tung, A. S., and Klinman, N. R. (1978) Hybrid plasmocytoma production: Fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. *Curr. Topics Microbiol. Immunol.* **81,** 495.
3. Hsu, H. T., Lee, I., Davis, R. E., and Wang, Y. C. (1990a) Immunisation for generation of hybridoma antibodies specifically reacting with plants infected with a mycoplasma like organism (MLO) and their use in detection of MLO antigens. *Phytopathology* **80,** 946–950.
4. Hsu, H. T., Wang, Y. C., Lawson, R. H., Wang, M., and Gonzalves, D. (1990b) Spenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies *Phytopathology* **80,** 158–162.

2

Synthetic Peptides as Antigens for Antibody Production

David C. Hancock and Nicola J. O'Reilly

Summary

The use of synthetic peptide immunogens as a means to generate specific immunological reagents for a variety of purposes has increased markedly in recent years. In this chapter, we outline some of the salient factors to be considered when designing peptide immunogens and describe basic methodologies for the conjugation of short synthetic peptides to immunogenic carrier proteins.

Key Words: Synthetic; peptide; antibody; antigen; polyclonal; immunogen; carrier; conjugation.

1. Introduction

There is an ongoing requirement in cell and molecular biology for the preparation of antibodies to use as probes for specific proteins. Two main strategies exist to raise appropriate antibodies. A complimentary deoxyribonucleic acid (cDNA), or gene sequence encoding the protein of interest can be expressed in a heterologous species,

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

usually bacteria, and the resultant purified protein used as an immunogen. Glutathione-*S*-transferase fusion proteins, for example, have been extensively used as immunogens. Alternatively, small synthetic peptides can be synthesized that contain amino acid sequences derived from the cDNA acid or gene. Such antipeptide antibodies crossreact with the corresponding intact native protein with surprisingly high frequency and have the additional advantage that the epitope recognized by the antibody is already well defined ***(1)***. In this way, antibodies can be raised against novel gene products that are specifically directed against sites of interest, for example, unique regions, highly conserved regions, active sites, extracellular domains, intracellular domains or regions of posttranslational modification, such as phosphorylation sites. Moreover, the ready availability of the peptide immunogen against which the antibody was raised means that sera can be rapidly and easily screened, for example, using an enzyme-linked immunosorbent assay for antipeptide activity. Free peptide can also be used to block antibody binding and so demonstrate immunological specificity, and it may be coupled to a solid support (e.g., agarose) to generate an affinity matrix for antibody purification. In this chapter, we describe the basic principles behind the design, synthesis, and use of synthetic peptides as immunogens and in this and the following chapter outline some of the basic methods used in our laboratories. These methods have been used for several years, with a considerable degree of success, by groups in our institute and elsewhere.

1.1. Choosing Peptide Sequences

Many peptide sequences can be immunogenic, but not all are equally effective at eliciting antibodies that crossreact with the intact cognate protein (we term these crossreactive peptides). There is no guarantee that antibodies raised against a particular synthetic peptide will crossreact with the intact protein from which the sequence is derived. In our experience the probability of generating a successful anti-protein antibody by the methods outlined is approx 50%. Many factors can influence the success of using peptide

immunogens to raise antiprotein antibodies. These include elements such as the number of peptides from one protein sequence to be used and the number of animals available for immunization (both of which may be determined by existing resources); the availability and accuracy of sequence data, the predicted secondary structure of the intact protein and finally, the ease of synthesis of specific sequences. Continual improvements to synthesis methodologies means that the latter aspect is less significant than in the past, although certain sequences can still be problematic (*see* **Subheading 1.2.**). Despite these potential reservations, there are a number of ways of improving one's chances of success (*see* **Subheading 1.1.1.–1.1.3**).

1.1.1. Predicted Structure of the Whole Protein

There is a wide range of predictive algorithms available that can provide data on antigenicity, hydrophilicity, flexibility, surface probability, and charge distribution over a given amino acid sequence. The algorithms of Chou and Fasman and of Robson and Garnier ***(2,3)*** have provided a basis for many secondary structure predictive algorithms that can give a good idea of where regions of particular secondary structure, such as α-helix, β-sheet, turns, and coils are likely to form. For example, the proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/) provides access to primary and secondary structure analysis tools via the Expert Protein Analysis System (ExPASy). Other prediction scales include the Turn scales of Pellequer and Westhof ***(4)***. These are based on the occurrence of amino acids within turns. The level of correctly predicted antigenicity using this program is high (70%), but the number of predicted antigenic sites per protein is smaller than for other programs. In general, however, there is rather poor correlation between amino acid type and secondary structure with similar folds able to be made by sequences with only 20% identity. The relative merits of different predictive scales is discussed in depth elsewhere ***(5)***.

Primary amino acid sequences can also indicate consensus sequences that may be sites of posttranslational modification (e.g.,

O- and *N*-linked glycosylation sites and sites of phosphorylation) and that may therefore be immunologically unavailable in the fully mature protein. Clearly, accessibility on the external surface of the intact protein is, overall, the most important requirement for a cross-reactive peptide. Very frequently, the C-terminus of a protein, although often not a region of strongly predicted secondary structure, is exposed, and this sequence makes a good first choice. However, the C-terminus occasionally forms the membrane anchoring region of some membrane-bound proteins and in these cases would generally be too hydrophobic to consider. The N-terminus of a protein can also prove to be a good candidate sequence, but in our experience is a less reliable choice than the C-terminus and may be modified or truncated. Regions with too high a charge or hydrophilicity are sometimes not as effective as might be expected, probably because almost all known antibody combining sites make contact with their epitope via polar and Van der Waal's bonds and not–ionic interactions. Hydrophilic α-helical regions can be good peptide epitopes because, provided the synthetic peptide is itself long enough to form a helix, it often assumes an identical conformation to that in the intact protein.

1.1.2. Specific Requirements

By their nature, antipeptide antibodies are site-directed probes for proteins. Both the sequence and position of the antibody epitope is predefined. Indeed, the technique of "epitope tagging" exploits the existence of an antibody with specificity for a given linear peptide epitope that can be expressed in the context of a fusion protein ***(6)***. It is, therefore, possible to target antipeptide antibodies to specific regions of interest in the intact protein, such as areas of high conservation to identify additional members of a protein family; or areas of hypervariability in order to unambiguously identify a particular family member. The increasing reliability of synthesis of, for example, phosphopeptides means that sites of posttranslational modification can also be analyzed. Antibodies that recognize both degenerate and specific consensus phosphorylation motifs are avail-

able commercially and antibodies raised against a specific phosphopeptide have been used as tools to recognize novel phosphorylation targets *(7,8)*. When selecting a peptide to produce a phosphospecific antibody, it is preferable to localize the phosphorylated residue close to the middle of the peptide to reduce the likelihood of producing an immunodominant epitope containing nonphospho amino acid sequence. Other functional or regulatory regions of a protein, such as binding sites, transmembrane domains or signal sequences may also be targeted. However, factors , such as hydrophilicity and secondary structure, may affect the success of any given peptide immunogen.

1.1.3. Immunological Requirements

Peptides of 10–20 amino acids are optimal as antigens and our standard is approx 15 residues. Short peptides (less than approx 7 residues) are probably of insufficient size to function as epitopes. Larger peptides may adopt their own specific conformation (that is often immunodominant over any primary structural determinants), which may not be reflected in the conformation of the sequence within the intact protein. Given the previous criteria, it is possible to say that almost all peptide sequences are immunogenic if presented to the immune system in the right way (*see* **Subheading 1.3.**), but that not all will generate cross-reactive antibodies. Probably the most important factors in optimizing one's chances of making useful antibodies to a protein of interest are to use several peptides from different regions of the protein sequence and to immunize more than one animal with each peptide. Different animals within the same group frequently respond differently to the same immunogen. In addition, a given antipeptide antibody may sometimes work well in one assay, for example, Western blotting, but not in another, for example, immunoprecipitation.

1.1.4. Synthesis Requirements

The chemical difficulties of synthesizing certain amino acid sequences can be complex. In general, hydrophilic sequences are

more soluble and easier to synthesize (and are more likely to be exposed on the surface of the intact molecule). There appears to be little requirement for a high degree of purity for peptide immunogens. Our experience is that peptides of 75% purity, or sometimes even less, generate effective polyclonal antisera, although criteria may need to be more stringent when making monoclonal antibodies.

1.2. Peptide Synthesis

Peptides can be purchased from several companies specializing in contract synthesis and if only a few are required, this is the most straightforward way to obtain the desired reagents. Custom synthesis of peptides can be expensive, with specific modifications costing even more. However, in-house synthesis is labor-intensive, requires significant knowledge of peptide chemistry and, if performed using an automated machine, involves large capital expenditure. In general, acquisition of an automated peptide synthesizer is probably best suited to laboratories or institutes with substantial and ongoing requirements for synthetic peptides, and preferably with their own dedicated personnel. An in-house peptide synthesis facility is a particularly attractive alternative to custom synthesis because it allows much greater flexibility in the design and production of peptides. This can be important if specially derivatized peptides are needed, or if, for example, chemically defined immunogens such as multiple antigen peptides are to be synthesized ***(9,10)***.

1.2.1. Principles of Peptide Synthesis

Solid-phase peptide synthesis is based on the sequential addition of protected amino acids onto an insoluble support. Addition proceeds from carboxy terminus to amino terminus. The first amino acid is attached to a solid support by a linker and, if necessary, side-chain amino acid function is protected throughout chain assembly. The carboxy group of the in-coming, acylating amino acid is activated for coupling while its amino group is protected temporarily for each coupling step and then deprotected for the next cycle. The

cycles of deprotection and coupling are continued until the amino acid chain is complete. The peptide is then cleaved from the solid support and the amino acid side-chains are deprotected to give the final peptide product. In general, once a peptide is made the sequence cannot be altered. Modifications such as acetylation, phosphorylation or the introduction of additional residues (e.g., for use in conjugation) should be planned before synthesis. The modifications can then be incorporated into the synthesis procedure. For example, phosphopeptides are synthesized using amino acid residues with specially derivatized side-chains. The final product is usually evaluated by reverse-phase high-performance liquid chromatography (C8 or C18 columns with water/acetonitrile gradients) and mass spectrometry. For antibody production, a suitable amount of peptide is about 50 mg. This allows for affinity purification of antibodies and antibody blocking experiments, to demonstrate antibody specificity, as well as immunization. The synthesis of most peptide sequences in the region of 20 residues in length is currently considered to be quite routine. There are, however, always exceptions. Certain sequences can be extremely difficult to synthesize and may require alternative synthetic strategies ***(11**,**12)***.

1.3. Conjugation of Peptides to Carrier Proteins

In general, short peptides are poor immunogens, so it is necessary to conjugate them covalently to immunogenic carrier proteins to raise effective anti-peptide antibodies. These carrier proteins provide necessary major histocompatibility complex class II/T-cell receptor epitopes while the peptides can then serve as B-cell determinants. Keyhole limpet hemocyanin (KLH) and thyroglobulin are examples of carriers that are commonly used to generate polyclonal anti-peptide antibodies. We generally avoid using bovine serum albumin because the high levels of anti-bovine serum albumin antibody generated can interfere with subsequent studies on tissue culture cells grown in media containing bovine sera.

The peptides are covalently conjugated to the carrier molecule using an appropriate bifunctional reagent—the most straightforward

coupling methodologies involve the amine or sulfhydryl groups of the peptide. Substantial antibody titres are also usually generated against determinants present on the carrier molecules. In general, such anticarrier antibodies present few problems in polyclonal antipeptide antibodies and may anyway, be adsorbed out on a matrix of carrier bound to agarose. When making monoclonal antibodies, however, the substantial anticarrier response may mask the frequently weaker anti-peptide response, resulting in few peptide-specific hybridomas being isolated. A variety of alternative approaches to the use of conventional peptide-carrier conjugates have been developed including, for example, the multiple antigenic peptide (MAP) system ***(9)***. The MAP system makes use of the epsilon-amino group of lysine residues to generate a branched core matrix that can be used as a scaffold for subsequent peptide synthesis. This system can be employed to deliver high densities of single, defined peptide antigens or to generate B-cell and T-cell epitopes attached to the same MAP scaffold ***(10)***. Nevertheless, MAP synthesis products can be difficult to analyze by high-performance liquid chromatography and mass spectrometry because of their large mass.

The most straightforward carrier-peptide conjugation procedure uses glutaraldehyde as the bifunctional reagent, which crosslinks amino groups on both carrier and peptide. In our experience, glutaraldehyde conjugation is reliable, easy and effective, and generates good antipeptide antibodies even with short peptides. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) can be used to crosslink the thiol group of cysteine on the peptide to an amino group on the carrier. The MBS method generates a somewhat better defined conjugate, but it involves a slightly more involved procedure and requires the presence of a reduced cysteine residue at one end of the peptide (this is frequently added to the sequence during synthesis specifically for conjugation purposes). If the chosen peptide sequence contains an internal cysteine residue, coupling via MBS should be avoided.

2. Materials

2.1. Conjugation of Peptides

1. KLH: purchased as a solution in 50% glycerol (Calbiochem) and stored at 4°C. If obtained as an ammonium sulfate suspension the KLH will require extensive dialysis against borate buffered saline (20 m*M* Na Borate/144 m*M* NaCl) containing 50% glycerol.
2. Glutaraldehyde (Sigma, Grade 1) stock: is a 25% solution divided into 1-mL aliquots. It is stored at –20°C and never re-frozen.
3. Sodium bicarbonate stock (10X): a 1-*M* solution adjusted to pH 9.6 with HCl.
4. Glycine ethyl ester hydrochloride (Sigma): make up as a 1-*M* stock and adjust to pH 8.0 with NaOH.
5. MBS "Sulfo-MBS" version of this reagent is water-soluble and, therefore, preferable (Pierce).
6. 0.1 *M* Sodium phosphate buffer, pH 6.0: mix 12 mL of 1 *M* disodium hydrogen phosphate with 88 mL of 1 *M* sodium dihydrogen phosphate and make up to 1 L with water.
7. Sephadex G25 (APBiotech).
8. Sodium borohydride.
9. Borate buffer: 0.1-*M* boric acid solution adjusted to pH 8.0 with NaOH.
10. 1 *M* HCl.
11. 1 *M* NaOH.
12. Acetone.
13. Saline: 0.9% NaCl.
14. Ammonium hydrogen carbonate, pH 7.5.

3. Methods

3.1. Glutaraldehyde Conjugation Method (see *Notes 1 and 2)*

1. Weigh out the peptide and an equal weight of KLH (or thyroglobulin) carrier. This gives an approximate ratio of 40–150 molecules of peptide to each molecule of carrier (2 mg of peptide per animal is ample).

2. Dissolve the peptide and carrier protein in 0.1 *M* (1X) sodium bicarbonate using 1 mL for every 2 mg of carrier protein.
3. Thaw out a fresh vial of glutaraldehyde and add to the peptide-carrier solution to a final concentration of 0.05%. Mix in a glass tube, stirring with a magnetic stirring bar; keep at room temperature overnight in the dark (wrap the tube in foil). The solution will usually turn a pale yellow color. Occasionally the solution will turn pale brown or orange—this reflects the fact that peptide preparations sometimes contain traces of chemical scavenger reagents used in the final cleavage of the peptide from the resin and is not a cause for concern.
4. Either: dialyze against double distilled water for 12 h and lyophilize the coupled carrier. Assess yield by weighing the lyophilized material to determine the percentage of peptide coupled.

   Or, because coupling efficiency is usually reasonable, and not too critical, it is easier to do the following: add 1 *M* glycine ethyl ester to a final concentration of 0.1 *M* and leave for 30 min at room temperature. Then, precipitate the coupled carrier with 4–5 vol of ice-cold acetone at –70°C for 30 min. Briefly warm at room temperature and pellet the protein at 10,000*g* for 10 min at room temperature, pour off the acetone, air dry the pellet, and redisperse it in saline at 1 mg carrier/mL. As the pelleted protein is rather sticky, this is best done using a Dounce™ homogenizer. Conjugates can be stored at –20°C and rehomogenized before use.

3.2. MBS Coupling Method (see *Notes 2–4)*

1. Dissolve 15–20 mg of carrier protein in a small amount of phosphate-buffered saline (about 1 mL).
2. Dissolve 5 mg of MBS in a small amount of dimethylformamide (about 0.75 mL) or for Sulfo-MBS, dissolve in a small amount of sterile water.
3. When crosslinker and carrier are completely dissolved, mix well and leave at room temperature for 1 h.
4. Desalt on a 20-mL Sephadex G25 column using 0.1 *M* sodium phosphate buffer, pH 6.0. Collect 2-mL fractions. Read the optical density (OD) of the fractions at 280 nm. Keep the two fractions with the highest OD_{280}.
5. Meanwhile, reduce the peptide.

a. Make up fresh 5 mg/mL solution of sodium borohydride and store on ice.
b. Dissolve 15–20 mg of peptide in minimum amount of 0.1 *M* borate buffer, pH 8.0.
c. Add 100 mL of sodium borohydride to the dissolved peptide, mix well, and stand on ice for 5 min.
d. Lower pH by adding 1 *M* HCl (approx five drops), mix, and leave on ice for a further 5 min.
e. Add equal number of drops of 1 *M* NaOH and check that the pH is between 6 and 7. If not, then adjust with 1 *M* NaOH or 1 *M* HCl. (10 mL is approx 0.5 of a pH unit).

6. Add desalted crosslinker/carrier to reduced peptide and leave overnight at room temperature.
7. If the conjugate becomes insoluble, precipitate completely with 4–5 vol of ice-cold acetone at –70°C for 30 min. Briefly warm at room temperature. Pour off the supernatant and air dry. Resuspend in saline as in **Subheading 3.1.4.** Alternatively, if the conjugate remains soluble, then desalt the solution on a 20-mL Sephadex G25 column using ammonium hydrogen carbonate buffer, pH 7.5. Collect 2-mL fractions and pool those of $OD_{280} > 0.4$. Conjugates can be stored at –20°C and rehomogenized before use.

4. Notes

1. It is often worth adding a cysteine residue at the C-terminus of the peptide to give the option of coupling via MBS as well as via glutaraldehyde.
2. During glutaraldehyde conjugation it is vital to exclude any buffers containing amino, imino (e.g., Tris-HCl), ammonium or azide moieties as these will inhibit the cross-linking reaction. If the peptide or carrier is insoluble in coupling buffer, sodium dodecyl sulphate may be added to 0.1% without affecting the conjugation. Occasionally, a peptide–carrier conjugate becomes less soluble as the conjugation reaction proceeds. This does not appear to affect the efficacy of the final product and is usually, therefore, no cause for concern.
3. If, during MBS coupling, the DMF concentration exceeds 30% the KLH will come out of solution. KLH concentrations in excess of 20 mg/mL will also lead to insolubility. Use the Sulfo-MBS derivative if possible.

4. If many conjugation reactions are required, the MBS activation of KLH can be scaled up and performed batchwise. Alternatively, if only a few conjugates are needed, Maleimide-activated KLH is commercially available (Calbiochem or Pierce). This leaves only the final addition of peptide to be performed.

References

1 Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., et al. (1983) Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA* **80,** 4949–4953.

2 Chou, P. Y. and Fasman, G. D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* **47,** 45–158.

3 Garnier, J., Osguthorpe, D. J., and Robson, B. (1978). Analysis of the accuracy and implications of simple methods of predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120,** 97–120.

4 Pellequer, J. L. and Westhof, E. (1993). PREDITOP: a program for antigenicity prediction. *J. Mol. Graph.* **11,** 204–210.

5. Van Regenmortel, M. H. V. and Muller, S. (1999) *Synthetic Peptides as Antigens*. Elsevier Press, Amsterdam.

6 Munro, S. and Pelham, H. R. B. (1986) An HSP70-like protein in the ER: identity with the 78kD glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46,** 291–300.

7 Astoul, E., Laurence, A. D., Totty, N., Beer, S., Alexander, D. R., and Cantrell, D. A. (2003) Approaches to define antigen receptor-induced serine kinase signal transduction pathways. *J. Biol. Chem.* **278,** 9267–9275.

8 Basu, S., Totty, N. F., Irwin, M. S., Sudol, M., and Downward, J. (2003). Akt phosphorylates the yes-associated protein, YAP, to induce interaction with 14–3-3 and attenuation of p73-mediated apoptosis. *Mol. Cell* **11,** 11–23.

9 Tam, J. P. (1988). Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA* **85,** 5409–5413.

10. Calvo-Calle, J. M., de Oliveira, G. A., Clavijo, P., Maracic, M., Tam, J. P., Lu, Y-A, et al. (1993) Immunogenicity of multiple antigen pep-

tides containing B and non-repeat T cell epitopes of the circumsporozoite protein of *Plasmodium falciparum*. *J. Immunity* **150,** 1403–1412.

11. Bodanszky, M. (1988). *Peptide Chemistry: A Practical Approach*. Springer-Verlag, Berlin, Heidelberg.
12. Chan W. C. and White P. D. (2000). *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*. Oxford University Press, Oxford.

3

Production of Polyclonal Antibodies in Rabbits

David C. Hancock and Nicola J. O'Reilly

Summary

The generation of polyclonal antibodies to an antigen of interest is an important technique applicable to many areas of biological research. In this chapter, we describe a basic immunization procedure designed to generate polyclonal antisera in rabbits and two methods that are commonly employed in the subsequent preliminary characterization of antipeptide antibodies raised in this way.

Key Words: Antibody; antigen; polyclonal; immunogen; immunization; peptide; ELISA.

1. Introduction

The past few years have seen a substantial increase in the commercial availability of antibodies to a wide variety of antigens. In addition, the increasing use of epitope tags as a means to identify proteins in gene expression studies sometimes bypasses the need for *de novo* generation of antibodies against a given antigenic target. Nevertheless, the production of specific antibodies to a protein of interest or to particular regions of a protein of interest remains an important tech-

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

nique for the generation of specific tools to aid the characterization of biological processes at the cellular and molecular level.

Immunization protocols often vary substantially between laboratories, and different protocols can often produce satisfactory results. Although there are few hard and fast rules, the methods described here are designed to give optimal results with minimal discomfort to the donor animal and they have been used successfully in our institute and elsewhere for a number of years ***(1–5)***. Despite the fact that the immunization of, for example, goats or sheep produces a greater volume of antiserum, polyclonal antisera raised in rabbits offers the advantage of the ready availability and general high quality of a wide variety of antirabbit secondary detection reagents. This can be a significant issue, especially if the antibodies are to be used for purposes more demanding than conventional Western blotting or immunoprecipitation.

Short peptides are generally poor immunogens and must be conjugated to carrier proteins, which provide the necessary helper T-cell epitopes for an efficient antibody response. Keyhole limpet hemocyanin (KLH) is a very common and effective carrier protein to use for this purpose, although other proteins, such as bovine serum albumin and thyroglobulin also work well. Purified proteins, for example, those isolated by means of bacterial expression, normally don't require coupling to a carrier before immunization. Nevertheless, conjugation may be worth considering should the protein be rather short or if it is thought to be only weakly immunogenic.

1.1. Immunizations

The methods described are suitable for use with immunogens derived from a variety of sources, for example, bacterially expressed fusion proteins, baculovirus-expressed proteins or synthetic peptides conjugated to suitable carrier proteins (*see* Chapter 2). Antibody responses to peptide immunogens often differ from those where the immunogen is a larger macromolecule in that maximal antipeptide titres (which arise rapidly after two to three immunizations) do not always coincide with maximal titres against the intact

protein (which tend to peak rather later at four to six immunizations). Although anti-peptide enzyme-linked immunosorbent assays (ELISAs) are useful for the monitoring of an immune response, there is no substitute for screening for immunoreactivity against the intact protein, for example, by immunoprecipitation or Western blotting.

Individual variation in antibody response is often significant, so it is advisable to immunize more than one animal (two or three rabbits per immunogen is recommended). Immune responses are generally poorer in specific pathogen-free rabbits, probably reflecting their greater immune naivity.

Adjuvants stabilize immunogens so that they induce the immune system persistently over a prolonged period. Oil–water adjuvants such as Freund's are extremely effective but must be prepared properly as stable emulsions. Such emulsions are very viscous, do not separate even after standing for long periods, and do not disperse if pipetted onto the surface of water. Well-prepared immunogens administered in Freund's adjuvant can persist for weeks and there is, therefore, little point in repeating immunizations too frequently. Freund's complete adjuvant (FCA), which contains bacterial cell wall components to induce nonspecific inflammation, potentiates the antibody response to immunogens. However, FCA can cause ulceration (resulting in loss of immunogen and discomfort to the animal) if administered in too large a bolus in one place or if given more than once. For this reason, subsequent immunizations are given using Freund's incomplete adjuvant (FIA), which contains no bacteria. An effective alternative to oil-based adjuvants is either to administer the immunogen on alum as a fine adsorbed suspension or to precipitate the immunogen with acetone. These treatments render proteins partially insoluble and thus more persistent immunogens (*see* **Note 1**).

1.2. ELISA for Anti-Peptide Antibodies

Because synthetic peptide immunogens used to generate antipeptide antibodies often are available in comparatively large

amounts, it is straightforward to check antibody titres by an enzyme-linked colorimetric assay. This procedure also is useful for monitoring recovery of antibody after purification by affinity chromatography. The target antigen is passively adsorbed to the walls of microtitre wells, either as free peptide or as peptide conjugated to an irrelevant carrier protein (e.g., bovine serum albumin, if the immunogen was a KLH conjugate). Usually, the free peptide makes a perfectly effective antigenic target, but occasionally important determinants on some peptides can be masked by adsorption to the plate, in which case peptide–carrier conjugates should be used. Thus, if antibody titre on free peptide is low it is a good idea to try conjugated peptide as the target. Antibodies bound to the adsorbed peptide are detected with an appropriate enzyme-linked second-layer reagent, typically peroxidase-linked anti-immunoglobulin, and the assay developed with a colorimetric substrate.

1.3. Affinity Purification of Anti-Peptide Antibody

It is sometimes advantageous to affinity purify an antibody preparation, in order to eliminate background "noise" in a given assay. In particular, it is frequently necessary to purify anti-phosphopeptide immunoglobulins in an anti-phophopeptide serum away from non-phosphopeptide immunoreactivity (*see* **Note 2**). With a ready source of immunogen in the form of a synthetic peptide, it is straightforward to purify anti-peptide antibodies by affinity chromatography. The peptide is covalently coupled to a matrix such as agarose and the crude antibody preparation passed down the column. Unbound material is washed away and the bound antibody eluted under denaturing conditions, for example, low pH (pH 2.5), high pH (pH 11.5), or 4 *M* $MgCl_2$. Immunoglobulins are unusually resistant to permanent denaturation by pH extremes or chaotropic reagents, although there are always exceptions (especially with monoclonal antibodies). Low pH followed by rapid neutralization is, perhaps, most straightforward. A 4-*M* $MgCl_2$ elution is milder and, therefore, should be used if low pH elution results in significant loss of activity.

The affinities of antibodies for their cognate peptides can be very high, so that it is sometimes difficult to quantitatively recover the higher-affinity antibodies in a polyclonal serum from the peptide-matrix. For this reason, it is important to use low concentrations of peptide on the matrix (typically 100–200 µg of peptide per milliliter of agarose gel). In addition, elution of bound antibody in the reverse direction to which it was run into the column, so as not to drive eluting antibodies into a further excess of antigen, enhances recovery. If both of these criteria are adhered to, recovery is usually of the order of 60–80% (*see* **Note 3**).

Suitable affinity resins are CNBr-activated Sepharose and similarly activated *N*-hydroxysuccinimide ester-based gels with spacer arms. These react with free amino groups on the peptide. If the peptide contains several lysine residues, coupling via amino groups can adversely affect antibody affinity for the peptide (although this is not necessarily inevitable). For this reason, a cysteine residue added to the N- or C-terminus of the synthetic peptide affords the option of coupling to agarose via the sulfhydyl group (*see* Chapter 2).

2. Materials

2.1. Immunizations

1. Freund's complete and incomplete adjuvants: FCA is a suspension of heat-killed *Mycobacterium tuberculosis* in oil. This should be shaken well before use. FIA is the oil without the bacteria. Freund's adjuvants should be mixed with the aqueous immunogen solution/suspension at a ratio between 1:2 and 2:1 and mixed until an emulsion is formed. This is most easily achieved by passage back and forth between two glass syringes connected by a three-way luer fitting. After a short time (1–5 min) the mixture should become noticeably "stiffer" and may then be administered.
2. Potassium aluminium sulphate (Alum) $AlK(SO_4)_2 \cdot 12\ H_2O$ precipitates: the aqueous immunogen solution/suspension is mixed with 0.3 vol 10% alum. The pH is then adjusted to about 8.0 with sodium hydroxide solution and the resultant precipitate washed in 0.154 *M* NaCl solution and administered.

3. Acetone precipitates: the aqueous immunogen is precipitated with 4.5 vol acetone at –20°C. The precipitate is collected by centrifugation at 10,000*g* at room temperature, washed in 80% acetone and air-dried. The pellet is resuspended in saline using a Dounce™ homogenizer and then administered directly or in association with alum or Freund's adjuvants.
4. Saline: 0.9% NaCl.
5. Glass syringes (2 and 10 mL).
6. Disposable three-way luer fitting taps.
7. Peptide-conjugate/purified protein immunogen.

2.2. ELISA

1. 10X Adsorption buffer: 1 *M* sodium bicarbonate, pH 9.6 (adjust with NaOH).
2. Peptide solution/suspension at 1 mg/mL in phosphate-buffered saline (PBS) stored at –20°C (*see* **Note 4**).
3. Antipeptide antibody.
4. "Immulon 2" high binding capacity 96-well microtiter plates (Dynatech).
5. Donkey anti-rabbit Ig-horseradish peroxidase conjugate (DaRIg-HRP).
6. Swine anti-rabbit Ig-horseradish peroxidase conjugate (SwaRIg-HRP) (APBiotech or DAKO).
7. Tris-buffered saline (TBS): 25 m*M* Tris-HCl/144 m*M* NaCl, pH 8.1.
8. Blocking buffer (TM): TBS containing 2% dried milk powder (Marvel).
9. Assay buffer (TMT): TBS containing 2% dried milk powder and 0.2% Tween-20.
10. Substrate solution for HRP: 1-m*M* ABTS (2,2' azinobis [3-ethylbenzthiazoline-6 sulphonic acid]) (Sigma) in 0.1 *M* sodium acetate pH 5.0. Add 1 µL of 30% hydrogen peroxide per 1 mL of ABTS solution just before use. Discard any old ABTS stocks that have a noticeable green color when dissolved in the absence of hydrogen peroxide.
11. Stop solution for peroxidase assays: 5% sodium dodecyl sulfate (SDS).

2.3. Affinity Purification

1. CNBr-activated Sepharose (APBiotech; *see* **Note 5**).
2. Sintered glass funnels.
3. Chromatography columns (APBiotech C series).
4. Peristaltic pump.
5. Concentrated HCl.
6. PBS, pH 7.2.
7. 100 m*M* Na acetate, pH 4.0.
8. 2 *M* NaCl in PBS.
9. TBS.
10. TBS containing 0.1% sodium azide (Sodium azide is toxic—consult MSDS data sheet).
11. TBS containing 0.1% NP40 (detergent).
12. 250 m*M* ethylene diamine tetraacetic acid, pH 8.0.
13. Saturated ammonium sulfate solution.
14. NP40 (detergent) stock solution: 10% in water.
15. 100 m*M* Na Citrate, pH 2.5.
16. 2 *M* Tris-HCl Base.
17. Saline: 0.9% NaCl in distilled water.
18. 4 *M* $MgCl_2$.
19. 10X TBS: 250 m*M* Tris-HCl, 1.44 *M* NaCl, pH 8.1.

3. Methods

3.1. Immunizations

3.1.1. Immunize Rabbits as Follows (see **Note 6**)

1. Subcutaneously with 50–200 µg of immunogen in FCA at multiple sites.
2. After 2–3 wk, immunize again subcutaneously with 50–100 µg of immunogen in FIA.
3. Repeat **step 2** at 2-wk intervals. Immunizing more frequently than twice weekly is not necessary and may even be detrimental. Intervals of longer than 2 wk are fine. Bleed 10 d after immunization. Test bleed (from the ear marginal vein) after the third and fifth im-

munizations, then completely ex-sanguinate after a further one to four immunizations as appropriate depending upon the efficacy of the antiserum.

3.2. ELISA for Anti-Peptide Antibodies

3.2.1. Adsorption of Peptide to Microtiter Plates

1. When using free peptide as an antigenic target, use at a final concentration of approx 50 pmol/mL in adsorption buffer (this corresponds to a dilution of 1 in 2000 of a 100 µg/mL stock solution of a 10-mer peptide). If using a peptide–carrier conjugate as the target, dilute the conjugate in adsorption buffer to a peptide concentration of about 10 µg/mL. The precise amount of peptide conjugate may need eventually to be titrated to give optimal signals.
2. Add 100 µL of diluted peptide solution to each well.
3. Leave at room temperature in a moist environment overnight (a sealable box containing a small amount of water is suitable).
4. Shake out any unadsorbed peptide, and wash the plate three times in TBS by immersion of the plate in TBS. Immerse the plate at an angle to avoid trapping air bubbles. Shake the plate dry.
5. Add 150 µL of TM buffer per well and leave at room temperature for at least 30 min. If required, store the plates at this stage at –20°C.

3.2.2. ELISA

1. Empty the wells by shaking the plate dry and add 100 µL of antibody diluted in TMT per well. A starting dilution of 1 in 50 is suitable for most antisera. Serially dilute antibody in doubling dilutions down one row of the microtitre plate (i.e., eight dilutions in all).
2. Leave for 30–60 min at room temperature.
3. Wash the wells three times in TBS as before.
4. Add 100 µL per well of appropriate second-layer reagent diluted in TMT. For most commercially available HRP-anti-Rabbit Ig conjugates a dilution of 1 in 200 should suffice, although titration may help to optimize signals.
5. Leave for 30–60 min at room temperature.
6. Wash three times in TBS as before.

7. Add 100 µL of substrate solution per well.
8. Incubate at room temperature. Peroxidase reactions take about 5–30 min to develop. Judge the reaction time by eye (*see* **Notes 7–9**). Reactions may be stopped by adding 100 µL of stop solution to each well. The SDS in the stop solution also solubilizes any precipitated products formed in the HRP reaction.
9. Read the optical density (OD) on an ELISA plate reader. Green ABTS reaction product should be read at OD_{406}.

3.3. Affinity Purification

3.3.1. Preparation of Peptide-Agarose

1. Mix 1.5 g of CNBr-Sepharose and 200 mL of 1 m*M* HCl and leave for 15 min at room temperature.
2. Collect the slurry on a sinter funnel, drain until a moist cake is formed. Add the cake (approx 5 mL volume) to 5 mL of PBS (pH 7.5–8.0) containing approx 500 µg of peptide. Agitate gently for about 2 h at room temperature. Note: Do not use a magnetic stirrer as this fragments the resin and generates fines that may slow or block the column flow.
3. Pour the slurry onto a sinter, wash sequentially with 20 mL of the following: PBS, pH 7.2; 100 m*M* sodium acetate, pH 4.0; 2 *M* NaCl in PBS; TBS, pH 8.0. Store as a 50% slurry in TBS containing 0.1% sodium azide at 4°C.

3.3.2. Preparation of Serum

1. Allow clot to form then ease away from sides of tube (to prevent clot adhering to the sides) and leave overnight at 4°C.
2. Aspirate the supernatant (serum) and clarify by centrifugation at 1000*g* for 5 min.
3. Add 5 m*M* ethylene diamine tetraacetic acid to the sample then add 0.82 vol of saturated ammonium sulfate solution while stirring and leave for 15 min at room temperature.
4. Collect the pellet by centrifugation (10 min, 10,000*g*, 4°C).
5. Redissolve the pellet in its original volume using TBS. Add 10% NP40 to a final concentration of 0.1% and spin in a microfuge to clarify.

3.3.3. Affinity Chromatography

1. Use an APBiotech reversible column. Pack 2 mL of affinity matrix into the column (keep moist) in running buffer (TBS containing 0.1% NP40). Wash with 20 mL of running buffer over 20 min.
2. Run in the antibody solution as prepared earlier. The flow-rate should be approx 1–2 mL/min (a peristaltic pump is useful to control the flow-rate). Run in the equivalent of about 1 mL of antiserum per mL of gel.
3. Wash with 10-column volume of running buffer.
4. Reverse the direction of flow through the column and wash with 10-column volume of TBS containing 0.1% NP40 for 10 min; five-column volume of TBS at the same flow-rate; and five-column volume of 0.9% NaCl.
5. Elution of antibody may be achieved by either one of the following two procedures.
   a. Low pH elution:
      1. Elute the bound antibody with four-column volume of 100 m*M* sodium citrate, pH 2.5, for 10 min.
      2. Collect the eluate and immediately neutralizing to pH 5.0–8.0 with 2 *M* Tris-HCl base. This can be achieved by prealiquoting into the collecting tubes the amount of Tris base necessary to neutral ize a given fraction volume.
      3. Assay fractions by ELISA (*see* **Subheading 3.2.**) diluted 1 in 5 to 1 in 50.
      4. Pool the most strongly positive fractions and adjust the pooled fractions to pH 6.0.
      5. Add 1 vol of saturated ammonium sulfate solution.
      6. Leave for 10 min at room temperature and pellet the antibody at 10,000*g* for 10 min at 4°C.
      7. Resuspend the antibody in water at approx 1–5 mg/mL (OD_{280} of IgG is about 1.4 for a 1 mg/mL solution). Either: dialyze against TBS containing 0.1% sodium azide or add one-tenth volume 10X TBS (*see* **Note 10**). Store in aliquots at –20°C.
   b. Elution with 4 *M* $MgCl_2$:
      1. Elute the bound antibody with four-column volumes of 4 *M* $MgCl_2$.

2. Dilute the eluate 10X with distilled water.
3. Add an equal volume of saturated ammonium sulfate and pellet the immunoglobulin at 10,000*g*.
4. Resuspend the pellet in water.
5. Either: dialyze against TBS containing 0.1% sodium azide or add one-tenth volume 10X TBS (*see* **Note 10**). Store in aliquots at –20°C.

4. Notes

1. These protocols are broadly applicable to the generation of polyclonal antisera in hosts other than rabbits, however if it is intended to generate mouse monoclonal antibodies (Mabs) the following should be noted: mouse responses are often best in F_1 crosses (e.g., BALB/c × C57Bl/6) rather than pure strains. Anticarrier antibodies often comprise a significant proportion of Mabs generated using conventional carriers and it is, therefore, worth considering the use of an alternative carrier, such as the purified protein derivative (PPD) of tuberculin. If PPD conjugates are used for peptide immunizations, the animals must first be primed with live attenuated bacteria (BCG strain), which express PPD on their surface. This priming step elicits a strong T-cell helper response against subsequent PPD-linked immunogens. Freund's complete adjuvant appears to interfere with this priming process and so should be avoided if using this method. Immunizations should be performed using FIA. Immunizations immediately prior to hybridoma fusions should not be done with persistent adjuvants such as Freund's, because in this case the aim is to induce a rapid and transient immune response whose early (lymphoproliferative) stage coincides with fusion to myeloma cells. Therefore, acetone precipitates are particularly useful for immunizations prior to spleen fusions for the development of Mabs.
2. A single-step affinity purification of anti-phosphopeptide antibodies on phosphopeptide matrix is normally sufficient to purify the antiserum to a significant degree. However, a second purification step on nonphosphopeptide matrix, to deplete residual non-phosphopeptide immunoreactivity, may be necessary.

3. Affinity purification may be performed using gravity flow through a disposable column (e.g., a Bio-Rad Poly-Prep chromatography column). In this case, flow reversal is not possible and recovery of high affinity antibodies may be compromised. Nevertheless, acceptable results are often achieved.
4. Not all peptides will be soluble at 1 mg/mL in PBS. Addition of SDS with gentle heating and shaking can aid solubilization.
5. NHS-activated Sepharose 4 Fast Flow incorporates a spacer arm and is a good alternative to CNBr-Sepharose.
6. A "Pre-immunization" serum sample from the test animal serves as an excellent negative control serum. This can be taken at the time of the first immunization.
7. Avoid contamination of substrate solutions by skin contact as this can sometimes increase background activity. Read HRP reactions immediately as atmospheric oxidation will gradually react with substrate in all wells.
8. β-Galactosidase conjugates are suitable alternatives to HRP conjugates as second layer reagents. Substrate solution for β-galactosidase: 4 mg/mL *o*-nitrophenyl-β-D-galactopyranoside dissolved in TBS containing 0.7% 2-mercaptoethanol and 1 m*M* $MgCl_2$. Stop solution for β-galactosidase is 1 *M* Na_2CO_3.
9. If the ELISA color development takes more than a few minutes, continue the incubation in the dark. β-galactosidase reactions tend to have a lower spontaneous background than peroxidase reactions but the yellow *o*-nitrophenyl-β-D-galactopyranoside reaction product can take longer to develop. β-galactosidase reactions can be speeded up by incubation at 37°C.
10. Ammonium sulfate precipitation of IgG inevitably leads to residual ammonium sulfate in the pellet. Dialysis will remove this, but may also lead to a significant loss of antibody. For most purposes, addition of 10XTBS to the resuspended pellet is an acceptable alternative.

References

1 Evan, G. I., Hancock, D. C., Littlewood, T. D., and Gee, N. S. (1986) Characterisation of human myc proteins. *Curr. Top. Micro. Immunol.* **132,** 362–374.

2 Waters, C. M., Hancock, D. C., and Evan, G. I. (1990) Identification and characterisation of the egr-1 gene product as an inducible, short-lived, nuclear phosphoprotein. *Oncogene* **5,** 669–674.

3 Fraser, A. G. and Evan, G. I. (1997) Identification of a *Drosophila* melanogaster ICE/CED-3-related protease, drICE. *EMBO J.* **16,** 2805–2813.

4. Iaccarino, I., Hancock, D., Evan, G., and Downward, J. (2003) c-Myc induces cytochrome c release in Rat1 fibroblasts by increasing outer mitochondrial membrane permeability in a Bid-dependent manner. *Cell Death Differ.* **10,** 599–608.
5. Basu, S., Totty, N. F., Irwin, M. S., Sudol, M., and Downward, J. (2003) Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14–3-3 and attenuation of p73-mediated apoptosis. *Mol. Cell* **11,** 11–23.

4

Making Hybridomas

Robert Burns

Summary

Hybridomas are derived from the fusion of spleen and myeloma cells and produce monoclonal antibodies. Each hybridoma cell line produces an antibody with a unique specificity allowing the production of highly defined reagents that can be used in many branches of immunochemistry.

Key Words: Monoclonal antibodies; cell fusion; hybridoma; immunization; cell culture; myeloma; splenocytes.

1. Introduction

In 1975, Kohler and Milstein reported ***(1)*** that immortal cell lines secreting antibody of a single specificity could be produced by the artificial fusion of splenocytes derived from an immune mouse and tumour cells derived from a murine myeloma. They called these cell lines hybridomas and the product from them monoclonal antibodies. The development of monoclonal antibodies opened up huge possibilities in all areas of antibody use because reagents could be created with specificity to a single domain (epitope) on the target

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

substance. Additionally, antibodies could be generated to compounds that had previously been regarded as impossible when using conventional serum production. Monoclonal antibodies should, however, be seen as complementary to those derived from animal serum because each has its place in immunochemistry. The unique specificity, defined affinity, and avidity of the monoclonal antibody are very desirable when looking at cell surface markers or single epitopes on a viral protein. In contrast, the broad specificity of polyclonal antibodies is a characteristic that may be desirable when screening for multiple strains of a virus or in techniques such as immunoaffinity purification.

The techniques for generating hybridoma cell lines have not changed much from the early work of Kohler and Milstein. Original work ***(2)*** used mice as donor animals and the mouse myeloma line NS-1 or its derivative NS-O (*see* **Note 1**) as the fusion partner. The murine system is probably still the most prevalent today although rats may be used for some antigens. Most murine hybridomas are produced from the physical fusion of spleen and tumour cells using low-speed centrifugation and polyethylene glycol ***(3)***. Recombinant hybridoma cells are selectively grown because only they have the necessary characteristics of immortality derived from myeloma cells and a salvage pathway for purine nucleotide (hypoxanthine-guanine phospho ribosyltransferase; HPRT) inherited from the splenocytes (*see* **Note 2**). Unfused spleen cells have a limited natural life span in tissue culture, and the myeloma cell line lacks the purine nucleotide salvage pathway that is necessary for survival in the presence of the purine biosynthesis inhibitors (Aminopterin) contained in the selective medium (hypoxanthine aminopterin thymidine; HAT).

Techniques such as electroporation and transfection have successfully been used for hybridoma production, but are much less commonly used than cell fusion assisted by polyethylene glycol.

Hybridomas may be unstable because the techniques used to create them are fairly crude and may lead to a loss of cellular and genetic integrity. Instability is characterized by cell death after a few divisions or change in specificity. It is vitally important to test the hybridomas repeatedly to ensure that loss of specificity or abil-

ity to secrete has not occurred. The desirable qualities of monoclonal antibodies hinge on the fact that they are the products of a cell line derived from, a single parent clone. To ensure that this is the case it is important to aggressively clone the cells until a cloning efficiency of close to 100%, based on the quality of the antibody is achieved.

The vast majority of hybridomas generated in laboratories are destined to be discarded because they will not have the desired qualities of antibody specificity, growth characteristics, or cloning ability required. In most cases, it is more practical to derive a new cell line rather than try to continue with one that is less than ideal. It is very important to have in mind the qualities of the cell line that are required along with the characteristics of the antibody that are needed before embarking on hybridoma production.

Other species of hybridomas, including human, have been produced but are generally created by the use of viruses conferring cellular immortality. Artificial immunization of the donor is often not practical or ethical and so cell lines are often derived from peripheral lymphocytes obtained from individuals naturally immune to the target substance. Some human monoclonal antibody secreting cell lines have been derived from spontaneously occurring myelomas, but this line of approach frequently is unrewarding as the probability that the antibody will be one of interest is remote.

Nonsecretory myeloma fusion partners with defective purine nucleotide biosynthesis pathways do now exist for a number of species, including humans, and so hybridoma production by cell fusion using polyethylene glycol (PEG) is now a possibility.

A number of non-hybridoma techniques have been developed for in vitro antibody production; a review of recombinant antibody production is covered in Chapter 6.

Hybridoma production can be broken down into four processes, immunization of donor animals, cell fusion, cell selection, and expansion. Each of these stages is important for the quality of the final product. Antigens used to immunize animals must be representative of the target substance (*see* **Note 3**) or the likelihood of producing cell lines with the correct specificity is remote. Cell

fusions must generate hybridomas but steps must be taken to ensure that neither too few nor too many are generated. Cell lines must be selected using strict criteria to ensure that desirable qualities in both cell growth and stability are present along with the specificity, avidity and affinity required for the final testing format. It is always advisable to screen primary cell lines using the assay format envisaged for the final test (*see* **Note 4**). Monoclonal antibodies may perform perfectly well in one assay format but may not for one reason or another convert to another.

2. Materials

1. Four female Balb-c mice (*see* **Note 5**).
2. NS-O myeloma cell line.
3. Media: basal RPMI 1640 medium requires supplementation with sodium pyruvate, L-glutamine, and penicillin/streptomycin before use. The supplements are supplied as concentrates of 50X or 100X, and the appropriate amounts should be added. Standard tissue culture media for hybridoma production contain 5%, 10%, or 15% fetal bovine serum (FBS; *see* **Note 6**). Sufficient quantities should be prepared in advance and a sterility check should be performed on them prior to use.
   All prepared media should be stored at –20°C until required and used within 28 d of thawing. L-Glutamine has an effective lifespan of about 28 d in prepared medium and will degrade giving rise to ammonia after this time.

Allogeneic mixed thymocyte medium ***(4)*** is a conditioned medium used to aid cells recover from the fusion process. It also encourages division of hybridoma cells after dilution cloning when colonies derived from single cells are required. Rat thymi are used for this purpose to minimise the number of animals required to produce adequate numbers of cells. The medium contains a number of "helper" cytokines produced by the thymocytes. It is important that the thymocytes used are derived from two different rat strains as this causes co-stimulation and enhanced cytokine secretion by the cells. Mixed thymocyte medium must always be diluted with RPMI 1640 medium containing 15% FBS and is usually used at a dilution of 10–15%.

2.1. HAT Medium

1. Add 5 mL of HAT supplement to 500 mL of RPMI 1640 medium containing 15% FBS.
2. Check sterility prior to use.
3. Store –20°C.

2.2 Allogeneic Mixed Thymocyte-Conditioned Medium

1. Obtain two 6-wk-old rats of different strains (e.g., Sprague and Wistar).
2. Kill them and remove the thymus glands aseptically.
3. Homogenize the thymus glands using the frosted ends of glass microscope slides (*see* **Subheading 3.3.** for method of spleen hemogenization) and suspend in 10 mL of phosphate-buffered saline (PBS).
4. Wash by centrifugation at 400*g* and resuspend in 10 mL of PBS.
5. Add the thymocyte suspension to 1 L of RPMI 1640 medium containing 15% FBS and distribute into 4X 225-cm T flasks.
6. Incubate the cells for 30 to 40 h at 37°C/5% CO_2 (it is important that incubation is not longer than 40 h or undesirable cytokines will be produced).
7. Harvest the conditioned medium is by centrifugation at 500*g*. The medium should be tested for sterility and stored at –20°C in 20-mL aliquots.

2.3. Freezing Medium

1. Add 50 mL of dimethyl sulfoxide to 500 mL of FBS.
2. Dispense into 20-mL aliquots.
3. Test sterility.
4. Store at –20°C.

2.4. PEG Medium for Cell Fusions (see Note 7)

1. Autoclave 20 g of PEG 4000 MW in a glass universal at 121°C/ 24 min.
2. When cool enough to handle quickly add 20 mL of RPMI 1640 without FBS.
3. Mix well and dispense in 2-mL aliquots.
4. Check sterility.

5. Store at room temperature in dark (*see* **Note 6**).
6. 96-Well tissue culture plates.
7. 24-Well tissue culture plates.
8. 25-cm "T" tissue culture flasks.
9. Cryovials.
10. Sterile glass Petri dish and one pair of sterile "frosted end" glass microscope slides.
11. 5% CO_2, 37°C incubator
12. Microbiological safety cabinet (class 2 preferably).

3. Methods

3.1. Immunizations

Mice should be obtained from a reputable source and should be maintained under standard conditions as required by local animal welfare legislation. Female mice can be housed together and it is usual to indelibly mark individuals by tattoo, ear punch or electronic chip so that each animal and its immune response can be monitored.

1. Prepare antigens at approx 1 mg/mL in physiological saline.
2. Inject the mice intraperitoneally with 0.05 mL of antigen mixed with an appropriate adjuvant on d 0, 14, 28, and 44. Take a test bleed on d 51 and assess the serum for the presence of circulating antibody to the antigen.
3. If the immune response is good (*see* **Note 8**) rest the animals for a minimum of 8–10 wk before cell fusion work. If the immune response is poor give another immunization on d 61 and assess the circulating antibody level on d 68.
4. Prior to carrying out a fusion, give the mouse with the highest antibody titer a booster dose of 0.05 mL of antigen without adjuvant intraperitoneally. Three days later, kill the mouse by cervical dislocation and remove spleen aseptically.
5. If all mice have responded well to the immunizations give all of them a booster dose, harvest the spleens and cryogenically store the splenocytes for use at a later date (*see* **Note 9**). Store the spleen cells cryogenically as described in **Subheading 3.6.**

3.2 Preparation of the Myeloma Cells

NS-O myeloma cells are normally kept stored cryogenically and so should be resuscitated 3 d before conducting the fusion.

1. Quickly thaw a frozen aliquot of NS-O cells either in a 37°C water bath or between the palms of the hands. Once the pellet has melted, add 1 mL of 37°C RPMI 1640 medium supplemented with 10% FBS and draw up into a Pasteur pipet. Transfer the cells to a 225-cm "T" flask and add 50 mL of RPMI 1640 medium containing 5% FBS and place in a tissue culture incubator 37°C/5% CO_2.
2. Inspect the cells on d 2, they should be semi-confluent and adherent to the flask base. Increase the volume of RPMI 1640/5% FBS medium to 75 mL and return to the incubator.
3. Inspect the cells on d 3, they should be almost fully confluent on the flask base. Discard the medium, add 10 mL of cold sterile PBS containing 0.02% ethylene diamine tetraacetic acid and leave for a few minutes to allow the cells to detach. Split the cells equally between two flasks and add 75 mL of RPMI 1640 medium/5% FBS to each flask and return to the incubator.
4. On d 4, remove the medium from each flask and replaced with 20 mL of cold sterile PBS containing 0.02% ethylene diamine tetraacetic acid. After a few minutes the cells will detach from the flask surface. Harvest the cells by gently tapping the flask and pouring the cell suspension in to sterile universal containers. Wash the cells by centrifugation at 300*g* and resuspend in 10 mL of cold PBS.

3.3. Preparation of Splenocytes

Keep the spleen on ice, in RPMI 1640 medium without FBS. Spleens can kept in this way for 1–2 h without significant loss of splenocyte viability.

1. Decant the spleen into the sterile glass Petri dish and gently dissociate by rubbing between the frosted ends of the glass microscope slides. It is sometimes necessary to break up the spleen a little before dissociating using either the ends of the microscope slides or a pair of dissecting scissors. After dissociation there will be fibrous tissue

remaining from the spleen capsule and a red liquid containing the splenocytes.
2, Aspirate the red liquid from the fibrous tissue and place into a sterile universal container. Allow the contents of the universal container to settle for a few minutes then aspirate or "pour off" the supernatant and retain it, as this contains the splenocyte (*see* **Note 10**).
3. Wash the splenocytes with PBS by centrifugation at 400*g* and resuspend the pellet in 10 mL of cold PBS.
4. Remove 2.5 mL of splenocyte suspension for cell fusion.
5. Harvest the remaining cells by centrifugation, resuspend in freezing medium (2 mL/spleen) and dispense into cryotubes in 0.5-mL aliquots. Freeze the cells and store cryogenically.

3.4. Cell Fusion

1. Mix the washed NS-0 cells harvested from the 2-T flasks with one quarter of the splenocytes from one spleen.
2. Pellet cells by centrifugation at 400*g* for 5 min.
3. Discard supernatant and slowly add 1 mL of PEG/RPMI mixture.
4. Gently resuspend the pellet by swirling.
5. Pellet cells by centrifugation at 250*g* for 5 min.
6. Slowly overlay cell/peg layer with 5 mL of RPMI 1640 with no FBS and then gently swirl to create a cell suspension.
7. Pellet cells by centrifugation at 400*g* for 5 min.
8. Discard PEG/RPMI mixture and replace with 10 mL of HAT medium. Do not disturb the cell pellet, then incubate the cell pellet for 5–10 min at 37°C.
9. Resuspend the cells by swirling and add 20 mL of HAT and 7.5 mL of allogeneic mixed thymocyte medium.
10. Dispense cells suspension into 96-well tissue culture plates (0.2 mL/well; *see* **Note 11**).
11. Place in 37°C/5% CO_2 incubator.
12. After 7 d examine the wells for the presence of hybridomas and given an additional 0.1 mL of HAT medium.
13. Test wells exhibiting growth for the presence of monoclonal antibodies when the hybridoma growth covers approx 25% of the base of the cell well. The assay system used should mimic the final test format required. Commonly, Triple Antibody Sandwich (TAS) ***(5)*** enzyme-linked immunosorbent assay (ELISA) is use for screening hybridomas for specific antibody. It is important when testing hybri-

domas that a suitable negative is included as background antibody activity from unfused spleen cells may give apparently positive results. Up to 0.2 mL of medium can be harvested from each cell well, which can be split to assay against specific antigen and a suitable negative.

3.5. Stabilizing Hybridomas

Wells testing positive for specific antibody should be assayed more than once to ensure that activity continues over the course of a few days. The health of the cells should be checked daily to ensure that overcrowding does not occur and once the cells are confluent in the cell well they should be subcultured into 24-well plates. The cells must also be cloned by limiting dilution at this stage and plated out at 1 cell/well. It is useful to test the isotype of the secreted antibody when the cells are growing in the 24-well plates. This can be accomplished using a number of "dip stick" assays giving results in a matter of a few minutes. The isotype of the antibody is important for development of the antibody if purification and reagent development will be required. Antibodies of the sub classes G1, G2a, and G2b are the most desirable for further development as they are readily purified and are relatively stable. Antibodies with the G3 isotype, most commonly produced from bacterial antigens may be inherently unstable and liable to spontaneous aggregation.

Approximately 50% of hybridomas will secret antibody of the M subclass. IgM is a large molecule that does not readily purify but may be used for TAS ELISA. Some antigens will only give rise to IgM secreting hybridomas and are know as anamnestic antigens. These substances are frequently highly glycosylated and do not invoke a "memory" response in the immune system. The result of this is that B-lymphocytes never mature beyond the production of IgM regardless of the number of immunizations the animal is given.

3.5.1. Limiting Dilution Cloning

1. Agitate the contents of the cell well using a Pasteur pipet and then using a pipet with a sterile tip, draw up 2X 0.01-mL aliquots of cells.

Add one aliquot to 1 mL of warm RPMI 1640 medium with 15% FBS. Add the other aliquot to 0.01 mL of Trypan blue dye and count the cells using a Neubauer counting chamber.

2. Calculate the cell count in the 1 mL tube using the formula: (total cell count/number of grid squares counted) $\times 10^4 \times 2$ = cells/mL.
3. Calculate the volume of cell suspension from the 1-mL tube that would contain 60 cells.
4. Transfer the appropriate volume of the medium and cells from the 1-mL tube to the 9 mL of cloning medium and dispense 0.15 mL/well in 60 wells of the cloning plate.
5. Place the cloning plate into the tissue culture incubator 37°C/5% CO_2 for 1 wk and then inspect for growth. Most cell wells should have single hybridoma colonies growing in them. It is important at this stage to record the wells containing single colonies. Give each of the cell wells 0.05–0.1 mL of cloning medium and reincubate until cells are between 30–50% confluent.
6. Test the wells that originally contained single colonies for specific activity, and record the numbers that are positive.
7. Subclone the cells using the previously mentioned method until 100% of the clones are positive for the specific antibody. Occasionally some cell lines will not achieve a cloning efficiency of 100% regardless of the number of subcloning attempts. In these cases a final clone should be selected, rapidly multiplied and cryogenically stored. Cells from these unstable stores should never be used to produce additional cell stocks without subcloning again.
8. Choose one of the clones from the final (100% efficiency) cloning as the master cell line, expand it in tissue culture and cryogenically preserve stocks of it as the master cell bank (at least 12 vials).

3.6. Preserving Cells Cryogenically

See also **Subheading 3.3.3.** in Chapter 5.

1. Keep 25-cm^2 "T" flasks of cells in log phase of growth by subdividing when almost confluent. Freezing should only be carried out when cells are less than confluent and appear healthy.
2. Make sure that caps are tightened on flasks and then sharply tap to dislodge cells.
3. Pour off cell suspension into a sterile plastic universal container.

4. Pellet cells at 400*g* for 5 min.
5. Pour off medium, add 0.2 mL of 1% sodium azide solution and retain for assessment of antibody activity.
6. Tap the cell pellet and add 0.5 mL of cold freezing medium.
7. Aspirate with Pasteur pipet to resuspend pellet then transfer to a 2-mL cryogenic vial.
8. Cells must be frozen at approx 1°C/min and this can be achieved by putting the cryovials into an expanded polystyrene container with a wall thickness of 0.5 cm and then placing them in a –70°C freezer.
9. Transfer the cells to liquid nitrogen storage within 3 d of freezing at –70°C, they will remain viable for many years.

4. Notes

1. It is preferable to use NS-0 as a fusion partner, it does not secrete a constitutive antibody but has the ability to produce monoclonal antibodies once fused to an immune spleen cell. NS-1 has a constitutive antibody, which may cause interference in screening assays for recombinant hybridoma cells.
2. Cell lines are available that have other defective pathways, aminopterin cannot be used to select for their growth, and an alternative agent must be used.
3. Antigens must be representative but do not need to be identical to the target substance. Synthetic peptides modelling single epitopes and fusion proteins containing homologous regions to the target can be used very effectively as antigens.
4. It is not always possible to use exactly the same format for screening as will be used in the final assay but it is advisable to attempt to ensure that the "position" that the monoclonal antibodies (MAb) will occupy in the final format is the position that is used for screening. For example MAbs which will eventually be conjugated to enzymes and used in Double Antibody Sandwich (DAS) ELISA should be screened for using TAS ELISA. This ensures that the epitope-binding portion of the antibody is in the same position as it will be in the final test format with the analyte bound to the coating antibody.
5. Balb-c mice are normally used for MAb production, partly because they are the strain of mouse that the NS-0 cell was derived from and also because they are easily handled and the females can be communally housed.

6. Batches of FBS vary enormously in their ability to support cell growth and it is important to establish that the batch you are buying will be suitable. Most suppliers will allow you to reserve quantities from a batch and will provide small samples for testing. Usually a few batches are tested at the same time and then the best performer is selected. Testing is carried out by cloning an established hybridoma line by limiting dilution in medium containing the test FBS and then observing numbers of resulting colonies.
7. Polyethylene glycol 4000 MW is normally used for cell fusions. Batches may vary in their ability to produce viable hybridomas and it is wise to test a few batches from different sources prior to undertaking hybridoma project work. A number of biochemical suppliers now produce ready-to-use PEG/media solutions in sterile ampoules, which workers may find is a more practical source.
8. If the antigen to be used is derived from fungal mycelium, bacterial cell walls or is known to be highly glycosylated then repeated immunization is of no value. Antigens of this type are known to be anamnestic and do not produce "memory" B-lymphocytes. The animal sees each immunization as a primary challenge and the likelihood of producing an IgG response is remote.
9. Frozen spleen cells work well for cell fusions and this approach allows a greater degree of flexibility in performing fusions than using splenocytes directly ex vivo. The vial of spleen cells should be rapidly thawed in a 37°C water bath or between the palms of the hands. One milliliter of PBS is then added to the vial and the contents aspirated and added to 5 mL of PBS. The cells are washed once by centrifugation at 400*g* and resuspended in 5 mL of cold PBS; they should then be stored on ice until required. Spleen cells that have been frozen may have a tendency to clump and the pellet will look very pale. This does not affect their ability to produce viable hybridomas.
10. Disposable cell strainers (Falcon) that fit into the top of universal containers may be used to remove large fragments of spleen tissue.
11. The volumes of media used during cell fusion and subsequent plating in to tissue culture wells do not need to be accurately measured. Disposable Pasteur pipets can be used to approximate volumes and most manufacturers publish specifications including drop volume for dispensing cells and medium.

References

1 Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256,** 495–497.
2 Galfre, G. and Milstein, C. (1981) Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol*. **73,** 3–46.
3. Kennet, R. H., Denis, K. A., Tung, A. S, and Klinman, N. R. (1987) Hybrid plasmocytoma production: fusions with adult spleen cells, monoclonal spleen cell fragments, neonatal spleen cells and human spleen cells. *Curr. Top. Microbiol. Immunol.* **81,** 485.
4. Micklem, L. R., McCann, M. C., and James, K. (1978) The use of rat mixed thymocyte-conditioned medium for hybridoma production, cloning and revival. *J. Immunol. Methods* **104,** 81–86.
5. Clark, F. M. and Adams, A. M. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Virol. Methods* **34,** 475–483.

5

Growing Hybridomas

Gary Entrican and Gareth Young

Summary

Hybridomas can be grown in a number of ways to produce stocks of monoclonal antibodies. Smaller volumes may be produced in static flask culture, but if larger amounts are required, a bioreactor may be used.

Key Words: Hybridoma; bioreactor; flask culture; monoclonal antibodies; culture medium; cryopreservation.

1. Introduction

The fusion of antigen-primed B-cells with transformed myeloma cells results in immortalized hybridomas that secrete antibodies (*see* Chapter 4). The subsequent cloning of the hybridomas gives rise to cell lines that secrete monoclonal antibodies (MAbs) of a single specificity ***(1)***. This technology has had a tremendous impact on research and medicine, with MAbs being used to identify and characterize the biological significance of myriads of molecules. The outcome has been the development of diagnostic tests and therapies for the detection and management of disease ***(2)***. The amount and purity of a MAb required for any given purpose can vary greatly. Here, we

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

describe the procedures involved in the maintenance and management of hybridomas and suggest techniques for maximizing yields.

2. Materials

1. RPMI 1640 1X liquid without L-glutamine, without HEPES, stored at 4°C (cat. no. 31870; Gibco Invitrogen Corporation, Paisley, UK).
2. 100 m*M* L-glutamine solution stored at –20°C (cat. no. G3126; Sigma, Poole, Dorset, UK).
3. 1 *M* HEPES solution stored at 4°C (cat. no. H0887; Sigma).
4. Nutrient Broth No. 2 (NB): 25 g dissolved in 1 L of sterile H_2O and stored at 4°C (cat. no. CM67; Oxoid Ltd., Basingstoke, Hampshire, UK).
5. Oxaloacetate, pyruvate insulin (OPI) media supplement solution: one vial dissolved in 10 mL of sterile H_2O stored at –20°C (cat. no. O5003; Sigma).
6. Penicillin/streptomycin solution, stored at –20°C: 10,000 IU/mL benzypenicillin sodium BP (cat. no. 301889; Britannia Pharmaceuticals, Surrey, UK) and 10,000 µg/mL streptomycin sulphate (cat. no. 11800-038; Gibco Invitrogen Corporation, Paisley, UK).
7. Fetal bovine serum (FBS), heat-inactivated at 56°C for 30 min to destroy complement and stored at –20°C (Labtech International, Ringmer, East Sussex, UK).
8. Costar 24-well tissue culture plates (cat. no. 3524; Corning Incorporated, Ithaca, NY).
9. Costar 25-cm^2, 75-cm^2, and 225-cm^2 vented sterile tissue culture flasks (cat. nos. 3056, 3376, 3001; Corning Incorporated).
10. Sterilin 5 mL, 10 mL, and 25 mL disposable sterile pipets (cat. nos. PP28033, PP28060, PP40125; Mackay and Lynn, Edinburgh, UK).
11. MiniPERM classic kit, molecular weight cutoff 12.5 kDa/vol 35 mL. Kit comprises: 12 production modules, single packed, sterile; four nutrient modules, reusable; four stands for the MiniPERM bioreactor (cat. no. IV-76001055; Vivascience, Sartorius AG Weender Landstrasse 94-108, 37075 Goettingen, Germany).
12. CellPROTECT, anti-shearing agent for high-density culture (cat. no. IV-76077041; Vivascience).
13. AntiFOAMa (cat. no. IV-76077320; Vivascience).
14. Universal turning device, which holds four miniPERM bioreactors (cat. no. IV-76001065; Vivascience).

15. Disposable hypodermic syringe, 50-mL Luer (cat. no. IV-76077137; Vivascience).
16. Disposable hypodermic syringe, 2-mL Luer (cat. no. IV-76077136; Vivascience).
17. Spare Luer-Lock screw caps for production modules: six pieces, single packed, sterile (cat. no. IV-76077027; Vivascience).
18. Neubauer hemocytometer (cat. no. MNK-420-010N; Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, UK).
19. Cryopreservation tubes (cat. no. 366656, Nunc A/S, DK-4000 Roskilde, Denmark).
20. DMSO Hybrimax (cat. no. D2650; Sigma).
21. Cryo 1°C freezing containers (cat. no. 5100; Nalgene, Nalge Europe Ltd, Rotherwas, Hereford, UK).
22. 2-Propanol (RH1018; Rathburn Chemicals, Walkerburn, Scotland, UK).
23. Nigrosin, dissolved to a final concentration of 0.1% w/v in phosphate-buffered saline containing 2% FBS, filter-sterilized, and stored at 4°C (BDH, Merck, Poole, Dorset, UK).
24. Supplemented RPMI culture medium: RPMI is used as the standard base medium for culturing hybridoma cell lines. The following supplements are added to each 500-mL bottle of RPMI: 10 mL of penicillin/streptomycin (*see* **Note 1**), 10 mL of glutamine (*see* **Note 2**), 5 mL of HEPES, and 50 mL of FBS (*see* **Note 3**). The contents should be mixed by swirling, not by inversion, thereby avoiding leakage and contamination. Hereafter this mixture is referred to as complete medium (CM). Two 1-mL aliquots of CM should be removed and added to two 5-mL aliquots of NB for sterility testing. Keep one NB at room temperature for 3 d and incubate the other at 37°C for 3 d. Check the NBs for evidence of microbial contamination prior to using the CM for the first time. Store CM at 4°C and use within 2 wk of preparation (*see* **Note 2**).

3. Methods

The methods in the following sections describe 1) the growth of cloned hybridoma cell lines, 2) high-density culture systems required for bulk production of MAbs, and 3) the generation of validated cell banks where hybridomas can be stored for future retrieval. All procedures that involve manipulation of cell lines are conducted

within the confines of a class II microbiological safety cabinet using sterile equipment. Cell cultures are maintained at 37°C in an atmosphere of 5% CO_2 in air and 100% humidity. It is preferable to use tissue culture flasks that have filter-vented caps to minimize the chances of contamination. Several hybridoma cell lines were used in the following procedures: 36F ***(3)***; VPM 20, VPM 21, VPM 22 ***(4)***; 73B ***(5)***; 3C2, and 8D8 ***(6)***. These cell lines have individual characteristics, but the procedures described are generic in nature and can be applied to all hybridoma cell lines, taking into account the comments in the Notes section.

3.1. Expansion of Cloned Hybridomas

1. Transfer hybridoma cells from the 96-well cloning culture plate to a 24-well culture plate in 1 mL of fresh CM (*see* **Note 4**) using gentle pipetting to dislodge the cells from the plastic (*see* **Note 5**).
2. Two days later, transfer the cells to a 25-cm^2 flask in 2 mL of fresh CM (*see* **Note 6**). The next day, add 3 mL of CM to feed the cells.
3. The following day, gently pipet the 5 mL of medium in the flask several times several to dislodge the cells and transfer the total volume to a 75-cm^2 flask. Add a further 5 mL of fresh CM immediately to the flask to feed the cells. Add 10 mL of fresh CM 24 h later. The subsequent day, transfer the 20 mL containing the cells to a 225-cm^2 flask using gentle pipetting as before, then add 30 mL of fresh CM immediately.
4. The next day, dislodge the cells from the flask by pipetting and add 10 mL of the resulting suspension to each of five 225-cm^2 flasks. Add 40 mL of fresh CM per flask. This protocol of passaging 10 mL of cell suspension at a 1:5 ratio can be followed for expansion of hybridomas either for generating cells for high density cultures (**Subheading 3.2.**) or for preparation of cell banks (**Subheading 3.3.**). Check the cells for *Mycoplasma* contamination (*see* **Note 7**) and analyze the supernatant for antibody production (*see* **Note 8**) before progressing to either of the next stages.

Fig. 1. The MiniPERM bioreactor. Reproduced with permission from Joachim Lücke.

3.2. High-Density Culture

3.2.1. The MiniPERM Bioreactor

The MiniPERM bioreactor is our method of choice for the high-density culture of hybridoma cells (**Fig. 1**). The modular system, consisting of a 40-mL disposable culture chamber and a 550-mL reusable nutrient module separated by a dialysis membrane (12.5 kDa molecular weight cutoff), allows for the production of a low-volume, high-density cell population with a correspondingly high antibody yield.

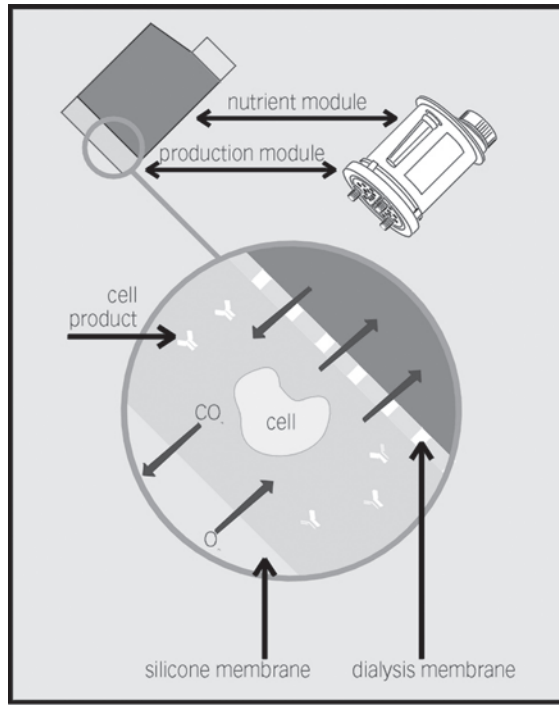


Fig. 2. Schematic view of the MiniPERM production module. Reproduced with permission from Joachim Lücke.

The hybridomas and secreted antibody are retained in the production module (**Fig. 2**). Depending on the cell line, cell densities can reach upwards of 1×10^7 cells/mL. The MiniPERM bioreactor is rotated on a universal turning device that allows four MiniPERM bioreactors to be run simultaneously for larger production runs of one hybridoma or production of different hybridomas. This motion speeds the distribution of nutrients to the cells and facilitates the removal of metabolic waste products and CO_2, processes that rely on passive diffusion in static bioreactors. Sample collection and harvesting is via Luer-Lock connections on the production module. The MiniPERM is a continuously fed culture system that allows cultures to be maintained for several weeks.

3.2.2. Inoculation of the Bioreactor

Assemble the MiniPERM bioreactor according to the manufacturer's instructions (*see* **Note 9**), disinfect the universal roller, and install in a CO_2 incubator. Media for the production module (production medium) and the nutrient module (nutrient medium) should be prepared before the inoculation (*see* **Notes 1–4**). The production medium is complete medium as described previously but supplemented with a further 5% FBS and 0.1% v/v CellPROTECT (*see* **Note 10**). The nutrient medium contains the same components as complete medium, with the exceptions of 5% FBS instead of 10% FBS and with the addition of 0.2% v/v AntiFOAMa (*see* **Note 11**). Both the nutrient media and the production media are prewarmed to 37°C to prevent expansion of the MiniPERM membrane when introduced into the incubator.

1. Detach the hybridoma cells by tapping and gentle pipetting from a 225-cm^2 tissue culture flask, wash by centrifugation at 300*g* for 10 min, and resuspend the resulting cell pellet in 5 mL of production medium.
2. Calculate the cell density by diluting 10 µL of suspension in 90 µL of nigrosin then count the cells using a haemocytometer. Adjust the density to 5×10^5 cells/mL in production medium and inoculate the reactor with 35 mL of this suspension using the following procedure (*see* **Note 12**).
3. Draw the suspension slowly up into a 50-mL hypodermic syringe (*see* **Note 13**). Unscrew the two Luer-Locks on the assembled MiniPERM and inject the cell suspension carefully into the production module while the module is slowly rotated (*see* **Note 14**). This ensures that displaced air is expelled through the open Luer-Lock.
4. Replace both Luer-Locks then add 400 mL of nutrient medium to the nutrient module. Close the cap and place the MiniPERM module inside an incubator on the Universal turning device set at 5 rpm (*see* **Note 15**).

3.2.3. Sampling and Harvesting

1. Remove 1-mL samples daily using a 2-mL syringe to assess the culture (*see* **Note 16**). To do this, put the MiniPERM on a stand and turn

it so that a Luer-Lock sampling port is at 12 o'clock position. Open the port and insert the 2-mL syringe (*see* **Note 17**). Rotate the device 120 degrees clockwise so that another port is at 12 o'clock and the sample port used for withdrawal is now at the 4 o'clock position.

2. Assess cell viability using nigrosin exclusion (*see* **Note 18**).
3. Harvesting is performed using the method described for sampling, with the exception that a larger quantity of medium is removed using a 50-mL syringe. Harvest 20 mL either when the cell viability falls below 50% or when the cell density exceeds 2×10^7 cells/mL. The remaining 15 mL of cell suspension allows for repopulation of the production module after the addition of 20 mL fresh production medium.
4. Change the medium in the nutrient module every 2 to 4 d (*see* **Note 19**). Proceed with harvesting and replacement of nutrient media in a cyclic manner.
5. After sampling and harvesting the Luer-Locks must be wiped down with 70% ethanol to prevent infection being introduced to the production module (*see* **Note 20**). Periodical measurement of cell density, viability, and antibody production during the course of the production run will help in the planning of future production runs.

3.3. Storage of Hybridomas

3.3.1. Cryopreservation

Prepare freezing mix (FM) in advance of the procedure. To do this, mix 10 mL of FBS with 8 mL of CM and 2 mL of dimethyl sulfoxide (DMSO) and store at 4°C (*see* **Note 21**).

1. Harvest the cells and supernatant from five 225-cm^2 flasks 1 d after being seeded (*see* **Note 22**) and pool the resulting cell suspension.
2. Remove 2 mL of this suspension and add to a 25-cm^2 flask. Supplement with 3 mL of fresh CM and place the flask in the incubator (*see* **Note 23**).
3. Centrifuge the remaining suspension at 300*g* for 10 min at 4°C. Retain the supernatant for antibody analysis. From this stage onwards all reagents should be used at 4°C. Recover the cell pellets, pool the cells, resuspend in 50 mL of CM, and wash by centrifugation at 300*g* as before.

4. Discard the wash supernatant and resuspend the cell pellet in 20 mL of fresh CM. Calculate the cell density by diluting 10 µL of suspension in 90 µL of nigrosin and counting the cells using a haemocytometer.
5. Centrifuge the cells as before then finally resuspend the pellet to a density of 5×10^6 viable cells/mL in FM and 1 mL added to each cryovial.
6. Place the vials were placed in a Nalgene freezing tub filled with isopropanol then place in a –70°C freezer overnight (*see* **Note 24**).
7. Transfer the vials to LN2 for long-term storage (*see* **Note 25**).

3.3.2. Resuscitation of Frozen Cells

1. Remove one vial from the cell bank in LN_2 after 24 h of storage and immediately thaw by immersion in a water bath at 37°C (*see* **Note 26**).
2. As the last of the ice disappears, transfer the suspension to a centrifuge tube (*see* **Note 27**). Slowly add 10 mL of CM at 4°C to the cells with gentle mixing and centrifuge the suspension at 300*g* for 10 min at 4°C.
3. Wash the cells by resuspending the pellet in 10 mL of CM at 4°C and then centrifuging as before.
4. Finally resuspend the cells in 1 mL of CM and count as described in **Subheading 3.3.1.**
5. Adjust the cell concentration to 1×10^6/mL in CM, add to a 25-cm^2 flask, and incubate as described in **Subheading 3.1.2.** (*see* **Note 28**).
6. Visually monitor the cells daily for growth and lack of contamination. Expansion of the cells can be taken as evidence of a viable cell bank and also that no microbial contamination of the cells occurred during the freezing/resuscitation process (*see* **Note 29**).

3.3.3. Management of a Cell Bank

Validated cell banks are essential for the long-term survival of hybridomas. Ideally, databases should be set up to manage the banks and to record information about the cells. An example of useful fields for a database is detailed in **Fig. 3**. One vial of cells should be resuscitated after 1 wk, 1 mo, 6 mo, and then annually and checked

Hybridoma identification code		
Specificity		
Isotype		
Cloning history		
Passage number		
Result of *Mycoplasma* screen		
Date frozen		
Cell density per vial		
Number of vials in bank		
	Date	**Viability**
Resuscitation (1 week)		
Resuscitation (1 month)		
Resuscitation (6 months)		
Resuscitation (1 year)		
Resuscitation (2 years)		
Resuscitation (3 years)		
Resuscitation (4 years)		
Resuscitation (5 years)		

Fig. 3. Recommended fields for the management of a hybridoma cell bank.

for viability after the procedure set out in **Subheading 3.3.2.** A new cell bank should be made if there is concern about the viability of the stock. It is recommended to have vials stored in more than one container to safeguard against loss as a result of mechanical failure of the storage system.

4. Notes

1. It is not desirable to routinely use antibiotics in tissue culture. Antibiotics may limit growth of contaminants without eradicating them, ultimately having a detrimental effect on the cells. Sound tissue culture technique conducted in suitable facilities with appropriate equipment is sufficient to maintain established cell lines. However, hybridomas are expensive to produce and are often unique, and therefore the inclusion of antibiotics in the early expansion stages acts as an added safeguard against contamination.
2. CM should be prepared at least 3 d before use to allow for sterility checking. CM can be stored for 2 wk at 4°C, after which time the components will deteriorate. Glutamine is notably labile, hence storage of the concentrated stock at –20°C. It is possible to purchase a formulation of glutamine supplement that is stable (Glutamax™, Gibco).
3. Selection of appropriate FBS is crucial for successful growth. Batches of FBS should be screened prior to selection for their ability to support hybridoma cloning and growth. Antibody content of FBS tends to be low, however batches of FBS may contain antibodies to ruminant pestiviruses and other transplacental pathogens. In the vast majority of cases this will not present a problem unless the hybridomas are producing MAbs to be used for pestivirus diagnosis or research. If the FBS contains pestivirus itself, this will create problems if the MAbs are used for research involving ruminant cells that can be infected by virus that then establishes a persistent infection and cannot be eradicated *(7)*.
4. Hybridomas vary in their growth characteristics and different lines will multiply at different rates. The expansion of hybridomas after they have been cloned requires careful monitoring to ensure that the cells have suitable growth conditions and an adequate supply of nutrients. Daily examination is desirable, with good note-keeping to record growth patterns. Cell populations are expanded by a gradual step-wise increase in the size of the culture vessel and volume of medium as the cell number increases. A change in the color of the medium from orange to pale yellow signifies a drop in pH as the cells metabolize. This is accompanied by an increase in cell density and indicates that the cells can be moved to a larger culture vessel with fresh medium. If the medium becomes bright yellow, the cells require urgent attention. CM should be warmed to 37°C before being

added to cells. If cells are at low density and/or showing signs of poor growth, CM can be temporarily supplemented with OPI solution to a final concentration of 2% to encourage cell growth.

5. Hybridomas vary in their rate of growth, ability to adhere to plastic, and to each other. It is important to avoid subjecting the cells to excessive mechanical stress such as vigorous pipetting or foaming of the medium that can cause damage and cell death when removing them from plastic or disrupting clumps.
6. Culture flasks with vented filter caps allow for gaseous exchange between the culture and the incubator while protecting the cells from airborne microbial contamination.
7. There are various methods for detecting *Mycoplasma* contamination of cell culture. A sensitive polymerase chain reaction test with broad specificity for *Mycoplasma* species is our method of choice ***(8)***. There are several products available for the eradication of *Mycoplasma* species from cell lines. The effectiveness of the treatment will depend on the cells and involves trail and error. This is because some cell lines are very sensitive to the chemicals used to eradicate *Mycoplasma* and may become static or die during treatment.
8. Once hybridomas cell lines have been cloned, they tend to have a stable phenotype. However, they do divide rapidly and can spontaneously change. Cells that do not produce antibody can arise from cloned populations, albeit at a low frequency. The growth properties of nonproducing cells may not be the same as that of the parent clone, in fact they are likely to outgrow the producing cells resulting in loss of the line. An important rule to remember is that different hybridoma lines should never be handled simultaneously in tissue culture. There is the risk of cross-contamination and if this does occur, it may not be immediately apparent if the lines are phenotypically similar (e.g. adherence properties). As with reverting clones, contamination may only become apparent when the supernatant is tested for MAb content. Hybridoma lines can be safeguarded by ensuring that cells are cryopreserved at an early stage after cloning (*see* **Subheading 3.3.**), by routine checking for MAb production and subcloning if required.
9. Preassembled sterile bioreactors are available from the manufacturer. With the classic kit, the production modules are sterile and can be used just once, the nutrient module needs to be assembled and sterilized.

10. Some hybridomas require a higher concentration of FBS, up to 15%. As the cells in the production module are subject to shearing forces owing to the rotation of the bioreactor, CellPROTECT can be used as an antishearing supplement.
11. Nutrient medium is supplemented with 5% fetal bovine serum. Low molecular weight serum proteins diffuse across the dialysis membrane between the nutrient and production modules. A reservoir of low molecular proteins is required in the nutrient module to maintain the equilibrium for hybridoma growth and survival. Accumulation of foam in the nutrient module can be a problem. To counteract foaming, do not exceed a concentration of 5% FBS in the nutrient module and add AntiFOAMa antifoaming agent. Do not fill the nutrient module with more than 400 mL of nutrient medium. An air space is required within the module to ensure successful hybridoma growth.
12. Hybridomas for inoculation into the MiniPERM bulk culture device should be greater than 90% viable and free from infection (mycoplasmas are a particular problem in high-density culture). Ideally, a concentration of 5×10^5 cells/mL is required for the 35-mL inoculum, although the density can be increased to 2×10^6 cells/mL if the culture fails to seed at the lower density.
13. Do not use a needle for loading the syringe because the shearing forces can damage the cells.
14. Work on the MiniPERM should be conducted promptly so that the cells do not settle and clump together impairing their viability. This is particularly important to bear in mind when high densities are reached because these populations will rapidly exhaust nutrients and oxygen when static, in addition the localized build up of metabolic products will have a toxic effect on the cells. A stand is available to aid this procedure.
15. Mouse hybridomas growing in the MiniPERM can be rotated at speeds of 5–20 rpm. Cells sensitive to shearing forces should be turned at lower rpm.
16. To maintain the optimum cell density, cell viability and maximize the antibody yield within the cell chamber it is necessary to sample the cell population at regular intervals. The frequency of harvesting is dependent on the growth properties of the hybridoma being cultured so sampling should initially be performed daily to assess the culture.
17. Pressure within the module can cause the cell-culture medium to spurt out when the Luer-Locks are opened. If the rubber membrane

of the production module is distended stand the MiniPERM upright and release the cap of the nutrient module and gently push the membrane into a flat position.

18. Cells often take a time to adjust to the bioreactor and a small initial drop in viability is normal. If the cells are not growing and the viability has dropped below 50%, and contamination has been ruled out, there are several options to try: 1) re-seed the bioreactor at a higher cell density; 2) adjust the rolling speed; 3) increase the serum concentration in the nutrient module; 4) add OPI at 1/50 to promote cell growth.
19. Low-density cultures (populations that reach 5×10^6 cells/mL) will require fresh nutrient media less frequently than high-density (2×10^7 cells/mL) cultures. A change in the color of the medium from orange to yellow indicates that the medium requires to be changed.
20. Replacement sterile caps for the nutrient module Luer-Locks can be used to reduce the chances of contamination.
21. The components of FM (FBS, CM, DMSO) are used in the ratio of 5:4:1. It is preferable to prepare fresh FM for each cell bank and chill to 4°C. DMSO can be purchased in 10-mL units. Once a vial has been opened, any unused DMSO can be stored at 4°C in a sealed container. Undiluted DMSO crystallizes at 4°C, so it is recommended to store aliquots in suitable working volumes to avoid repeat thawing of the stock.
22. Cells are in optimal condition for cryopreservation when they are in log-phase growth. This occurs when the cells are at low-to-medium density in CM that is not exhausted of nutrients.
23. It is advisable to maintain a small culture of cells until the viability of the cell bank is validated. This ensures survival of the line should the freezing procedure fail.
24. To avoid cell damage as a result of the rapid formation of ice crystals a freezing rate of 1°C per minute is optimal. If Nalgene tubs are not available, vials can be wrapped loosely in cotton wool to achieve a similar effect, but this has a more variable success rate.
25. Appropriate safety procedures must always be followed when dealing with LN2 storage. Cells can be stored in liquid phase or vapour phase, or in ultracool freezers. Prevention of ice-crystal formation is essential for successful long-term storage and the vials should be protected from temperature fluctuations wherever possible. Temperature fluctuations can damage cells when frozen, even if they do not result in the contents of the vial thawing out.

26. Cells are vulnerable in the transition period from freezing to resuscitation. Rapid thawing in a water bath is advisable to limit cell damage. The vial should never be submerged to the level of the seal on the cap, otherwise contamination will occur.
27. It is important not to allow the cells to remain in the water bath since they need to be washed free of freezing mix as soon as possible after they have thawed.
28. It is desirable to have a cell bank with containing a high percentage of viable cells upon thawing. If the viability is less than 70%, another cell bank can be produced from the cells that have been kept in culture (**Subheading 3.3.1.**, *see* **Note 10**). Alternatively, low viability cell populations can be resuscitated into culture plates at a concentration of 1×10^6 total cells/mL, allowed to settle for 24 h, and the CM changed to remove detrimental intracellular components released by dead cells that can inhibit the growth of the surviving cells.
29. If microbial contamination is observed, another vial should be resuscitated to confirm that the contamination is not unique to one vial and may be something affecting the entire bank, in which case the bank should be discarded.

Acknowledgments

The authors thank Joachim Lücke for permission to reproduce the miniPERM images. This work was supported by Scottish Executive Environment and Rural Affairs Department.

References

1 Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256,** 495–497.
2 Milstein, C. (2000) With the benefit of hindsight. *Immunology Today* **21,** 359–364.
3 Mackay, C. R., Hein, W. R., Brown, M. H., and Matzinger, P. (1988) Unusual expression of CD2 in sheep: implications for T cell interactions. *Eur. J. Immunol.* **18,** 1681–1688.
4 Dutia, B. M., Entrican, G., and Nettleton, P. F. (1990) Cytopathic and noncytopathic biotypes of border disease virus induce polypeptides of different molecular weight with common antigenic determinants. *J. Gen. Virol.* **71,** 1227–1232.

5 Dutia, B. M., Ross, A. J., and Hopkins, J. (1993) Comparison of workshop CD45R monoclonal antibodies with OvCD45R monoclonal antibodies in sheep. *Vet. Immunol. Immunopathol.* **39,** 121–128.
6. Entrican, G., Deane, D., Maclean, M., Inglis, L., Thompson, J., McInnes, C., et al. (1996) Development of a sandwich ELISA for ovine granulocyte macrophage-colony stimulating factor. *Vet. Immunol. Immunopathol.* **50,** 287–298.
7. Nettleton, P. F. and Entrican, G. (1995) The ruminant pestiviruses—a review. *Br. Vet. J.* **151,** 615–642.
8. Pruckler, J. M., Pruckler, J. M., and Ades, E. W (1995) Detection by polymerase chain reaction of all common *Mycoplasma* in a cell culture facility. *Pathobiology* **63,** 9–11.

6

Phage Display Vectors for the In Vitro Generation of Human Antibody Fragments

Michael Hust and Stefan Dübel

Summary

A major source of human antibodies are phage display libraries, which are constructed from various genetic sources. Antibodies are expressed as scFV and Fab antibody fragments using various vector systems. This review offers a comprehensive overview of M13 phage display antibody vectors and discusses their applications.

Key Words: Antibody (Ab); fragment antigen binding (Fab); fragment variable (Fv); single-chain Fv (scFv); variable part of heavy chain; variable part of light chain; wild type.

1. Introduction

The production of polyclonal antibodies by the immunization of animals is a method that has been used for more than a century ***(1)***. Hybridoma technology was the next development, allowing the production of monoclonal antibodies ***(2)***. However, hybridoma technology has some limitations, some resulting from the instability of the aneuploid cell lines, but most of all, difficulties in producing human antibodies especially to toxic or highly conserved antigens ***(3)***.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

In the past decade, the in vitro selection of antibodies from recombinant antibody repertoires has proven to bypass these limitations. In vivo libraries offer the capability of generating human antibodies in transgenic mice carrying the human IgG loci. Here, antigens are injected into the humanized mouse, followed by conventional hybridoma technology for selection and production of antibodies ***(4–6)***.

To be able to raise human antibodies against toxic, highly pathogenic, or nonimmunogenic antigens, the method of choice is the use of libraries and an in vitro selection process. The first antibody repertoires were generated and screened in phage Lambda ***(7,8)*** with limited success. The phage display method most commonly used today is based on the groundbreaking work of Smith ***(9)***. Here, the genotype and phenotype of a polypeptide are linked by fusing short gene fragments to the minor coat protein III gene of the filamentous bacteriophage M13. This results in the expression of this fusion protein on the surface of phage and allows affinity purification of the gene by the peptide binding. Shortly after this breakthrough, another method was developed in which antibodies were presented on the surface of M13, fused to pIII ***(10–15)***. By uncoupling antibody gene replication and expression from the phage life cycle by locating them on a separate plasmid (phagemid), genetic stability, propagation, and screening of antibody libraries was greatly improved ***(11,12,14,15)***. To date, "single-pot" antibody libraries with a theoretical diversity of up to 10^{11} independent clones have been assembled ***(16)*** to serve as molecular repertoires for phage display selections. An overview of antibody libraries is given by Hust and Dübel ***(17)***.

The novel way of isolating antibodies by their binding activity in vitro is called "panning," which refers to the method used by gold prospectors ***(18)***. The antigen is immobilized to a solid surface, such as nitrocellulose ***(19)***, magnetic beads ***(20)***, a column matrix ***(12)*** or, the most widely used, plastic surfaces as polystyrole tubes ***(21)*** or 96-well plates ***(11)***. The antibody phage are incubated with the

surface-bound antigen, followed by thorough washing to remove the excess nonbinders. The bound antibody phage can subsequently be eluted and amplified by infection of *Escherichia coli*. This method allows the detection of a single antibody phage by panning, and as it can be selected by its resistance marker, it can give rise to a bacterial colony after elution. This illustrates the tremendous sensitivity of the method. This selection cycle can be repeated, by infecting the resulting *E. coli* colonies with a helper phage to produce new antibody phage, which can then be used for a new round of panning, and so on. The number of antigen specific antibodies will increase with every panning round. Usually two to six panning rounds are necessary to get specifically binding antibodies.

Other display techniques have been developed and successfully applied for antibody selection. Techniques requiring intracellular antibody expression, such as the two-hybrid system ***(22)***, are of restricted use because of the limited folding of antibody fragments in the reducing milieu of the cytoplasm ***(23)***. Bacterial surface display techniques were developed, which rely on fusing antibodies to peptidoglycan-associated lipoprotein ***(24,25)***. Recently, fusions to lipoprotein NlpA have been used ***(26)***, but a universal method has not yet been developed. To display antibodies on yeast, antibodies were fused with the Aga2p mating adhesion receptor embedded in the cell wall ***(27)***. An extravagant technique completely avoiding the use of living cells in the process uses the linkage of messenger ribonucleic acid (mRNA) to the translation product (protein). In ribosomal display, mRNA and the related protein (antibody) complexed to the ribosome are selected by antigen binding of the attached antibody fragment. Then, the antibody mRNA is amplified by reverse transcription polymerase chain reaction and a further round of selection can be started ***(28,29)***. A related concept is the puromycin linked display ("profusion"). The 3' end of the mRNA is fused to puromycin, which serves as a peptidyl acceptor. After translation, the resultant polypeptide is covalently linked to mRNA by the puromycin ***(30)***, allowing selection as previously described.

2. Phage Display

Because of its robustness and ease of use, phage display has been the method most widely used in the past decade. This review therefore focuses on libraries using this selection principle. For an overview of available strategies and protocols, *see* McCafferty et al. ***(31)*** and Kontermann and Dübel ***(32)***. Display systems that require the insertion of antibody genes into the phage genome have been developed for phage T7 ***(33)***, phage Lambda ***(7,34,35)***, and the Ff class (genus *inovirus*) of the filamentous phage f1, fd, and M13 ***(10)***. Being well established for peptide display, the phage T7 is not well suited for antibody phage display because protein assembly takes place in the reducing milieu of the cytoplasm, thus leaving most antibodies unfolded ***(33)***. In contrast, the oxidizing milieu of the bacterial periplasm allows antigen binding fragments of antibody heavy and light chain to be folded and assembled properly ***(36)***. The Ff class nonlytic bacteriophage are assembled in this cell compartment and allow the production of phage without killing the host cell. This is a major advantage compared with the lytic Lambda phage ***(7)***. In addition, filamentous phage allow the production of soluble proteins by introducing an amber stop codon between the antibody gene and gene III. In an *E. coli* supE suppressor strain, the fusion proteins will be produced, whereas soluble antibodies are made in a nonsuppressor strain ***(37,38)***. As a result the members of the Ff class are the phage of choice for antibody phage display.

To achieve surface display, five of the M13 coat proteins have been used in fusion to foreign protein fragments. In the most widespread system the antibody is coupled to the N-terminus or second domain of the minor coat protein pIII ***(11,12,14)***. The naive function of the three to five copies of the pIII, in particular their N-terminal domain, is to provide binding of the phage to the f-pili of *E. coli* to initiate infection ***(39)***. The major coat protein (pVIII) has been used as an alternative fusion partner, with only very few successes reported in the past decade ***(40)***. This fusion technique is more useful for the display of short peptides ***(41,42)***. Fusions to pVI have been tried, but not yet with antibodies ***(43)***. pVII and pIX were

used in combination by using the variable part of light chain domain to pIX and the variable part of heavy chain domain to pVII, which allowed the expression of a fragment variable (Fv) on the phage surface. This particular method offers the potential for heterodimeric display ***(44)***. However, the fusion with pIII remains the most widely used system for phage display and is the only system of practical relevance so far.

Two different systems have been developed for the expression of the antibody/pIII fusion proteins. First, the fusion gene can be inserted directly into the phage genome substituting the wildtype pIII ***(10)***. Second, the fusion gene can be provided on a separate plasmid with an autonomous replication signal, promoter, resistence marker and phage morphogenetic signal, allowing this "phagemid" to be packaged into assembled phage particles. A helper phage, usually M13K07, is necessary for the production of the antibody phage to complement the phage genes not encoded on the plasmid. Because of its mutant origin, the M13K07 helper phage genome is not efficiently packaged during antibody phage assembly when compared with the phagemid ***(45)***, thus increasing the selection of the phagemid of interest during panning.

What are the practical differences of the vectors described so far? In the system using direct insertion into the phage genome, every pIII protein on a phage carries an antibody fragment. This is of a particular advantage in the first round of panning, where the desired binder is diluted by millions of phage with unwanted specificity. The oligovalency of these phage improves the chances of a specific binder being enriched because of the improved binding provided by the avidity effect. This advantage, however, has to be weighed against a number of disadvantages. The transformation efficiency of phagemids is two to three orders of magnitude better than phage vectors, thus facilitating the generation of large libraries in phagemids. Second, the additional protein domains fused to pIII may reduce the function of pIII during reinfection. In a phagemid system, the vast majority of the pIII assembled into phage are wildtype (wt) proteins, thus providing normal pilus binding. This may explain why only two "single-pot" antibody libraries ***(38,46)*** have

ever been made using phage vectors. In phagemid systems, by contrast, both replication and foreign fusion protein expression are independent from the phage genome. Because the propagation of the phagemid occurs in the absence of helper phage, there is no selection pressure from this end. The fusion protein can be produced in adjustable quantities by the use of the amber stop/suppressor system for switching expression to antibody (Ab) fragments without a pIII domain Finally, despite not usually being derived from highest copy plasmids, the dsDNA of phagemids is more easy to handle than phage DNA, facilitating both cloning and analysis. Therefore, most pIII display systems use the phagemid approach. There is, however, a disadvantage that originates from the two independent sources for the pIII during phage packaging. During assembly, the wt pIII of the phage are inserted into the phage particles with much higher rate than the pIII fusion protein. As a result, the vast majority of resulting phage particles carry no antibody fragments at all. The few antibody phages in these mixtures are therefore mainly monovalent, with phage carring two or more antibodies being extremely rare. This allows the selection of antibodies with high monovalent affinity, since avidity effects which would decrease the dissociation rate from the panning antigen can be avoided. In the first panning round, however, when a few binders have to be fished out of a huge excess of unwanted phage, the fact that only a few percent of the phage carry antibodies hampers the efficiency of the system ***(11,12,14,46–48)***. Recently, this problem has been solved by using a helper phage (Hyperphage), which avoids the need to introduce wt pIII into the packaging process, thus leaving the phagemid as the sole source of pIII and therefore offering multivalent display for phagemid vectors as well. This method improves Ab display by two orders of magnitude and vastly improves panning efficiency ***(49)***. Multivalent display has however been achieved by integrating two amber stop codons into the gIII gene of the helper phage genome. This allows the production of a functional helper phage (ex-phage) in an *E. coli* suppressor strain. In the associated phagemid pIGT3, the Ab/pIII fusion occurs without an amber stop codon and the Ab phage is produced in an *E. coli* nonsuppressor strain ***(50)***. However,

the deletion of the amber stop codon in the phagemid makes it imperative that the antibody gene is subcloned or that a protease is used to produce soluble antibodies. This is in contrast to the Hyperphage system where the amber stop/supressor system can be used for switching expression to Ab fragments without a pIII domain.

An elegant application of phage display is selective infective phage technology. Here, antibodies are fused to the C-terminal domain of pIII by cloning into the phage genome; therefore, every pIII carries an antibody and deletion of the pIII aminoterminal region makes the phage noninfective. Antigen is then fused to the C-terminal end of separately produced soluble pIII N-terminal domain. The functional, f-pili binding pIII is reconstituted when the antibody phage binds to the antigen, allowing only the correct antibody phage to infect *E. coli* and be propagated ***(51)***. However, because of the fast kinetics of pIII/pilin interactions and very low concentrations of the three reaction partners if not co-expressed in the same cell, this method does not lend itself to the rapid panning of larger libraries.

3. Phage Display Vectors

A large number of different phage display vectors have been constructed. With pretending to be complete, **Table 1** lists a selection of phage display vectors. Some of them have not been used for the construction of a library up to now but have been included because they offer possible alternatives. For example, one of the systems allows the success of antibody gene cloning to be monitored by the expression of green fluorescent protein ***(52)***.

A variety of different promoters have been used for the expression of antibody genes. Widely used is the lacZ promoter (lacZ) derived from the lactose operon ***(53)***. The gIII promoter (gIII) from the bacteriophage M13 ***(9)***, the tetracycline promoter (1X $tet^{o/p}$; **ref. *54***) and the phoA promotor of the *E. coli* alkaline phosphatase ***(47)*** also have been used successfully. It appears that very strong promoters, for example, the synthetic promoter PAI/04/03 ***(55)***, are

Table 1
Phage Display Vectors for Cloning of Antibody Genes in Alphabetical Order

Phage display vector	Promoter	Secretion	Antibody format used by reference	C-domains in vector	Sites heavy chain	Sites light chain	Tags	gIII	Expression of soluble Ab	Reference
pAALFab	1x lac Z, 2x RBS	2x pelB	Fab	no	*EcoR*I – *BstP*I	*Spe*I - *Xho*I	-	truncated	subcloning	77
pAALFv	1x lac Z, 2x RBS	2x pelB	Fv	no	*EcoR*I – *BstP*I	*Spe*I - *Xho*I	-	truncated	subcloning	
pAALSC	1x lac Z, 1x RBS	1x pelB	scFv	no	*EcoR*I – *BstP*I	*Spe*I - *Xho*I	-	truncated	subcloning	
pAK100	1x lac Z, 1x RBS	1x pelB	scFv	no	*Sfi*I (tet resistance will be removed)		FLAG, myc	truncated	amber, supE strain	78
pAPIII$_e$ scFv	1xphoA, 1x RBS	1x OmpA	scFv	no	*Hind*III – *Sal*I		FLAG, His	truncated	*Sal*I – KI digest, deletion of gIII	79
pCANTAB3his	1x lac Z, 1x RBS	1x g3p	scFv	no	*Nco*I/*Sfi*I – *Not*I		His, myc	full	amber, supE strain	59
pCANTAB5his/ pCANTAB 6	1x lac Z, 1x RBS	1x cat	scFv	no	*Nco*I/*Sfi*I – *Not*I		His, myc	full	amber, supE strain	
pCANTAB 5 E	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Not*I		E tag	full	amber, supE strain	www.amershambiosciences.com
pCES1	1x lac Z, 2x RBS	1x gIII (L) 1x pelB (H)	Fab	yes	*Sfi*I – *Pst*I/*BstE*II (VH)	*ApaL*1 – *Asc*I (L chain), *ApaL*1 – *Xho*I (VL)	His, myc	full	amber, supE strain	73
pComb3	2x lac Z, 2x RBS	2x pelB	Fab	no	*Xho*I – *Spe*I	*Sac*I - *Xba*I	-	truncated	*Nhe*I – *Spe*I digest, deletion of gIII	11
pComb3H	1x lac Z, 2 x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	*Xho*I – *Spe*I	*Sac*I - *Xba*I	-	truncated	*Nhe*I – *Spe*I digest, deletion of gIII	80
pComb3X	1x lac Z, 2 x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	*Xho*I – *Spe*I	*Sac*I - *Xba*I	His, HA	truncated	amber, supE strain	
pCW93/H, pCW99/L[1]	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Nhe*I	*Sac*I – *Bgl*II	myc	truncated	amber, supE strain	81
pDAN5	1x lac Z, 1x RBS	undiscribed leader	scFv	no	*Xho*I – *Nhe*I	*BssH*II – *Sal*I	SV5, his	full	amber, supE strain	16
pDH188	2x phoA, 2x RBS	2x stII	Fab	no	n.d.	n.d.	-	truncated	subcloning	47
pDN322	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Not*I		FLAG, His	full	amber, supE strain	82
pDNEK	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Not*I		FLAG, His	full	amber, supE strain	83
pEXmide3	1x lac Z, 2x RBS	2x pelB	Fab	yes	*Sfi*I/*Nco*I – *Kpn*I/*Apa*I	*Eag*I/*Not*I – *Nhe*I/*Spe*I	-	full	amber, supE strain	84
pEXmide4	1x lac Z, 1x RBS	1x pelB	scFv	CH1	*Nco*I – *Sal*I		-	full	amber, supE strain	85
pEXmide5	1x lac Z, 1x RBS	1x pelB	scFv	?	*Nco*I – *Sal*I		-	full	amber, supE strain	86
pFAB4	2x lac Z, 2x RBS	2x pelB	Fab	no	*Sfi*II – *Not*I		-	truncated	amber, supE strain	87
pFAB4H	1x lac Z, 2x RBS	2x pelB	Fab	CH1	*Sfi*II – *Not*I		-	truncated	subcloning	88

pFAB5c	2x lac Z, 2x RBS	2x pelB	Fab	no	*Sfi*I – *Not*I		-	truncated	amber, supE strain	87
pFAB5c-His	1x lac Z, 2x RBS	2x pelB	scFv	no	*Sfi*I – *Not*I		his	truncated	amber, supE strain	89
pFAB60	1x lac Z, 1x RBS	2x pelB	Fab	CH1	*Sfi*I – *Spe*I (VH) *Sfi*I – *Not*I (Fd)	*Nhe*I – *Asc*I (L chain)	his	truncated	*Eag*I digest, deletion of gIII	90
pFAB73H	1x lac Z, 1x RBS	2x pelB	Fab	CH1	*Nhe*I – *Apa*I (VH)	*Sfi*I – *Asc*I (L chain)	his	truncated	*Eag*I digest, deletion of gIII	91
pGP-F100	1x tet^{op}, 1x RBS	1x pelB	scFv	no	*Sfi*I (GFPuv will be removed)		myc	truncated	TEV protease site	52
pGZ1	1x tet^{op}, 1x RBS	1x pelB	scFv	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	54
pHEN1	1x lac Z, 1x RBS	1x pelB	scFv, Fab, Fd, LC	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	14
pHEN1-Vλ3	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Xho*I	Vλ3 anti-BSA Ab chain	myc	full	amber, supE strain	92
pHEN2	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Xho*I	*ApaL*I – *Not*I	his, myc	full	amber, supE strain	http://www.mrc-cpe.cam.ac.uk
pHENIX	1x lac Z, 1x RBS	1x pelB	scFv	no	*Sfi*I/*Nco*I – *Sal*I/*Xho*I	*ApaL*1 – *Not*I	myc	full	amber, supE strain	93
pHG-1m/A27Jκ1	1x lac Z, 1x RBS	1x pelB	scFv	no	*ApaL*I – *Sfi*I	A27Jκ1 (VL)	his, myc	full	amber, supE strain	94
phh3mu-γ1	2x lac Z, 2x RBS	2x pelB	Fab bidirectional	yes	*Xho*I – *EcoR*I	*Sac*I – *Hind*III	-	truncated	subcloning	95
pIG10	1x lac Z, 1x RBS	1x OmpA	scFv	no	*EcoR*V – *EcoR*I		myc	full	amber, supE strain	96
pIGT2 (vector)	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	50
pIGT3 (vector)	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Sfi*I		myc	full	subcloning	
pIT2	1x lac Z, 1x RBS	1x pelB	scFv	no	SfiI/*Nco*I – *Xho*I	*Sal*I – *Not*I	His, myc	full	amber, supE strain	97
pLG18	1x phoA, 2x RBS	2x stII	Fab	yes	*Bss*HII – *Nco*I (CDR2-3)	*Bst*EII – *Asp*718 (CDR1-3)	-	truncated	subcloning	98
pM834, pM827 [1]	2x lac Z, 2x RBS	2x pelB	Fab	no	*Xho*I – *Spe*I	*Sac*I – *Xba*I	-	full	amber, supE strain	99
pMorph series [2]	1x lac Z ?	1x phoA	scFv	no	*Xba*I - *EcoR*I		FLAG ?	?	subcloning	100
pScUAGΔcp3	1x lac Z, 1x RBS	1x pelB	scFv with Cκ	Cκ	*Xho*I – *Nhe*I	*Sst*I – *Bgl*II	-	truncated	amber, supE strain	101
pSEX	1x PA1/04/03 1x RBS	1x pelB	scFv	no	-		Yol1/34	full	subcloning	12
pSEX20	1x PA1/04/03 1x RBS	1x pelB	scFv	no	-		Yol1/34	full	subcloning	75
pSEX81	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Hind*III	*Mlu*I – *Not*I	Yol1/34	full	subcloning	74

[1]Cre/lox recombination. [2]λ recombination. [3]Construction of the HuCAL library is described, but the pMorph vectorsystem is unpublished.

rather a disadvantage. To our knowledge, a systematic comparison of the different promotors has not been conducted to date.

The targeting of antibodies to the periplasm requires the use of signal peptides. The pelB leader of the pectate lyase gene of *Erwinia carotovora* ***(56)*** is commonly used. The gIII leader ***(9)***, the phoA leader of the *E. coli* alkaline phosphatase, and the ompA leader of *E. coli* outer membrane protein OmpA have also been used, being common to many protein expression vectors ***(57,58)***. Further examples are the heat-stable enterotoxin II (stII) signal sequence ***(47)*** and the bacterial chloramphenicol acetyltransferase (cat) leader ***(59)***.

Because of the general inability of *E. coli* to assemble complete IgG, with only one exception documented ***(60)***, smaller antibody fragments are used for display. In particular, fragment antigen binding (Fabs) and single-chain Fvs (scFvs) have been shown to be the antibody fragments of choice. In Fabs, the fd fragment and light chain are connected by a disulfide bond. In scFvs, the V_H and V_L are connected by a 15–25 amino acid linker ***(61–63)***. Soluble scFvs tends to form dimers, in particular when the linker is reduced to 3 to 12 amino acid residues. Without or with only a few amino acid linker, diabodies or tetrabodies can be found, with high variability in the behaviour of different V regions (**refs. *64–66***, Schmiedl and Dübel, unpublished). The dimerization can cause problems with binding because of possible avidity effects of the antibody complex ***(67)***. Furthermore, some scFvs have a reduced affinity of up to one order of magnitude compared with Fabs ***(62)***, only in rare cases, scFvs with a higher affinity than the associated Fab have been found ***(68)***. Small antibody fragments like Fv and scFv can easily be produced in *E. coli*. The yield of functional Fvs expressed in *E. coli* is higher than the yield of the corresponding Fabs, because of a lower folding rate of the Fabs ***(69,70)***. In one example, the stability in long-term storage was much higher for Fabs than for scFvs. After 6 mo, the functionality of scFvs stored at 4°C was reduced by 50%; Fabs, however, showed no significant loss of functionality after 1 yr ***(71)***. The overall yield of Fvs expressed in *E. coli* vary from 0.5 to 10 mg/L culture compared with 2 to 5 mg Fabs/L culture ***(72)***.

Therefore, the choice of the antibody fragment, scFv or Fab, depends on the desired application.

For the expression of Fabs in *E. coli*, two polypeptide chains have to be made. To achieve this, there are two strategies. In the monocistronic systems, for example, pComb3, the antibody genes are under control of two promoters and each has its own leader peptide ***(11)***. In plasmids like pCES1 with a bicistronic Fab operon, both chains are under control of a single promoter, leading to mRNA with two ribosomal binding sites ***(73)***.

Two variants of pIII-fusions to antibody fragments have been made. Either full-size pIII or a truncated version of pIII have been used. The truncated version is made by deleting the pIII N-terminal domain. This domain mediates the binding to the f-pili of *E. coli*. Infection is provided by wt pIII because only a small percentage of phage in phagemid-based systems are carrying an antibody. These truncated vectors are therefore not compatible for use with either Hyperphage or Ex-phage, as the full-size pIII is necessary to provide infections ***(49,50)***. Some vectors, for example, pSEX81 ***(74)***, allow an elution during panning by protease digestion instead of pH shift. This is possible because of a protease cleavage sequence between pIII and scFv and allows a complete recovery of bound phage for infection, even in case of very strong antigen binding.

Most of the described phagemids have an amber stop codon between the antibody gene and gIII. This allows the production of soluble antibodies after transformation of the phagemid to a non suppressor bacterial strain like HB2151 ***(38)***. For phagemids like pComb, it is necessary to delete the gIII by digestion followed by religation of the vector before tranformation into *E. coli* ***(11)***. In the case of vectors like pSEX81, the selected antibody genes have to be subcloned into a separate expression vector like the pOPE series ***(12,75,76)***.

4. Outlook

The vast variety of available methodologies and constructs have not been systematically compared for their efficiency. This is mainly

Table 2
Human Single-Pot Phage Display Libraries

Library vector	Library type	Antibody type	Library cloning strategy	Library size	Reference
fdDOG-2lox,pUC19-2lox	Semisynthetic	Fab	PCR with random CDR3 Primers, Cre-lox	6.5×10^{10}	***38***
fdTet	Naive	scFv	Recloning of a naive library[1]	5×10^{8}	***46***
pAALFab	Semisynthetic (anti-hen egg white lysozyme Ab framework)	Fab	PCR with random CDR Primers, assembly PCR	2×10^{8}	***77***
pAP-III_6 scFv	Naive	scFv	Assembly PCR	n.d.	***79***
pCANTAB 6	Naive	scFv	Assembly PCR	1.4×10^{10}	***102***
pCES1	Naive	Fab	Three-step cloning (Lchain, VH)	3.7×10^{10}	***73***
pComb3	Semisynthetic (anti-tetanus Ab framework[2])	Fab	PCR with random CDR H3 Primers	5×10^{7}	***103***
pComb3	Semisynthetic (anti-tetanus Ab framework[2])	Fab	PCR with random CDR H3 Primers	$>10^{8}$	***104***
pDAN5	Naive	scFv	Cre-lox	3×10^{11}	***16***
pDN322	Semisynthetic (VH DP47 and VL DPK22 V-genes)	scFv	Random CDR3 Primer, assembly PCR	3×10^{8}	***82***

pDNEK (ETH2 library)	Semisynthetic (VH DP47, Vl DPL16 and Vκ DPK 22 V-genes)	scFv	Random CDR3 Primer, assembly PCR	5×10^8	***83***
Semisynthetic (germline pEXmide5	VH-DP47 and VL-DPL3 framework)	scFv	Assembly PCR, CDR shuffling	9×10^6	***86***
pFAB5c-His (n-CoDeR library)	Semisynthetic (germline VH-DP47 and VL-DPL3 framework)	scFv	Assembly PCR, Assembly PCR CDR shuffling	2×10^9	***89***
pHEN1	Naive	scFv	Assembly PCR	$10^7 \times 10^8$	***15***
pHEN1	Naive	scFv	Assembly PCR	$2 \times 10^5 / 2 \times 10^6$	***37***
pHEN1	Naive	scFv	Assembly PCR	6.7×10^9	***106***
pHEN1-Vλ3	Semisynthetic (Vλ3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 Primers	10^7	***92***
pHEN1-Vλ3	Semisynthetic (Vλ3 anti-BSA Ab light chain)	scFV	PCR with random CDR H3 Primers	$>10^8$	***107***
pHEN1-Vκ3	Semisynthetic (VH)/ naive (VL)	scFv	Three-step cloning, PCR with random CDR H3 Primers	3.6×10^8	***108***
pHEN2 (Griffin 1 library)	Semisynthetic	scFv	Recloning of the lox library in scFv format[3]	1.2×10^9	www.mrc-cpe.cam.ac.uk

(continued)

Table 2 (*Continued*)
Human Single-Pot Phage Display Libraries

Library vector	Library type	Antibody type	Library cloning strategy	Library size	Reference
pIT2 (Tom I/ J library)	Semisynthetic (3x VH and 4x Vκ V-genes)	scFv	PCR with random CDR2 and CDR3 Primers	1.47×10^8 /1.37×10^8	***97***
pLG18	Semisynthetic (anti-HER2 Ab framework)	Fab	PCR with random CDR Primers, 2 step cloning	$2–3 \times 10^8$	***98***
pMorph series (HuCAL library)	Synthetic	scFv	Two-step cloning + 2 step CDR3 replacement	2×10^9	***100***
pScUAGDcp3	Semisynthetic	scFv connected to Ck	Three-step cloning with random CDR3 Primers	1.7×10^7	***101***
pSEX81	Naive	scFv (with N-terminus of CH1 and CL)	Two-step cloning	4×10^7	***109***
pSEX81	Naive	scFv (with N-terminus of CH1 and CL)	Two-step cloning	4×10^9	***110***
pSEX81	Naive	scFv (with N-terminus of CH1 and CL)	Four-step cloning	1.6×10^7/1.8×10^7/4×10^7	***111***

[1](106), [2](8), [3](38).

owing to the fact that experimental strategies allowing evaluation of the performance of any given vector other than using it for library construction and panning, is hard to formulate. The proof of the pudding is in the eating so, we have to analyze the resulting libraries produced by the various methods. However, this is not an easy task because different methods are used for each type of panning. When comparing successfully used human "single-pot" libraries (**Table 2**), it is evident that most of them use scFv fragments, but this is because the construction of scFv phage display vectors is less complicated compared to Fab phage display libraries. More fine tuning is necessary when expressing two chains in the case of Fab fragments, because the ratio of light chain to heavy chain must be harmonized, as in the case of scFv fragments.

Various leader sequences, promoters, and cloning strategies have been successfully used, with none of them providing a perfect construct. In conclusion, there are many different ways to success with many factors that can be altered depending on individual applications. Two factors are probably more important than the others and these are the careful control of phage biology during the panning process and secondly the use of an initial antibody library as large as possible.

References

1 Von Behring, E. and Kitasato, S. (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. *Deutsche Medizinische Wochenzeitschrift* **16,** 1113–1114.

2. Köhler, G. and Milstein, C. (1975) Continous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256,** 495–497.

3 Winter, G. and Milstein, C. (1991) Man-made antibodies. *Nature* **349,** 293–299.

4 Jakobovits, A. (1995) Production of fully human antibodies by transgenic mice. *Curr. Opin. Biotechnol.* **6,** 561–566.

5 Lonberg, N. and Huszar, D. (1995) Human antibodies from transgenic mice. *Int. Rev. Immunol.* **13,** 65–93.

6 Fishwild, D. M., O'Donnel, S. L., Bengoechea, T., Hudson, D. V., Harding, F., Bernhar, S. L., et al. (1996) High-avidity human IgG

kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat. Biotech.* **14,** 845–851.

7 Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., et al. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246,** 1275–1281.

8 Persson, M. A. A., Caothien, R. H., and Burton, D. R. (1991) Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc. Natl. Acad. Sci. USA* **88,** 2432–2436.

9 Smith, G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228,** 1315–1317.

10 McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990) Phage antibodies: filamentous phage displaying antibody variable domain. *Nature* **348,** 552–554.

11. Barbas III, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phages surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* **88,** 7987–7982.

12 Breitling, F., Dübel, S., Seehaus, T., Kleewinghaus, I., and Little, M. (1991) A surface expression vector for antibody screening. *Gene* **104,** 1047–1153.

13 Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* **352,** 624–628.

14 Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl. Acids Res.* **19,** 4133–4137.

15 Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. (1991) By-passing immunization: human antibodies from V-gene libraries diplayed on phage. *J. Mol. Biol.* **222,** 581–597.

16 Sblattero, D. and Bradbury, A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat. Biotech.* **18,** 75–80.

17 Hust, M. and Dübel, S. (2004) Mating antibody phage display to proteomics. *Trends Biotechnol.* **22,** 8–14.

18 Parmley, S. F. and Smith, G. P. (1988) Antibody selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* **73,** 305–318.

19 Hawlisch, H., Müller, M., Frank, R., Bautsch, W., Klos, A., and Köhl, J. (2001) Sitespecific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. *Anal. Biochem.* **293,** 142–145.

20 Moghaddam, A., Borgen, T., Stacy, J., Kausmally,L., Simonsen, B., Marvik, O. J., et al. (2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. *J. Immunol. Meth.* **280,** 139–155.

21 Hust, M., Maiss, E., Jacobsen, H.-J., and Reinard, T. (2002) The production of a genus specific recombinant antibody (scFv) using a recombinant Potyvirus protease. *J. Virol. Meth.* **106,** 225–233.

22 Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H., and Cattaneo, A. (1999) Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proc. Natl. Acad. Sci. USA* **96,** 11,723–11,728.

23 Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P., and Cattaneo, A. (1995) Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Bio/Technology* **13,** 1110–1115.

24 Fuchs, P., Breitling, F., Dübel, S., Seehaus, T., and Little, M. (1991) Targeting recombinant antibodies to the surface of E. coli: fusion to a peptidoglycan associated lipoprotein. *Bio/Technology* **9,** 1369–1372.

25 Fuchs, P., Weichel, W., Dübel, S., Breitling, F., and Little, M. (1996) Specific selection of E. coli expressing functional cell-wall bound antibody fragments by FACS. *Immunotechnology* **2,** 97–102.

26. Harvey, B. R., Rogers, G. K., Iverson, B. I., and Georgiou, G. (2002) Anchored periplasmic expression (APEx): a new platform for library screening and affinity maturation. Conference book IBC's 13th international conference, Antibody Engineering.

27 Boder, E. T. and Wittrip. K. D. (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotech.* **15,** 553–558.

28 Mattheakiss, L. C., Bhatt, R. R., and Dower, W. J. (1994) An in vitro polysome display system for identifying ligands form very large peptide libraries. *Proc. Natl. Acad. Sci. USA* **91,** 9022–9026.

29 Hanes, J. and Plückthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA* **94,** 4937–4942.

30 Roberts, R. W. and Szostak, J. W. (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc. Natl. Acad. Sci. USA* **94,** 12,297–12,302.

31. McCafferty, J., Hoogenboom, H. R., and Chiswell, D. J. (1996) *Antibody Engineering: A Practical Approach*. Oxford University Press, Oxford, UK.

32. Kontermann, R. E. and Dübel, S. (eds.) (2001) *Antibody Engineering*. Springer Lab Manuals, Berlin, Germany.

33 Danner, S. and Belasco, J. G. (2001) T7 phage display: A novel genetic selection system for cloning RNA-binding protein from cDNA libraries. *Proc. Natl. Acad. Sci. USA* **98,** 12,954–12,959.

34 Mullinax, R.L, Gross, E. A., Amberg, J. R., Hay, B. N., Hogreffe, H. H., Kubitz, M. M., et al. (1990) Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage λ immunoexpression library. *Proc. Natl. Acad. Sci. USA* **87,** 8095–8099.

35 Kang, A. S., Jones, T. M., and Burton, D. R. (1991) Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc. Natl. Acad. Sci. USA* **88,** 11,120–11,123.

36 Skerra, A. and Plückthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240,** 1038–1041.

37 Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M., and Winter, G. (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. *Bio/Technology* **10,** 779–783.

38 Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W., et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13,** 3245–3260.

39 Crissman, J. W. and Smith, G. P. (1984) Gene 3 protein of filamentous phages: evidences for a carboxyl-terminal domain with a role in morphogenesis. *Virology* **132,** 445–455.

40 Kang, A. S., Barbas, C. F., Janda, K. D., Bencovic, S. J., and Lerner, R. A. (1991a) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc. Natl. Acad. Sci. USA* **88,** 4363–4366.

41 Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. USA* **87,** 6378–6382.

42 Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., and Cesareni, G. (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* **222,** 301–310.

43 Jespers, L. S., Messens, J. H., de Keyser, A., Eeckhout, D., van den Brande, I., Gansemans, Y. G., et al. (1995) Surface expression and ligand based selection of cDNAs fused to filamentous phage gene VI. *Bio/Technology* **13,** 378–381.

44 Gao, C., Mao, S., Lo, C. H., Wirsching, P., Lerner, R. A., and Janda, K. D. (1999) Making artificial antibodies: a format for phage display of combinatorial heterodimeric arrays. *Proc. Natl. Acad. Sci. USA* **96,** 6025–30.

45 Vieira, J. and Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* **153,** 3–11.

46. O'Connel, D., Becerril, B., Roy-Burman, A., Daws, M., and Marks, J. D. (2002) Phage versus phagemid libraries for generation of human monoclonal antibodies. *J. Mol. Biol.* **321,** 49–56.

47 Garrard, L. J., Yang, M., O'Connel, M. P., Kelley, R., and Henner, D. J. (1991) Fab assembly and enrichment in a monovalent phage display system. *Bio/Technology* **9,** 1373–1377.

48 Lowman, H. B., Bass, S. H., Simpson, N., and Wells, J. A. (1991) Selecting High-Affinity binding proteins by monovalent phage display. *Biochemistry* **30,** 10,832–10,838.

49 Rondot, S., Koch, J., Breitling, F., and Dübel, S. (2001) A helperphage to improve single chain antibody presentation in phage display. *Nat. Biotechnol.* **19,** 75–78.

50 Baek, H., Suk, K. H., Kim, Y. H., and Cha, S. (2002) An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res.* **30,** e18.

51 Spada, S. and Plückthun, A. (1997) Selectivity infective phage (SIP) technology: A novel method for in vivo selection of interacting protein-ligand pairs. *Nat. Med.* **3,** 694–696.

52 Paschke, M., Zahn, G., Warsinke, A., and Höhne, W. (2001) New series of vectors for phage display and prokaryotic expression of proteins. *BioTechniques* **30,** 720–726.

53 Jacob, F. and Monod J. (1961) Genetic regulatory mechanism in the synthesis of proteins. *J. Mol. Biol.* **3,** 318–356.

54 Zahn, G., Skerra, A., and Höhne, W. (1999) Investigation of a tetracycline-regulated phage display system. *Protein Eng.* **12,** 1031–1034.

55 Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahimi, I., et al. (1987) A T5 promoter-based transcription-translation system for the analysis of proteins in vitro and in vivo. *Methods Enzymol.* **155,** 416–433.

56 Lei, S.-P., Lin, H.-C, Wang, S.-S., Callaway, J., and Wilcox, G. (1987) Characterization of the *Erwinia caratovora pelB* gene and its product pectate lyase. *J. Bacteriol.* **169,** 4379–4383.

57 Skerra, A., Pfitzinger, I., and Plückthun, A. (1993) The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. *Bio/Technology* **9,** 273–278.

58 Skerra, A. and Schmidt, T. G. M. (1999) Applications of a peptide ligand for streptavidin: the Strep-tag. *Biomol. Engineer.* **16,** 79–86.

59 McCafferty, J., Fitzgerald, K. J., Earnshaw, J., Chiswell, D. J., Link, J., Smith, R., and Kenten, J. (1994) Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage-display. *Appl. Biochem. Biotech.* **47,** 157–173.

60 Simmons, L. C., Reilly, D., Klimowski, L., Raju, T. S., Meng, G., Sims, P., et al. (2002) Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. *J. Immunol. Methods* **263,** 133–147.

61 Bird, R. E., Hardman, K. D., Jacobsen, J. W., Johnson, S., Kaufman, B.M, Lee, S. M., et al. (1988) Single-chain antigen-binding proteins. *Science* **242,** 423–426.

62 Bird, R. E. and Walker, B. W. (1991) Single chain variable regions. *Trends Biotech.* **9,** 132–137.

63 Huston, J. S., Levinson, D., Mudgett, H. M., Tai, M. S., Novotny, J., Margolies, M. N., et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digosin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85,** 5879–5883.

64 Kortt, A. A., Lah, M., Oddie, G. W., Gruen, L. C., Burns, J. E., Pearce, L. A., et al. (1997) Single chain Fv fragments of anti-neurominidase antibody NC10 containing five and ten residue link-

ers form dimers and with zero residue linker a trimer. *Prot. Eng.* **10,** 423–428.

65 Arndt, K. M., Müller, K. M., and Plückthun, A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry* **37,** 12,918–12,926.

66 Le Gall, F., Kipriyanov, S. M., Moldenhauer, G., and Little, M. (1999) Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. *FEBS Lett.* **453,** 164–168.

67 Marks, J. D., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1992) Molecular evolution of proteins on filamentous phage. *J. Biol. Chem.* **267,** 16,007–16,010.

68 Iliades, P., Dougan, D. A., Oddie, G. W., Metzger, D. W., Hudson, P. J., and Kortt, A. A. (1998) Single-chain Fv of anti-idiotype 11-1G10 antibody interacts with antibody NC41 single-chain Fv with a higher affinity than the affinity for the interaction of the parent Fab fragments. *J. Protein. Chem.* **17,** 245–254.

69. Plückthun, A. (1990) Antibodies from *Escherichia coli*. *Nature* **347,** 497–498.

70. Plückthun, A. (1991) Antibody engineering: advances from the use of *Escherichia coli* expression systems. *Bio/Technology* **9,** 545–551.

71 Kramer, K., Fiedler, M., Skerra, A., and Hock, B. (2002) A generic strategy for subcloning antibody variable regions from the scFv phage display vector pCANTAB 5 E into pASK85 permits the economical production of Fab fragments and leads to improved recombinant immunoglobulin stability. *Biosensors Bioelectronics* **17,** 305–313.

72 Ward, E. S. (1993) Antibody engineering using *Escherichia coli* as host. *Adv. Pharmacol.* **24,** 1–20.

73 De Haardt, H. J., van Neer, N., Reurst, A., Hufton, S. E., Roovers, R. C., Henderikx, P., et al. (1999) A large non-immunized human fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J. Biol. Chem.* **274,** 18,218–18,230.

74 Welschof, M., Terness, P., Kipriyanov, S., Stanescu, D., Breitling, F., Dorsam, H., et al. (1997) The antigen binding domain of a human

IgG-anti-F(ab')2 autoantibody. *Proc. Natl. Acad. Sci. USA* **94,** 1902–1907.

75. Dübel, S., Breitling, F., Fuchs, P., Braunagel, M., Klewinghaus, I., and Little, M. (1993) A family of vectors for surface display and production of antibodies. *Gene* **128,** 97–101.
76 Schmiedl, A., Breitling, F., and Dübel, S. (2000) Expression of a bispecific dsFv-dsFv' antibody fragment in *Escherichia coli*. *Protein Eng*. **13,** 725–734.
77 Iba, Y., Ito, W., and Kurosawa, Y. (1997) Expression vectors for the introduction of higly diverged sequences into the six complementarity-determining regions of an antibody. *Gene* **194,** 35–46.
78 Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H. R., and Plückthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J. Immunol. Meth.* **201,** 35–55.
79 Haidaris, C. G., Malone, J., Sherrill, L. A., Bliss, J. M., Gaspari, A. A., Insel, R. A., et al. (2001) Recombinant human antibody single chain variable fragments reactive with *Candida albicans* surface antigens. *J. Immunol. Meth.* **257,** 185–202.
80. Barbas III, C. F., Burton, D. R., Scott, J. K., and Silverman G. J. (2001) *Phage Display: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
81 Tsurushita, N., Fu, H., and Warren, C. (1996) Phage display vectors for in vivo recombination of immunoglobulin heavy and light chain genes to make large combinatorial libraries. *Gene* **172,** 59–63.
82 Pini, A, Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P., and Neri, D. (1998) Design and use of a phage display library. *J. Biol. Chem.* **273,** 21,769–21,776.
83 Viti, F., Nilsson, V., Demartis, S., Huber, A., and Neri, D. (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. *Meth. Enzymol.* **326,** 480–497.
84. Söderlind, E., Lagerkvist, A. C.S., Duenas, M., Malmborg, A.-C., Ayala, M., Danielsson, L., and Borrebaeck, C. A. K. (1993) Chaperonin assisted phage display of antibody fragments of filamentous bacteriophages. *Bio/Technology* **11,** 503–507.
85 Kobayashi, N. Söderlind, E., and Borrebaeck, C. A. K. (1997) Analysis of assembly of synthetic antibody fragments: Expression of functional scFv with predifined specificity. *BioTechniques* **23,** 500–503.

86 Jirholt, P., Ohlin, M., Borrebaeck, C. A. K., and Söderlind (1998) Exploiting sequences space: shuffling in vivo formed complementarity determining regions into a master framework. *Gene* **215,** 471–476.

87. Ørum, H., Andersen, P. S., Øster, A., Johansen, L. K., Riise, E., Bjørnevad, M., et al. (1993) Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucl. Acids Res.* **21,** 4491–4498.

88 Dziegiel, M., Nielsen, L. K., Andersen, P. S., Blancher, A., Dickmeiss, E., and Engberg, J. (1995) Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. *J. Immunol. Meth.* **182,** 7–19.

89. Söderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A.-M., et al. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat. Biotech.* **18,** 852–856.

90 Johansen, L. K., Albrechtsen, B., Andersen, H. W., and Engberg, J. (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Protein Eng.* **8,** 1063–1067.

91 Engberg, J. Andersen, P. S., Nielsen, L. K., Dziegiel, M., Johansen, L. K., and Albrechtsen, B. (1996) Phage-display libraries of murine and human Fab fragments. *Mol. Biotechnol.* **6,** 287–310.

92 Hoogenboom, H. R. and Winter, G. (1992) By-passing immunisation: Human antibodies from synthetic repertoires of germline V_H gene segments rearranged in vitro. *J. Mol. Biol.* **227,** 381–388.

93 Finnern, R., Pedrollo, E., Fisch, I., Wieslander, J., Marks, J. D., Lockwood, C. M., and Ouwehand, W. H. (1997) Human autoimmune anti-proteinase 3 scFv from a phage display library. *Clin. Exp. Immunol.* **107,** 269–81.

94 Rojas, Gertrudis, Almagro, J. C., Acevedo, B., and Gavilondo, J. V. (2002) Phage antibody fragments library combining a single human light chain variable region with immune mouse heavy chain variable regions. *J. Biotech.* **94,** 287–298.

95 Den, W., Sompuram, S. R., Sarantopoulos, S., and Sharon, J. (1999) A bidirectional phage display vector for the selection and mass transfer of polyclonal antibody libraries. *J. Immunol. Meth.* **222,** 45–57.

96. Ge, L., Knappik, A., Pack, P., Freund, C., and Plückthun, A. (1995) Expressing antibodies in *Escherichia coli*, in *Antibody Engineering* (Borrebaeck, C. A. K., ed.), Oxford University Press, Oxford, UK.

97 Goletz, A., Cristensen, P. A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G., and Karsten, U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J. Mol. Biol.* **315,** 1087–1097.

98 Garrard, L. J. and Henner, D. J. (1993) Selection of an anti-IGF-1 Fab from a Fab phage library created by mutagenesis of multiple CDR loops. *Gene* **128,** 103–109.

99 Geoffroy, F., Sodoyer, R., and Aujame, L. (1994) A new phage display system to construct multicombinatorial libraries of very large antibody repertoires. *Gene* **151,** 109–113.

100 Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., et al. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus framework and CDRs randomized with trinucleotides. *J. Mol. Biol.* **296,** 57–86.

101 Akamatsu, Y., Cole, M. S., Tso, J. Y., and Tsurushita, N. (1993) Construction of a human Ig combinatorial library from genomic V segments and synthetic CDR3 fragments. *J. Immunol.* **151,** 4651–4659.

102 Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., et al. (1996) Human antibodies with subnanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotech.* **14,** 309–314.

103 Barbas III, C. F., Bain, J.D,, Hoekstra, M., and Lerner, R. A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problems. *Proc. Natl. Acad. Sci. USA* **89,** 4457–4461.

104 Barbas III, C. F., Amberg, W., Simoncsitis, A., Jones, T. M., and Lerner, R. A. (1993) Selection of human anti-hapten antibodies from semisynthetic libraries. *Gene* **137,** 57–62.

105 Desiderio, A., Franconi, R., Lopez, M., Villani, A. E., Viti, F., Chiaraluce, R., et al. (2001) A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J. Mol. Biol.* **310,** 603–615.

106 Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., et al. (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc. Natl. Acad. Sci. USA* **95,** 6157–6162.

107 Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., et al. (1994) Antibody fragments from a

"single pot" phage display library as immunochemical reagents. *EMBO J.* **13,** 692–698.

108 De Kruif, J. Boel, E., and Logtenberg, T. (1995) Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J. Mol. Biol.* **248,** 97–105.

109. Dörsam, H., Rohrbach, P, Kürschner, T. Kipriyanov, S., Renner, S., Braunnagel, M., et al. (1997) Antibodies to steroids from a small human naive IgM library. *FEBS Lett.* **414,** 7–13.

110. Little, M., Welschof, M., Braunagel, M., Hermes, I., Christ, C., Keller, A., et al. (1999) Generation of a large complex antibody library from multiple donors. *J. Immunol. Meth.* **231,** 3–9.

111. Schmidt, A., Müller, D., Mersmann, M., Wüest, T., Gerlach, E., Garin-Chesa, P., et al. (2001) Generation of human high-affinity antibodies specific for the fibroblast activation protein by guided selection. *Eur. J. Biochem.* **268,** 1730–1738.

7

Purification of Antibodies and Preparation of Antibody Fragments

Graham D. Kemp

Summary

Antibodies may be purified from serum, ascitic fluid, and tissue culture supernatant by a number of methods. Purified antibodies can be treated to produce fragments that may have enhanced properties for use in immunochemistry.

Key Words: Serum; immunoglobulin; protein A; chromatography; Fab fragment; affinity.

1. Introduction

The binding between an antibody and its target ligand or antigen is among the strongest interactions known in biology. One of the consequences of this is that the specific antibody–ligand interaction will predominate and in many instances the presence of other molecules in the antibody preparation is of little consequence and prior purification is therefore not necessary. In some instances, however, purification is necessary or desirable as the other proteins in serum may interfere.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

Successful protein purification results from the exploitation of one or more of the many characteristics responsible for the wide diversity within the protein population, such as charge, size, hydrophobicity, or biological affinity. Fortunately, in some properties immunoglobulins are distinctive and as a consequence an acceptable degree of purity can often be achieved using relatively straightforward procedures.

One property readily exploited is the high isoelectric point (IEP) associated with the immunoglobulin family. They have an IEP in the region of 8.6 in contrast to most proteins, which have values typically in the region of pH 6.0–7.0, while that of the predominant serum protein and major "contaminant," serum albumin has an IEP less than 5. Therefore, at most pH values, the charge carried by immunoglobulins will differ from the majority of the other serum proteins, and this will allow the use of high capacity techniques, such as ion-exchange chromatography.

The exploitation of biological activity through affinity chromatography can be an extremely powerful purification method. A number of bacteria produce immunoglobulin-binding proteins as part of their defence against antibodies. Such proteins, the best-known of which is protein A from *Staphylococcus aureus*, bind to the constant region of the antibody molecule and thus their usefulness is independent of antibody specificity ***(1)***. In some circumstances the antigen, or a close structural analog, may be used as the basis for purification, but here the strong binding affinity may be counter productive because it is often difficult to displace the antibody without the use of denaturing conditions.

Other conventional methods of protein purification also have their place. Precipitation techniques, such as ammonium sulfate or polyethylene glycol fractionation, do not give a high degree of purity on their own but do concentrate the protein and in combination with other techniques, such as ion-exchange chromatography, can be very useful. Separation on the basis of size is not a high capacity technique but can be applicable in the purification of IgM, which,

with a molecular weight approaching a million, is much larger than the majority of other serum proteins.

There is no one procedure or combination of procedures that is applicable to all cases. The class of antibody to be purified has to be considered. Conventional procedures for the production of polyclonal antisera will generally result in immunoglobulin G, and procedures for the generation of monoclonal antisera may result in any of the immunoglobulin classes. Immunoglobulins from different species have broadly similar properties, but protein A, for example, will not bind all of the subclasses of human IgG, nor is it particularly effective in the purification of IgG from rat, sheep, or goat ***(2)***. Thus a certain amount of trial and error may be required to reach the best protocol for a particularly demanding application, but the methods described here should provide an adequate purity for most purposes.

2. Materials

2.1. Precipitation Methods

1. Saturated ammonium sulphate.
2. 0.05 *M* sodium phosphate, pH 7.4 (*see* **Note 1**).
3. 20% (w/v) polyethylene glycol in 0.05 *M* sodium phosphate, pH 7.4.
4. 0.06 *M* sodium acetate, pH 4.0.
5. Caprylic acid (octanoic acid).

2.2. Chromatographic Techniques

2.2.1. Ion-Exchange Chromatography

1. Diethylaminoethyl–agarose (e.g., DEAE-Sepharose Fast Flow).
2. 0.05 *M* Sodium phosphate, pH 6.3 (*see* **Note 1**).
3. 0.05 *M* Sodium phosphate, 1 *M* NaCl, pH 6.3 (*see* **Note 2**).
4. 0.05 *M* Tris-HCl, pH 8.5.
5. 0.05 *M* Tris-HCl, 1M NaCl, pH 8.5.
6. Gradient maker (*see* **Note 3**).
7. 0.02% Sodium azide.

2.2.2. Thiophilic Chromatography

1. Thiophilic adsorption chromatography matrix (*see* **Note 4**).
2. 0.1 *M* Tris-HCl, 0.1 *M* K_2SO_4, pH 8.0.
3. 0.05 *M* Tris-HCl, pH 8.0.

2.2.3. Affinity Chromatography

1. Sepharose 4B, cyanogen bromide-activated-Sepharose, or protein A, G, or L Sepharose.
2. 0.1 *M* Sodium bicarbonate, 0.5 *M* NaCl, pH 8.3.
3. 0.1 *M* Sodium carbonate, pH 10.5.
4. Cyanogen bromide (**Caution:** cyanogen bromide is extremely toxic, and advice should be sought on local practices regarding its safe use. The minimum amount necessary should be purchased).
5. 5 *M* NaOH.
6. 0.05 *M* Sodium phosphate, 0.1 *M* NaCl, pH 7.4 (*see* **Note 5**).
7. 0.001 *M* HCl.
8. Protein A, protein G, or protein L.
9. 1 *M* Ethanolamine or 0.5 *M* glycine.
10. 0.1 *M* Sodium acetate, pH 4.
11. 0.1 *M* Glycine-HCl, pH 2.5.
12. 1 *M* Tris-HCl.

2.3. Preparation of Fragment Antigen Binding (Fab) and $(Fab')_2$ Fragments

1. 1 *M* Sodium citrate, pH 3.2.
2. Pepsin.
3. 5 *M* Tris-HCl.
4. 1 *M* Sodium acetate, pH 5.0.
5. 1 *M* Cysteine (*see* **Note 6**).
6. 0.05 *M* Ethylene diamine tetraacetic acid (EDTA).
7. Papain.
8. Iodoacetamide.
9. Matrices for gel filtration (e.g., Sephacryl 400HR, Sepharose 4B, or Superdex 200).
10. 0.05 *M* Sodium phosphate, 0.15 *M* NaCl, pH 7.4.

3. Methods

3.1. Preparatory Methods

The starting point for the purification of antibodies is usually serum, ascitic fluid, or tissue culture supernatant. If it is necessary to prepare serum from an immunised animal, then the following procedure can be used.

3.1.1. Preparation of Serum From Whole Blood (see also Chapter 3)

1. Blood should be allowed to clot at 37°C for 30 to 60 min. After this time the clot should be solid (*see* **Notes 7** and **8**).
2. Leave at 4°C overnight to allow retraction to take place.
3. If necessary, gently detach the clot from the wall of the container using a blunt instrument such as a sealed Pasteur pipet, or a plastic rod, and remove the serum.
4. More serum may be recovered from the clot by centrifugation at 2500*g* for 30 min or by gently pressing the clot with a flat instrument, although this has to be conducted with care if hemolysis is to be avoided.
5. The serum from **steps 3** and **4** can be combined and centrifuged at 2500*g* to remove any cell and clot debris.
6. The serum should be stored in suitable aliquots at –20°C or at 4°C with the addition of a suitable antibacterial agent such as 0.02% (w/v) sodium azide.

3.2. Precipitation Techniques

The theory behind such techniques is complex and not clearly understood, but the techniques themselves are straightforward. To say that an increase in protein–protein interactions leads to precipitation whereas protein–solvent interaction favors solubility is a simplistic but nonetheless useful paradigm. Precipitation techniques do not, by themselves, achieve a great increase in the purity of a protein solution, but generally they result in an increase in concentration and have a role to play in many protein purification protocols.

The most common protein precipitation technique involves the use of ammonium sulphate, which has been the subject of a recent review ***(3)***. The widespread use of ammonium sulfate can be ascribed to the fact that it is very soluble (saturated solutions have a concentration in the region of 4 *M*), the density of solutions do not compromise collection of precipitates by centrifugation and its use does not promote denaturation of proteins. The addition of ammonium sulfate will cause a neutralization of the surface charge of the protein and a decrease in the effective concentration of water leading to a decrease in protein solvent interactions.

Polyethylene glycol also has a long history of use as an agent for protein precipitation ***(4)***. It shares some of the positive attributes of ammonium sulphate in having a low heat of solution and not promoting denaturation of proteins. It appears that after the addition of polyethylene glycol, proteins are excluded from the space occupied by the hydrated polymer, and their effective concentration is increased to a level incompatible with solubility. It is less effective in the purification of IgG but is useful for the isolation of the larger IgM.

A number of other precipitating agents have been used in the purification of proteins. In some instances, the use of sodium sulfate can result in a purer antibody preparation, but generally it does not offer advantages over ammonium sulphate. Caprylic (octanoic) acid, however, offers a different approach and also has a long history of use ***(5)***. Conditions can be created where this short chain fatty acid will effectively precipitate the majority of serum proteins with the exception of the immunoglobulins.

3.2.1. Ammonium Sulphate Fractionation

The majority of the immunoglobulins in serum will precipitate at ammonium sulphate concentrations equivalent to 50% saturation. This concentration of ammonium sulphate can be achieved either by adding the required weight of ammonium sulphate or by adding an equal volume of a saturated solution of the salt. The former procedure can be quicker in that the prior preparation of a saturated

solution is not required. With the latter method, it is easier to avoid the localized occurrence of high concentrations (>50% saturation) of ammonium sulfate, which may result in the precipitation of additional protein species (*see* **Note 9**).

1. If necessary, clarify the serum by centrifugation at 4000*g* for 30 min at 4°C.
2. While gently stirring the serum, slowly add an equal volume of saturated ammonium sulfate. Alternatively, add 3.1 g of solid ammonium sulphate per 10 mL of serum. Again, the ammonium sulphate should be added slowly with gentle stirring. Continue stirring the solution at 4°C for at least 1h (*see* **Note 10**).
3. Centrifuge at 3000*g* for 30 min at 4°C and retain the precipitate.
4. The precipitate can be kept at 4°C if not required immediately or at –20°C for longer-term storage. Alternatively, the precipitate should be resuspended in a 50% volume of a suitable buffer, then dialyzed overnight at 4°C (*see* **Note 11**).

3.2.2. Polyethylene Glycol Precipitation

There is a range of polyethylene glycol (PEG) preparations available categorized by the molecular weight of the polymer. Protein solubility in the presence of PEG is influenced by the molecular weight of the PEG used, but viscosity will also increase with increasing size. As is often the case, a compromise is necessary and the most useful molecular weight range for immunoglobulin purification is in the region of 6000–10,000. There is some confusion over nomenclature in the preparations from different suppliers, with one renaming PEG 6000 as PEG 8000.

1. Clarify the serum by centrifuging at 3000*g* for 15 min at 4°C. Discard any precipitate and add 2 vol of 0.06 *M* sodium acetate, pH 4.0, at room temperature.
2. Cool 20% (w/v) solution of PEG on 0.05 *M* sodium phosphate, pH 7.4 to 4°C.
3. Stir the serum gently, slowly add an equal volume of the PEG solution, and continue stirring for 30 min.
4. Centrifuge at 3000*g* for 10 min at 4°C.

Table 1
Immunoglobulin Purification Using Caprylic Acid

Species	Volume (μL) Caprylic Acid Required/mL Serum
Human	70
Horse	55
Mouse	40
Sheep	70
Rabbit	80
Goat	80
Cow	70

5. Discard the supernatant, drain the pellet, and carefully wipe the inside of the tube to remove any remaining supernatant.
6. Resuspend the pellet in a suitable buffer and, if necessary, dialyze the solution against at least two changes of a suitable buffer at 4°C (*see* **Note 11**).

3.2.3. Caprylic (Octanoic) Acid Precipitation

At moderately acidic pH values, the short chain fatty acid, caprylic acid, is an effective protein precipitant. It has found an application in immunoglobulin purification as conditions have been developed that result in the precipitation of the majority of non-immunoglobin proteins in the serum leaving the antibodies in solution. The concentration of caprylic acid required differs for the serum of different species as shown in **Table 1** (*see* **Note 12**).

1. If necessary, clarify the serum by centrifuging at 3000*g* for 15 min at 4°C. Discard any precipitate and add 2X vol of 0.06 *M* sodium acetate, pH 4.0.
2. While stirring, add the required volume of caprylic acid (*see* **Table 1**) dropwise, then leave stirring for 30 min at room temperature.
3. Centrifuge at 3000*g* for 30 min at 4°C.
4. Carefully remove supernatant and discard the precipitate.
5. Dialyze the supernatant overnight at 4°C against a suitable buffer.

3.3. Chromatographic Techniques

3.3.1. Ion-Exchange Chromatography

The isoelectric pH of immunoglobulins (approx 8.6) is higher than the majority of the other serum proteins. As a consequence, they will be among the few proteins that will not bind to anion exchange media at neutral pH, providing a rapid an effective means of purification, particularly if combined with precipitation methods such as ammonium sulphate fractionation.

Agarose-based exchangers are the most convenient to use because of their good flow properties and the most versatile of these is the diethylaminoethyl (DEAE or DE) derivative. The cellulose-based equivalent can also be used but in addition to having lower flow-rates/higher back-pressures it is advisable to precycle these before use (*see* **Note 13**). The subsequent protocol describes a column-based procedure, but a batch method could also be used, using filtration or centrifugation to separate the antibody containing supernatant from the ion-exchange medium.

3.3.1.1. Isocratic Method

1. Dialyse the serum, or preferably, the immunoglobulin-containing fraction resulting from one of the precipitation techniques previously described, against 0.05 *M* phosphate, pH 6.3 (*see* **Note 11**).
2. Wash the exchanger twice with a 10-fold excess of 0.05 *M* phosphate buffer pH 6.3 and pack into a suitable column (*see* **Note 14**). Use a volume of resin at least 2X the volume of Ig containing fraction. Equilibrate the column by passing through at least 10X the column volume of phosphate buffer.
3. Apply the sample and elute with 0.05 *M* phosphate, pH 6.3. The immunoglobulins should pass straight through; collect the eluate until the absorbance at 280 nm is less than 0.1.
4. Proteins bound to an agarose-based column can be removed by passing through 10 column volumes of 0.05 *M* phosphate, 1 *M* NaCl, pH 6.3, whereupon the column may be regenerated by passing through a further 10-column volumes of the phosphate buffer without NaCl.

Regeneration of cellulose-based columns is most reliably achieved by using the acid/alkali precycling procedure described (*see* **Note 13**).

3.3.1.2. Gradient Method

Immunoglobulins will bind to anion-exchangers if the pH is raised to between 8.0 and 9.0 and a higher degree of purity can often be achieved on subsequent elution using a salt gradient. Such methods can be readily adapted for use with high-resolution systems such as high-performance liquid chromatography or fast-proein liquid chromatography (*see* **Note 15**).

1. Dialyse the serum, or preferably, the immunoglobulin-containing fraction resulting from one of the precipitation techniques described earlier, against at least two changes of a 20- to 50-fold excess of 0.05 *M* Tris-HCl, pH 8.5.
2. Pack the exchanger (DEAE-agarose) into a suitable column (*see* **Note 16**) and equilibrate with 10-column volumes of 0.05 *M* Tris-HCl, pH 8.5.
3. Apply the sample and wash with two-column volumes of 0.05 *M* Tris-HCl, pH 8.5.
4. The majority of the immunoglobulins will bind to the column and can be eluted by the application of a NaCl gradient. The eluting buffer is progressively changed from 0.05 *M* Tris-HCl, pH 8.5, to 0.05 *M* Tris-HCl, 0.5 *M* NaCl, pH 8.5 (*see* **Note 3**). Collect fractions and monitor the absorbance at 280 nm to detect the protein containing fractions (*see* **Note 17**).
5. Finally elute the column with five-column volumes of 0.05 *M* Tris-HCl, 1 *M* NaCl, pH 8.5 This should remove any remaining proteins and allow the column to be re-equilibrated with 0.05 *M* Tris-HCl, pH 8.5, for re-use or washed with 0.02 (w/v) sodium azide for storage if reuse is not imminent.

3.3.2. Thiophilic Chromatography

Thiophilic chromatography depends on the binding of proteins to sulphur atoms contained in a particular chemical context. During the development of methods for the preparation of affinity supports

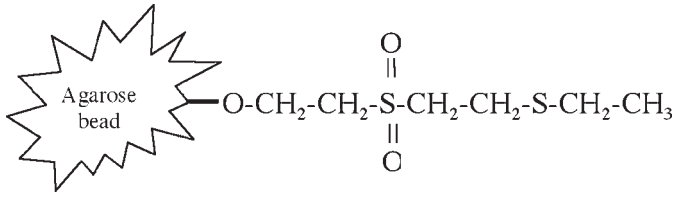


Fig. 1 Structure of the thiophilic ligand created by the reaction of 2-mercaptoethanol with divinyl sulfone-activated agarose. Immunoglobulins bind to the adjacent sulfone and thioether groups.

it was noticed by Porath and colleagues ***(6)***, that agarose activated with divinylsulfone and capped with mercaptoethanol had a strong affinity for proteins (**Fig. 1**).

This affinity was greatest in the presence of a high concentration of lyotropic ions such as sulphate and was reversed at lower salt concentration (*see* **Note 18**). This provides a useful method for the purification of immunoglobulins as they have a stronger affinity than most other serum proteins. This method has found particular application for the purification of IgM and chicken immunoglobulin (IgY) and is described in more detail by Boschetti ***(7)***.

1. Equilibrate the thiophilic matrix (*see* **Note 4**) with five-column volumes of equilibration buffer (0.05 *M* Tris-HCl, 0.5 *M* potassium sulphate, pH 8.0). Commercial thiophilic matrices have a capacity of 15–20 mg protein per milliliter of gel.
2. Slowly add an equal volume of 0.1 *M* Tris-HCl, 1 *M* potassium sulphate, pH 8.0, to the gently stirring immunoglobulin sample (*see* **Note 19**).
3. To clarify the solution, centrifuge at 4000*g*, at 4°C for 20 min.
4. Apply the supernatant to the column and wash with 10-column volumes of equilibration buffer or until the absorbance of the eluate at 280 nm is less than 0.05.
5. Elute the immunoglobulin from the column with 0.05 *M* Tris-HCl, pH 8.0. Collect the eluate in suitable sized fractions (2 mL) and check the absorbance of these fractions at 280 nm. Pool the protein-containing fractions and dialyse against a suitable buffer (*see* **Note 11**).

3.3.3. Affinity Chromatography

There are several bacterially derived proteins that have a strong affinity for the constant regions of immunoglobulins that can form the basis of affinity purification schemes (*see* **Table 2**). Of these, protein A and G have found the widest application. Protein A binds well to IgG from humans (apart from IgG3), rabbit, and guinea pig ***(1)***. It is less useful for the purification of mouse IgG as it has a lower affinity for IgG1 and IgG3 from that species (*see* **Table 2**). Protein G is produced by some strains of streptococci and this protein will bind to all the human IgG subclasses and also has a stronger affinity for mouse IgG (*see* **Note 20** and **ref. *8***). Both protein A and G bind predominantly to the Fc region of IgG, but they do also bind to sites in the variable domain of the heavy chain and so will also have some affinity for antibody fragments. However protein L, produced by *Peptostreptococcus magnus*, binds exclusively to the kappa light chain ***(9)*** and may be more useful in specific cases and particularly for the purification of antibody fragments (*see* **Subheading 3.3.**). Jacalin, a lectin isolated from the seeds of the jack fruit (*Artocarpus heterophyllus*), has an affinity for D-galactose and has been shown to bind to IgA1. Although this has been used to develop an affinity purification scheme for IgA1 ***(10)***, jacalin will also bind to other serum proteins, such as haemopexin and C1-inhibitor ***(11)***.

Agarose-based chromatography media provide a suitable basis for the preparation of affinity chromatography supports and of the many choices available Sepharose 4B is suitably versatile. Protein ligands can be readily attached to form suitable matrices for affinity chromatography by activation of the agarose with cyanogen bromide producing a derivative, which will couple to the amino groups of proteins at basic pH. The activation process is not difficult but requires access to an efficient fume-hood as cyanogen bromide is toxic, forming hydrogen cyanide at acid pH, and thus it may be prudent to purchase pre-activated resin. Protein A agarose and protein G agarose can also be purchased. The protocol (*see* **Subheading 3.2.3.1.**) is suitable for protein A, G, or L. Purification of antibodies by affinity chromatography has been reviewed recently by Huse et al. ***(12)***.

Table 2
Binding of Protein A, G, and L to the Immunoglobulin Classes and Subclasses of Different Species

		Protein A	Protein G	Protein L[a]
Human	IgG1	+++++	+++++	+++
	IgG2	+++++	+++++	+++
	IgG3	++	+++++	+++
	IgG4	+++++	+++++	+++
	IgM	+	–	+++
	IgE	++	–	+++
	IgA	+	–	+++
	IgD	–	–	+++
Mouse	IgG1	++	++++	++++
	IgG2a	+++++	+++++	++++
	IgG2b	+++++	+++++	++++
	IgG3	+++++	+++++	++++
Rabbit	IgG	+++++	++++	+
Goat	IgG	+	+++	–
Sheep	IgG	++	++++	–
Dog	IgG	+++++	++	+
Pig	IgG	+++++	++++	++++
Rat	IgG	+	++	++++
Cow	IgG	++	++++	–
Horse	IgG	++	++++	+/–
Guinea pig	IgG	+++++	++++	+++
Hamster	IgG	+++	++++	++++
Donkey	IgG	+++	+++++	–
Chicken	IgY	–	–	–/+

Strong binding, +++++; weak binding +; no binding -.

[a]Protein L binds to the k light chain and thus will not bind to all classes of immunoglobulin.

3.3.3.1. Preparation of Cyanogen Bromide-Activated Agarose

It should be stressed that cyanogen bromide is very toxic and thus it is particularly important to consult and adhere to local safety regulation regarding its use, the decontamination of containers and equipment and the disposal of solutions used. Purchase the mini-

mum amount of cyanogen bromide needed for the procedure. The procedure should be conducted in an efficient fume cupboard. The activated agarose prepared by this method is not stable and the ligand coupling should be conducted immediately.

1. Prepare the ligand solution in advance by dissolving the protein A/G/L at a concentration of 2–5 mg/mL (using 5 mg per mL of agarose) in coupling buffer (0.1 *M* bicarbonate buffer, 0.5 *M* NaCl, pH 8.3; *see* **Note 21**).
2. Wash Sepharose 4B with a 100-fold excess of water, resuspend in water, and allow to settle for 30 min. Note the settled volume and remove the excess water.
3. Add an equal volume of 0.1 *M* sodium carbonate, pH 10.5, and mix gently while monitoring the pH with an electrode.
4. In a fume cupboard, rapidly weigh out 0.5 g of cyanogen bromide per 5 mL of settled volume of Sepharose.
5. Add the cyanogen bromide to the stirring Sepharose and maintain the pH between 10.5 and 11.0 by the addition of 5 *M* NaOH (*see* **Note 22**).
6. Once the pH has stabilized, filter the reaction mixture using a Buchner funnel and wash the Sepharose with ice-cold PBS (or the appropriate buffer for the subsequent ligand-coupling step). Note that the filtrate and washings will contain cyanogen bromide and must be disposed of in an appropriate manner (*see* **Note 23**).
7. Proceed to **step 5** of the next section.

3.3.3.2. Coupling of Protein to Cyanogen Bromide-Activated Agarose

If purchased, cyanogen bromide-activated agarose will be in a dried form in the presence of stabilizers and will require swelling and washing before use. The activated groups are liable to hydrolysis at alkaline pH and thus the swelling and washing is carried out at acid pH.

1. If necessary swell the activated-agarose for 30 min at room temperature using 200 mL of 1 m*M* HCl per gram of agarose. One gram of dry weight will result in approx 3.5 mL of swollen gel.
2. Remove the acid using a sintered glass filter and a Buchner flask and wash with a further 200 mL of 1 m*M* HCl.

3. Dissolve the protein at a concentration of 2–5 mg/mL (using 5 mg per mL of agarose) in coupling buffer (0.1 *M* bicarbonate buffer, 0.5 *M* NaCl, pH 8.3; *see* **Note 21**).
4. Wash the activated agarose with 500 mL of coupling buffer. Remove the buffer by filtration, taking care not to allow the agarose to dry out.
5. Quickly add the ligand solution and mix gently for 2 h at room temperature, or overnight at 4°C (*see* **Note 24**).
6. Any unreacted groups on the agarose can be blocked by the addition of an excess of amino groups (1 mL of 1 *M* ethanolamine or 0.5 *M* glycine per milliliter of gel) and continuing to mix gently for another 2 h at room temperature, or overnight at 4°C.
7. Wash successively with 500 mL of coupling buffer, 0.1 *M* acetate buffer, pH 4.0, and coupling buffer to remove the unbound protein and store the agarose at 4°C until required (*see* **Note 25**).

3.3.3.3. Purification of Immunoglobulin Using Protein A, G, or L-Agarose

1. Equilibrate column with five-column volumes of 0.05 *M* phosphate, 0.1 *M* NaCl, pH 7.4, and apply the sample (*see* **Note 26**).
2. Wash the column with 10-column volumes of 0.05 *M* phosphate, 0.1 *M* NaCl, pH 7.4, or until the absorbance of the eluate at 280 nm is less than 0.05.
3. Elute the bound immunoglobulin by washing the column with 0.1 *M* glycine/HCL pH 2.5. Collect the eluate into 1 *M* Tris-HCl (0.1 mL per milliliter of eluate) in order to neutralize the solution and minimize denaturation of the protein.
4. Dialyze the immunoglobulin fractions against a suitable buffer and store at 4°C (*see* **Note 11**).

3.4. Preparation of Fab and (Fab')$_2$ Fragments From IgG

As well as their function in binding to antigen, immunoglobulins have other roles within the immune system that necessitate interactions with other proteins such as those of the complement and phagocytic systems. In techniques such as immunofluorescence or in in vivo applications, these interactions can be problematic but can be solved by the use of selective proteolysis to remove parts of the molecule. The polypeptide chains making up the IgG molecule

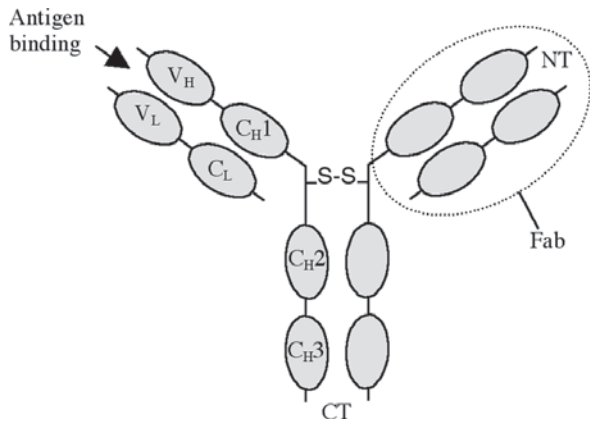


Fig. 2. Diagram of a molecule of IgG. The molecule is symmetrical, containing two identical heavy (H) chains and two identical light (L) chains. The V_L and V_H domains form antigen binding sites at the end of either arm. The Fc region is composed of the CH_2 and CH_3 domains from each of the larger heavy chains and is separated from the two Fab regions by the flexible hinge region which contains the disulphide bonds linking the two heavy chains. Different immunoglobulin classes (G, M, A, E, and D) have different heavy chains. There are two types of light chain (κ and λ) N, amino terminus; CT, carboxy terminus.

fold into a set of distinct domains (**Fig. 2**) with the two identical antigen binding domains being constituted by the V_H and V_C domains towards the N-terminus of the arms.

The other interactions are mainly associated with the C_H2 and C_H3 domains and these are separated from the rest of the molecule by a flexible region known as the hinge region, which is less compact allowing access of proteolytic enzymes. Papain will cleave the N-terminal side of the disulphide bond cluster creating two identical antigen-binding fragments (Fab) and one Fc fragment. Pepsin will cleave the C-terminal side of the disulphide bonds, and also within the Fc part to leave one major product, divalent with respect to antigen, known as $(Fab')_2$.

3.4.1. Preparation of $(Fab')_2$

1. Add 1/10 vol of 1 *M* sodium citrate, pH 3.2, to an IgG solution of approx 5 mg/mL (*see* **Notes 27** and **28**) and check that the resulting pH is below 3.8. If not adjust by the careful addition of 1 *M* HCl.
2. Add 5 µg of pepsin per mg of IgG and incubate at 37°C for 18 h (*see* **Note 29**).
3. Add a 1/10 vol of 5 *M* Tris-HCl to stop the reaction.
4. Purification of the $(Fab')_2$ fragment can be achieved by the use of size exclusion chromatography (**Subheading 3.3.3.**).

*3.4.2. Preparation of Fab (see **Note 30**)*

1. Add 1/10 vol of 1 *M* sodium acetate, pH 5.0, to an IgG solution of approx 5 mg/mL and check that the resulting pH is between 5.0 and 5.5. If not adjust by the careful addition of 0.1M HCl.
2. Add 1/100 vol of 1 *M* cysteine and a 1/50 vol of 50 m*M* EDTA.
3. Add 10 µg of papain per mg of IgG and incubate at 37°C for 6–12 h (*see* **Note 27**)
4. Inactivate the papain by the addition of iodoacetamide to a final concentration of 25 m*M*.
5. Fab can be purified using protein A agarose (*see* **Subheading 3.2.3.3.**). Fc and any undigested antibody should bind to the column matrix. If further purification is required, anion exchange chromatography on DEAE–Agarose (**Subheading 3.2.1.2.**) or size-exclusion chromatography (**Subheading 3.3.3.**) are options.

3.4.3. Purification of $(Fab')_2$ by Gel-Filtration Chromatography

Gel filtration chromatography (also known as size or molecular exclusion chromatography) separates molecules based on their ability to penetrate into the pores or channels in agarose or dextran beads. As a mixture of molecules in a fluid permeate through the beads of gel the volume available for diffusion is determined by their diameter and the size of the channels in the gel beads. The

smaller molecules have a greater volume available to them and therefore take longer to pass through the column. There is no interaction between the molecules to be separated and the column matrix and as a consequence this is a gentle technique with no harsh or potentially denaturing conditions necessary for elution. Indeed virtually any buffer system may be used and, as large protein molecules will be separated from small ions and molecules, the technique can be used to transfer the protein from one buffer to another, or to effect a change in salt concentration. The protein will end up in the buffer in which the column has been equilibrated. However, because diffusion is at the center of the separation process, the protein will become more dilute (by as much as a factor of 10), and the capacity (the amount of protein that can be loaded) is low. As a consequence it is a technique normally used in the final stages of a purification protocol when the protein is in a relatively pure state and there is a sufficient difference in molecular size in the remaining components and as such is suited to the separation of $(Fab')_2$ from contaminating peptides arising from the proteolytic digestion (*see* **Notes 31** and **32**).

3.4.3.1. Column Packing

1. The gel filtration matrix is supplied as a suspension and usually contains a preservative such as ethanol. Wash the matrix (*see* **Note 33**) with water on a filtration funnel to remove the ethanol and then wash with the eluting buffer (0.05 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4; *see* **Note 34**).
2. Transfer the matrix to a suitable container and allow it to settle. Add or remove water to give a volume equivalent to the settled volume plus 25%.
3. De-gas the slurry under vacuum.
4. Set up the column (*see* **Note 35**) vertically, add 5–10 mL of eluting buffer to the column, and allow approx 5 mL of this to flow through to remove air bubbles, then close off the outlet.
5. Attach a packing reservoir to the top of the column of sufficient volume to allow all of the matrix to be added at the one time.

6. Pour the matrix gently into the reservoir, taking care to avoid air bubbles (pour down a glass rod, one end of which is in contact with the inside of the reservoir).
7. Ensure that the operating pressure is less than the maximum indicated by the manufacturer (*see* **Note 36**) and allow the liquid to flow through until the level of the packed matrix is stable (*see* **Note 37**).
8. Once the column level is stable, remove the reservoir and equilibrate the column by passing through two-column volumes of the eluting buffer.

3.4.3.2. Sample Application and Chromatographic Separation

1. Carefully remove the liquid from the top of the column and apply the sample without disturbing the gel bed (*see* **Note 38**).
2. Allow the sample to run in and, taking care not to allow air to enter the gel bed, wash in with approx 1 mL of eluting buffer (*see* **Note 39**).
3. Layer 2–3 mL of eluting buffer on to the column, reinstate the eluting buffer delivery line and adjust the pressure head, or pump to give a flow rate of 0.5 mL/min (*see* **Note 40**).
4. Collect 1–2 mL fractions and monitor the absorbance at 280 nm.

4. Notes

1. 0.05 *M* Sodium phosphate buffer is conveniently made by mixing solutions of 0.05 *M* Na_2HPO_4 and 0.05 *M* NaH_2PO_4 to obtain the correct pH.
2. This is most simply prepared by the addition solid NaCl to 0.05 *M* phosphate buffer pH 6.3. This will alter the pH and concentration of the phosphate, but in this case it will not affect the procedure because the critical factor is the sodium chloride concentration.
3. Gradient makers are available commercially, but instructions for making a simple but effective device are given by Flurkey ***(13)***.
4. Suitable matrices are available from a number of suppliers and include T-gel adsorbent (Pierce). Amersham Biotech offers "HiTrap IgM" and "HiTrap IgY" aimed at the purification of IgM and the chicken immunoglobulin, IgY. These are based on 2-mercaptopyridine as the thiophilic adsorbent.

5. Prepare as recommended in **Note 2**. Any change in pH or phosphate concentration will not be relevant.
6. Use free cysteine rather than the hydrochloride salt. Cysteine solutions will oxidize readily and should be freshly prepared daily.
7 Glass containers are preferable as glass is more effective at promoting coagulation than plastic.
8. If the starting material is plasma rather than serum it is advisable to remove the fibrinogen as subsequent steps may remove the anticoagulant and initiate clotting. If the anticoagulant is EDTA or citrate add 0.1 mL of 0.5 *M* $CaCl_2$ and 1 IU of thrombin per mL of plasma and incubate at 37°C for 15 min. If the plasma is heparinized then first add 10 µL of 5 mg/mL protamine sulphate per mL of plasma before adding the calcium chloride and thrombin as previously explained.
9. Other salts, particularly sodium sulphate, can be used for the precipitation of immunoglobulins. Sodium sulphate can give a purer preparation of human and rabbit immunoglobulins, but the yield may be less than that obtained using ammonium sulphate.
10. In some instances 50% saturation may precipitate an unacceptable level of other serum proteins. If this is the case then reduce the amount of saturated ammonium sulphate added as the majority of the immunoglobulins are precipitated at less than 45% saturation.
11. Dialysis should be conducted at 4°C against a 50- to 100-fold volume excess of the appropriate buffer for at least 8 h with one change of buffer. If the volume of material to be dialysed makes this volume excess impracticable, then a smaller volume may be used and additional buffer changes incorporated.
12. It must be kept in mind that the immunoglobulin population is a heterogeneous one and although the values in **Table 1** will precipitate the bulk of the serum proteins from the given species, it is advisable to test for the yield of the desired specificity and make minor adjustments to the volume added if necessary. Further information is given by McKinney and Parkinson ***(14)***.
13. This is achieved by washing the exchanger in a 10X volume excess of 0.1 *M* HCl followed by extensive water washing until the pH of the wash approaches 6.0. This can be carried out using a Buchner filter, by centrifugation or by decanting the supernatant after allowing the slurry to settle. The exchanger is then washed in a 10x volume excess of 0.1 *M* NaOH and the water-washing repeated until the pH of the wash falls below 8.0.

14. The immunoglobulin containing fraction can be recovered by centrifugation or filtration rather than using a column method. If a column is used, then its geometry is not important and a 'short fat' column will provide a better flow rate and thus speed up the process.
15. Fast-protein or high-performance liquid chromatography systems using columns such as Mono Q, Poros HQ or TSK DEAE 5PW are suitable for the purification of immunoglobulins following this protocol.
16. In methods involving a gradient elution, the column geometry has more of an influence and it is preferable to have a length to diameter ratio of at least 5.
17. The total volume of the gradient should be 10–20-column volumes. In this method, albumin elutes immediately following the immunoglobulin fraction. The separation can be improved if necessary by applying a shallower gradient.
18. The affinity is influenced by salt concentration in accordance with the Hofmeister series. It is increased in the presence of lyotropic anions such as sulphate and decreased in the presence of chaotropic anions at the other end of the series such as thiocyanate. Anions from the middle of the scale such as chloride have little effect on binding.
19. If the immunoglobulin is to be purified from a dilute source such as a tissue culture supernatant, use solid K_2SO_4. Add this slowly, with gentle stirring, to prevent localized high concentrations of the salt, which may lead to precipitation.
20. Protein G contains several binding sites for the constant region of the heavy chain of IgG and also a binding site for serum albumin ***(15)***. There are recombinant versions of protein G where the albumin-binding site has been removed and these are preferable for use in purification procedures.
21. Coupling at slightly alkaline pH is more efficient, but can result in a ligand that is more constrained and therefore reduced in its ability to bind its target protein. Coupling at a lower pH (e.g., using 0.1 *M* phosphate, 0.5 *M* NaCl, pH 7.0, or 0.1 *M* sodium citrate, pH 6.5) may result in a lower yield, but this can be offset by higher binding efficiencies.
22. Do not allow the pH to rise higher than 11.5 because the activated groups are not stable above that pH, and the binding capacity of the matrix will be severely reduced.
23. Glassware that has been in contact with cyanogen bromide should be decontaminated before reuse. It is advisable to take advice on local

procedures, although soaking overnight in 2 *M* NaOH has been suggested as has the use of hypochlorite, although care should be exercised in using hypochlorite with protein containing solutions.

24. Avoid the use of magnetic stirrers because damage to the resin beads can result. Rotary ("Ferris wheel")-type mixers are ideal.
25. Incorporate 0.05% (w/v) sodium azide for long-term storage.
26. Yield can be increased by recirculating the unbound material back through the column two or three times.
27. This procedure should be performed with purified IgG.
28. The absorbance at 280 nm of a 1 mg/mL solution of human IgG is 1.36, and the values for other mammalian IgG are similar ***(16)***.
29. It can be beneficial to perform a trial digestion because the optimum time can vary with different Ig preparations.
30. A fragment with very similar properties to Fab, Fab' can be prepared by gentle reduction of $(Fab')_2$ with 10 m*M* cysteine in 0.1 *M* sodium bicarbonate, pH 8.2 at 25°C for 2 h. The sulfhydryl groups are then alkylated by the addition of iodoacetamide to a final concentration of 12 m*M* and incubation at 25°C in the dark for 2 h.
31. Gel filtration matrices are based on agarose, dextran, polyacrylamide or combinations of these. The most extensive range is supplied by Amersham Biosciences under the trade names of Sepharose, Superose, Sephacryl, Superdex, and Sephadex, and there are several versions of each brand with a variety of pore sizes and therefore a range of molecular weights for which they are most applicable. Sephadex has an upper limit of around 100 kDa, and thus its usefulness in immunoglobulin purification is as an alternative to dialysis for desalting applications. Superdex and Superose have better flow properties, permitting much faster separations and higher resolution, but are significantly more expensive than Sepharose or Sephacryl and require a pump capable of dealing with back pressures beyond the capabilities of a peristaltic pump. If such equipment is available, then Superdex 200 is a suitable choice for the purification of $(Fab')_2$. Prepacked Superdex columns are available commercially, but columns can be packed in house. If more modest equipment is all that is available and self-packing is to be attempted then Sephacryl S300 HR, or Sepharose 6B (or CL-6B) may be used.
32. Gel filtration can also have a role in a final "polishing" step in the purification of IgG for which the matrices suggested earlier for $(Fab')_2$ purification would be suitable. It has can also be used as a

main step in the purification of IgM, which has a significantly greater molecular weight than the majority of other proteins. For IgM purification, the matrices, which would be applicable would be Superdex 200, Sephacryl 400HR, or Sepharose 4B.

33. The amount of matrix required is approx 110 to 120% of the final column volume.
34. In gel filtration the buffer has little effect on the elution of the proteins in the sample and the limitations are imposed by the stability of the matrix. Sepharose is stable within the pH range of 4.0–9.0, CL-Sepharose, between pH values of 3.0 and 14.0, and Sephacryl from pH 3.0–11.0. There is the possibility of some ionic interaction between the matrix and the protein at very low ionic strength and therefore it is usual to maintain a salt concentration of approx 0.1–0.15 *M* to prevent this.
35. Column length has a significant influence on gel filtration whereas column diameter does not influence the resolution but, does affect the amount of protein that can be loaded. In this instance, column lengths between 30 and 60 cm and diameters between 1 and 3 cm would be suitable. Some columns are supplied with flow adaptors, which eliminate the dead volume above the gel bed.
36. Operating pressure is the height differential between the level of liquid in the reservoir and the end of the outlet tube. Sephacryl has better flow properties than Sepharose and can be packed at higher pressures. A peristaltic pump may be used to create a flow rate of 1 mL/min in a column of 1.6 cm diameter or 2.5 mL per min in a column of 2.5 cm in diameter.
37. It is important to ensure that the column does not "run dry," that is, that no air should be allowed to enter the packed gel bed.
38. The volume of sample applied should be 1–2% of the column volume; greater than 5% the resolution is adversely affected. The concentration of the sample is less critical unless this increases the viscosity, but this is unlikely to be a consideration unless the concentration exceeds 20 mg/mL.
39. Use of a flow adaptor can facilitate sample application. The sample may also be layered under the buffer after increasing the density by the addition of sucrose or glycerol.
40. Separation by gel filtration depends on the lateral diffusion of the solute molecules into the pores of the gel, and thus the resolution can be impaired by high flow rates.

References

1 Lindmark, R., Thoren-Tolling, K., and Sjoquist, J. (1983) Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *J. Immunol. Methods* **62,** 1–13.

2. Harlow, E. and Lane, D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

3 Kent, U. M. (1999) Purification of antibodies using ammonium sulfate fractionation or gel filtration. *Methods Mol. Biol.* **115,** 11–18.

4 Warrington, R. E. and Morgan, D. O. (1971) Foot-and-mouth disease virus in cattle and pigs: use of polyethylene glycol or dextran for purifying 19S gamma-M immunoglobulin from sera. *Arch. Gesamte Virusforsch* **33,** 134–144.

5 Steinbuch, M. and Audran, R. (1969) The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* **134,** 279–284.

6 Porath, J., Maisano, F., and Belew, M. (1985) Thiophilic adsorption–a new method for protein fractionation. *FEBS Lett.* **185,** 306–310.

7 Boschetti, E. (2001) The use of thiophilic chromatography for antibody purification: a review. *J. Biochem. Biophys. Methods.* **49,** 361–389.

8 Ohlson, S., Nilsson, R., Niss, U., Kjellberg, B. M., and Freiburghaus, C. (1988) A novel approach to monoclonal antibody separation using high performance liquid affinity chromatography (HPLAC) with SelectiSpher-10 protein G. *J. Immunol. Methods* **114,** 175–180

9 Bjorck, L. (1988) Protein L. A novel bacterial cell wall protein with affinity for Ig L chains. *J. Immunol.* **140,** 1194–1197

10 Gregory, R. L., Rundegren, J., and Arnold, R. R. (1987) Separation of human IgA1 and IgA2 using jacalin-agarose chromatography. *J. Immunol. Methods* **99,** 101–106.

11 Kabir, S. (1998) Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-derived lectin of versatile applications in immunobiological research. *J. Immunol. Methods* **212,** 193–211.

12 Huse, K., Bohme, H. J., and Scholz, G. H. (2002) Purification of antibodies by affinity chromatography. *J. Biochem. Biophys. Methods* **51,** 217–231.

13 Flurkey, B. (2000) An inexpensive gradient maker for the biochemistry laboratory. *J. Chem. Ed.* **185,** 1041.

14 McKinney, M. M. and Parkinson, A. (1987) A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. *J. Immunol. Methods* **96,** 271–278.

15. Bjorck, L., Kastern, W., Lindahl, G., and Wideback, K. (1987) Streptococcal protein G, expressed by *streptococci* or by *Escherichia coli*, has separate binding sites for human albumin and IgG. *Mol. Immunol.* **24,** 1113–1122.

16. Johnstone, A. P. and Thorpe R. C. (1996) *Immunochemistry in Practice*, 3rd ed. Blackwell Publishing, Oxford, UK.

8

Conjugation of Antibodies to Alkaline Phosphatase

G. Brian Wisdom

Summary

Alkaline phosphatase is coupled to immunoglobin G antibody in a one-step procedure using the homobifunctional reagent glutaraldehyde, which reacts with amino groups in the two proteins. The procedure is simple to perform and requires minimal equipment.

Key Words: Antibody; alkaline phosphatase; conjugation; labeling; glutaraldehyde.

1. Introduction

Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa has proven its worth as an enzyme label for many years. It is stable, has a moderate size (140 kDa), a high turnover number, and can be assayed using a variety of different substrates. Its activity is easily detected by eye in, for example, immunoblots, and it can be quantified by changes in absorbance, fluorescence, or luminescence for use in enzyme-linked immunosorbent assays.

The most common method of labeling immunoglobin G (IgG) antibody with this enzyme uses the homobifunctional reagent glu-

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

taraldehyde. The chemistry of glutaraldehyde is complex; it reacts with the amino groups and, to a lesser extent, the thiol groups of proteins and when two proteins are mixed in its presence, stable conjugates are produced without the formation of Schiff bases. Excessive self-coupling can be minimized by mixing the proteins at appropriate concentrations. In the method ***(1)*** described, there is usually little self-coupling of the enzyme or the IgG antibody, however, the size of the conjugate is large (>10^6 Da) because several molecules of each component are linked. This is the simplest enzyme labeling procedure to perform and, although the yields of enzyme activity and immunoreactivity are relatively small, the conjugates obtained are stable and practical reagents.

Alkaline phosphatase may also be coupled to antibody using heterobifunctional reagents containing the *N*-hydroxysuccinimide and maleimide groups, for example, succinimidyl 4-(*N*-maleidomethyl)-cyclohexane-1-carboxylate. However, because the enzyme has no free thiol groups, this approach is usually used for the labeling of Fab' fragments of IgG via their thiols ***(2)***. Alternatively a three-step procedure may be used in which thiol groups are introduced into either the enzyme or the antibody prior to the reactions of the maleimide and *N*-hydroxysuccinimide groups of the heterobifunctional linker.

2. Materials

1. Alkaline phosphatase from bovine intestinal mucosa, 2000 U/mg or greater (with 4-nitrophenyl phosphate as substrate); this is usually supplied at a concentration of 10 mg/mL. If ammonium sulfate, Tris-HCl, or any amine is present, it must be removed (*see* **Note 1**). There are numerous commercial sources of labeling-grade enzyme.
2. Antibody: this should be the pure IgG fraction or, better, affinity-purified antibody from an antiserum or pure monoclonal antibody (*see* Chapter 7).
3. Phosphate-buffered saline (PBS): 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
4. Glutaraldehyde (**Note:** glutaraldehyde is toxic).
5. 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$, 0.02% NaN_3 (**Note:** toxic), and 2% bovine serum albumin (BSA).

3. Methods

1. Add 0.5 mg of IgG antibody in 100 µL of PBS to 1.5 mg of alkaline phosphatase.
2. Add 5% glutaraldehyde (approx. 10 µL) to give a final concentration of 0.2% (v/v), and stir the mixture for 2 h at room temperature.
3. Dilute the mixture to 1 mL with PBS and dialyze against PBS (2 L) at 4°C overnight (*see* **Note 1**).
4. Dilute the solution to 10 mL with 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$, 0.02% NaN_3, and 2% BSA (*see* **Notes 2–4**).

4. Notes

1. The dialysis of small volumes can be conveniently performed in narrow dialysis tubing by placing a short glass tube, sealed at both ends, in the tubing so that the space available to the sample is reduced. Transfer losses are minimized by conducting the subsequent steps in the same dialysis bag. There are also various microdialysis systems available commercially, for example, the Slide-A-Lyzer units from Pierce Biotechnology (Rockford, IL).
2. The conjugates are stable for several years at 4°C because the NaN_3 inhibits microbial growth and the BSA minimizes denaturation and adsorption losses. These conjugates should not be frozen.
3. Purification of the conjugates is usually unnecessary; however, if there is evidence of the presence of free antibody it can be removed by gel filtration in Sepharose CL-6B (Amersham Biosciences, Chalfont St. Giles, UK) or a similar medium with PBS as solvent.
4. The enzyme-labeled antibody can be evaluated by enzyme-linked immunosorbent assay. Immobilize the appropriate antigen on the wells of a microtiter plate or strip (at a concentration of 2–10 µg/mL), incubate various dilutions of the conjugate for a few hours, wash the wells, add substrate, and measure the amount of product formed (*see* Chapter 15). This approach may also be used for monitoring conjugate purification in chromatography fractions.

References

1. Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-

labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109,** 129–135.

2. Mahan, D. E., Morrison, L., Watson, L., and Haugneland, L. S. (1987) Phase change enzyme immunoassay. *Anal. Biochem.* **162,** 163–170.

9

Conjugation of Antibodies to Horseradish Peroxidase

G. Brian Wisdom

Summary

Horseradish peroxidase is coupled to IgG antibody in a two-step procedure. In the first step monosaccharide residues in the enzyme are oxidized with periodate to produce aldehyde groups. Then, in the second step, the aldehyde groups are allowed to react with amino groups in the IgG antibody. The Schiff bases formed are reduced and the conjugate is purified by gel filtration.

Key Words: Antibody; horseradish peroxidase; conjugation; labeling; periodate oxidation.

1. Introduction

Horseradish peroxidase (HRP; EC 1.11.1.7) is the most widely used enzyme label for antibodies. It is relatively small (44 kDa), stable, and has a broad specificity that allows is to be measured by absorption, fluorescence, and luminescence. Several of its products are intensely colored, which makes the enzyme convenient to use for immunocytochemistry and immunoblotting applications.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

The most commonly used method ***(1)*** for labeling immunoglobin G (IgG) antibody molecules with HRP exploits the glycoprotein nature of the enzyme. The saccharide residues are oxidized with sodium periodate to produce aldehyde groups that can react with the amino groups of the IgG molecule, and the Schiff bases formed are then reduced to give a stable conjugate. The conjugates are of a high molecular weight ($0.5–1 \times 10^6$) because several enzyme molecules can bind to each IgG molecule and also crosslink the latter. Peroxidase has very few free amino groups so self-coupling is not usually a significant problem.

IgG antibody may be labeled with HRP using glutaraldehyde in a two-step procedure ***(2)***; this produces small conjugates, but their activity is low. Heterobifunctional reagents such as succinimidyl 4-(*N*-maleidomethyl)-cyclohexane-1-carboxylate may also be used to link the thiol group of Fab' fragments to an amino group in the enzyme ***(3)***.

2. Materials

1. Horseradish peroxidase, 1000 U/mg or greater (with 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] as substrate; *see* **Note 1**).
2. Antibody: this should be the pure IgG fraction or, better, affinity purified antibody from an antiserum or pure monoclonal antibody (*see* Chapter 7).
3. 0.1 *M* Sodium periodate.
4. 1 m*M* Sodium acetate buffer, pH 4.4.
5. 10 m*M* Sodium carbonate buffer, pH 9.5.
6. 0.2 *M* Sodium carbonate buffer, pH 9.5.
7. Sodium borohydride, 4 mg/mL (freshly prepared).
8. Sepharose CL-6B (Amersham Biosciences, Chalfont St. Giles, UK) or a similar gel filtration medium.
9. Phosphate-buffered saline (PBS): 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
10. Bovine serum albumin (BSA).

3. Methods

1. Dissolve 2 mg of peroxidase in 500 µL of water.
2. Add 100 µL of freshly prepared 0.1 *M* sodium periodate and stir the solution for 20 min at room temperature protecting it from light. (The color changes from orange to green).
3. Dialyze the modified enzyme against 1 m*M* sodium acetate buffer, pH 4.4 (2 L) overnight at 4°C (*see* **Note 2**).
4. Dissolve 4 mg of IgG in 500 µL of 10 m*M* sodium carbonate buffer, pH 9.5.
5. Adjust the pH of the dialyzed enzyme solution to 9.0–9.5 by adding 10 µL of 0.2 *M* sodium carbonate buffer, pH 9.5, and immediately add the IgG solution. Stir the mixture for 2 h at room temperature.
6. Add 50 µL of freshly prepared sodium borohydride solution (4 mg/mL) and stir the mixture for 2 h at 4°C.
7. Fractionate the mixture by gel filtration on a column (approx 1.5 × 85 cm) of Sepharose CL-6B in PBS. Determine the A_{280} and A_{403} (*see* **Note 3**).
8. Pool the fractions in the first peak (both A_{280} and A_{403} peaks coincide), add BSA to give a final concentration of 5 mg/mL, and store the conjugate in aliquots at –20°C (*see* **Notes 4** and **5**).

4. Notes

1. Preparations of HRP may vary in their carbohydrate content and this can affect the oxidation reaction. Free carbohydrate can be removed by gel filtration. Increasing the sodium periodate concentration to 0.2 *M* can also help, but further increases lead to inactivation of the peroxidase.
2. Dialysis of small volumes can be conveniently done in narrow dialysis tubing by placing a short glass tube, sealed at both ends, in the tubing so that the space available to the sample is reduced. Transfer losses are minimized by carrying out the subsequent steps in the same dialysis bag. There are also various microdialysis systems available commercially, for example, the Slide-A-Lyzer units from Pierce Biotechnology (Rockford, IL).

3. The absorbance at 403 nm is caused by the peroxidase's heme group. The enzyme is often specified in terms of its RZ value; this is the ratio of A_{403} to A_{280}, and it provides a measure of the heme content and purity of the preparation. Highly purified peroxidase has an RZ of approx 3. Conjugates with an RZ of 0.4 perform satisfactorily.
4. BSA improves the stability of the conjugate and minimizes loses as a result of adsorption and denaturation. NaN_3 should not be used with peroxidase conjugates because it inhibits the enzyme. If an antimicrobial agent is required, 0.2% sodium merthiolate (thimerosal) should be used.
5. The performance of the conjugate can be evaluated by enzyme-linked immunosorbent assay as described in **Note 4** of Chapter 8 and in Chapter 15.

References

1. Wilson, M. B. and Nakane, P. P. (1978) Recent developments in the periodate method of conjugating horse radish peroxidase (HRPO) to antibodies, in *Immunofluorescence and Related Staining Techniques* (Knapp, W., Holubar, K., and Wick, G., eds.), Elsevier/North Holland Biomedical, Amsterdam, pp. 215–224.
2. Avrameas, S. and Ternynck, T. (1971) Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry* **8,** 1175–1179.
3. Uto, I., Ishimatsu, T., Ueda, S., Tsuruta, J., and Kambara, T. (1991) Determination of urinary Tamm-Horsfall protein by ELISA using a maleimide method for enzyme-antibody conjugation. *J. Immunol. Methods* **138,** 87–94.

10

Conjugation of Antibodies to Fluorescein or Rhodamine

G. Brian Wisdom

Summary

The isothiocyanate derivative of the fluorophore, fluorescein or rhodamine, is coupled to the amino groups of IgG antibody in a one-step procedure and excess label is removed by gel filtration.

Key Words: Antibody; fluorescein; rhodamine; conjugation; labeling.

1. Introduction

Antibodies labeled with fluorescent molecules have several applications, particularly in cytochemistry and cell sorting. There are many fluorochromes used in labeling ***(1)***, such as coumarin derivatives, phycobiliproteins, and rare earth chelates; however, fluorescein and rhodamine (**Table 1**) are the most commonly used.

The isothiocyanate derivatives of fluorescein and rhodamine are widely used to label antibodies. They react with the amino groups of the immunoglobin G (IgG) molecule under alkaline conditions and a molar excess of about 20 is usually optimal. More recently, *N*-

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

Table 1
Properties of Fluorescein and Rhodamine

Fluorochrome	Molecular weight*	Excitation (nm)	Emission (nm)
Fluorescein	389	495	520
Tetramethyl-rhodamine	443	555	575

*The molecular weight given is that of the isothiocyanate derivative

hydroxysuccinimide derivatives have become available for the same purpose and they use milder conditions. In both cases, the reaction is quenched by the addition of ethanolamine and excess labeling reagent is removed by gel filtration. The extent of conjugation may be determined by absorbance measurements.

2. Materials

1. Fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (*see* **Note 1**) dissolved in dimethyl sulfoxide at a concentration of 1 mg/mL.
2. Antibody: this should be the pure IgG fraction or, better, affinity-purified antibody from an antiserum or pure monoclonal antibody (*see* Chapter 7).
3. 0.1 *M* Sodium carbonate buffer, pH 9.0.
4. 0.1 *M* Ethanolamine, pH 8.5.
5. Phosphate-buffered saline (PBS): 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
6. Sephadex G-25 (Amersham Biosciences, Chalfont St. Giles, UK; *see* **Note 2**).
7. Bovine serum albumin (BSA).
8. Sodium azide (**Note:** sodium azide is toxic).

3. Methods

1. Prepare 1 mg of the IgG antibody in 1 mL of 0.1 *M* sodium carbonate buffer, pH 9.0.
2. Prepare the solution of the isothiocyanate immediately prior to use.
3. Add 50 µL of the isothiocyanate solution to the antibody slowly with stirring at room temperature and incubate the mixture at 4°C for 8 h in the dark.
4. Terminate the reaction by adding 0.1 mL of 0.1 *M* ethanolamine, pH 8.5, and incubate at room temperature for 15 min.
5. Remove the excess fluorochrome derivative by gel filtration in Sephadex G-25 equilibrated with PBS (*see* **Note 2**). Measure the A_{280}.
6. Pool the fractions in the first (colored) peak, store the conjugate in aliquots at –20°C or in the dark at 4°C with BSA (0.1% final concentration) and NaN_3 (0.05% final concentration; *see* **Notes 3** and **4**).

4. Notes

1. 5(6)-Carboxyfluorescein-*N*-hydroxysuccinimide ester (Roche Diagnostics, Lewes, UK; Molecular Probes, Eugene, OR) and 5(6)-carboxytramethylrhodamine-*N*-hydroxysuccinimide ester (Molecular Probes, Eugene, OR) are alternative reagents. They can be conjugated to IgG in PBS at room temperature in about 1 h.
2. Ready-made Sephadex G-25 columns are available for use with gravity feed (PD-10) or syringe (HiTrap) from Amersham Biosciences. The PD-10 columns with a bed volume of approx 9 mL are very suitable for processing samples of 1-2 mL. Similar cross-linked dextran devices, Presto and Kwik columns, are available from Pierce (Rockford, IL).
3. The BSA minimizes losses due to adsorption and denaturation.
4. The extent of the conjugation can be determined by absorbance measurements. The absorbance maxima of fluorescein or rhodamine (495 and 555 nm, respectively) are related to the IgG absorbance at 280

nm. Ratios of 0.4–1.0 are appropriate for fluorescein conjugates and 0.3–0.9 for rhodamine conjugates. (These measurements must be made before the addition of BSA.)

References

1. Garman, A. J. (1997) *Non-radioactive Labelling: A Practical Introduction*. Academic Press, San Diego, CA.

11

Conjugation of Antibodies to Biotin

James D. Hirsch and Rosaria P. Haugland

Summary

Antibodies can be conjugated to biotin by a number of chemical means. They can then be used in immunochemical procedures in conjunction with secondary reagents coupled to biotin-binding protein proteins such as avidin.

Key Words: Biotin; avidin; antibody; labeling; affinity; Fab fragments; fluorescence.

1. Introduction

To exploit the extraordinary specificity and high affinity of biotin binding to biotin-binding proteins like streptavidin, avidin, and their derivatives, researchers often need to conjugate biotin to one or more of their reaction components in vitro (biotinylation). Such biotinylated molecules can then serve as targets in many detection protocols using biotin-binding proteins or antibiotin antibodies labeled with fluorescent, enzyme, radioactive, or other reporter groups. Also, biotinylated molecules can be isolated and purified

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

based on their highly specific, predictable, and with appropriate reagents, reversible interactions with immobilized biotin-binding proteins. Researchers have a wide range of options available for such affinity-based applications. The reader is referred to **refs. *1–8*** for detailed explanations of these subject areas.

Virtually any type of biomolecule can be biotinylated using a variety of reactive biotin derivatives that react covalently with specific molecular targets. These include, but are not limited to: 1) succinimidyl esters of biotin that label primary amines found on protein lysine residues and at the N-terminus; 2) biotin maleimides and other derivatives that react with endogenous or introduced thiol groups; 3) biotin hydrazides that label carbohydrate moieties under appropriate conditions; and 4) photoreactive biotin compounds that label biomolecules nonspecifically in the presence of light at the appropriate wavelength. Proteins, nucleic acids, carbohydrates, lipids, and conjugates thereof can be efficiently biotinylated in vitro with these reagents. Biotinylation of whole cells is also possible with some of these compounds (e.g., FluoReporter® Cell Surface Biotinylation Kit, Molecular Probes, Inc., Eugene, OR).

This chapter describes optimized protocols for conjugating antibodies to biotin using representative examples of the reagents mentioned previously. We will emphasize covalent biotinylation of polyclonal and monoclonal IgG molecules, but other types of immunoglobulins as well as immunoglobulin fragments can be biotinylated using the methods described here. Covalent biotinylation of antibodies on their primary amines is the most popular and simplest method, so we will emphasize this approach. Detailed explanations of the many available chemical biotinylation reagents, the chemistry of their reactive groups, and the huge number of biotinylated antibody-based applications are beyond the present scope, so the reader is referred to **refs. *1–8*** for further information.

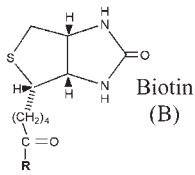
However, researchers should be aware that a new noncovalent method for biotinylating antibodies has recently become available. Zenon™ technology from Molecular Probes, Inc., is a unique immunolabeling system that enables researchers to attach biotin,

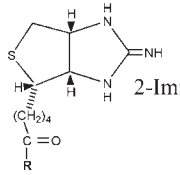
desthiobiotin, and other tags to mouse, rabbit, and human antibodies on their respective Fc regions. Zenon technology requires the use of anti-Fc F(ab) fragments as carriers for biotin, desthiobiotin, or many other reporter molecules . It is designed to rapidly biotinylate or otherwise label submicrogram amounts of poly- and monoclonal antibodies, and unlike the covalent biotinylation methods described here, the antibody targets can be labeled in ascites fluids or in the presence of extraneous proteins like bovine serum albumin or gelatin. Extensive descriptions and numerous applications of Zenon technology are available at www.probes.com/Zenon on the Internet.

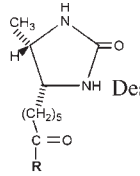
2. Materials

1. Poly- or monoclonal antibody or antibody fragments (*see* **Note 1**).
2. Reactive biotin derivative (*see* **Note 2** and **Tables 1–3**). These reagents are available from several companies, including Molecular Probes, Inc., Eugene, OR; Pierce Chemical Co., Rockford, IL; and Sigma/Aldrich, St. Louis, MO. Three kits containing all of the components necessary (except buffers) to biotinylate or desthiobiotinylate antibodies (and other proteins) are available from Molecular Probes, Inc. The FluoReporter® Mini-Biotin-XX Protein Labeling Kit (F-6347) contains sufficient reagents for five biotinylations of 0.1–3 mg each of antibody. The FluoReporter® Biotin-XX Labeling Kit (F-2610) is formulated for researchers who want to biotinylate five or more 5–20 mg amounts of protein and determine their degrees of labeling. The DSB-X™ Biotin Protein Labeling Kit (D-20655) is designed for five desthiobiotinylations with 0.5–3 mg each of an antibody or another protein.
3. Reaction buffer: 100 m*M* bicarbonate buffer (pH 8.0–8.3) is preferred for labeling antibody amines (*see* **Note 3**). The optimum reaction pH values for labeling antibodies with other reactive biotin derivatives are summarized in **Table 3**.
4. Other necessary materials:
   a. High-quality, heavy metal-free, well-washed dialysis tubing (e.g., Spectrum Laboratories Inc., Compton, CA or
   b. Gel filtration (size-exclusion) chromatography matrix and column (*see* **Note 4**).

Table 1
Structures of Reactive Biotin Derivatives


Biotin
(B)


2-Iminobiotin
(I)


Desthiobiotin
(D)

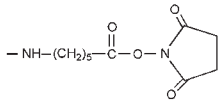
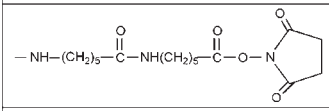
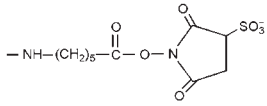
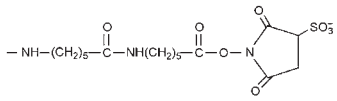
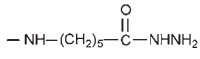
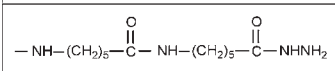
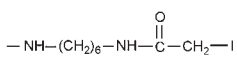
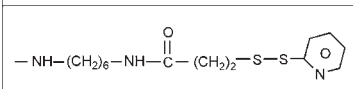
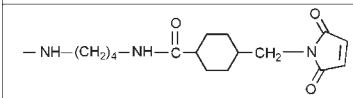
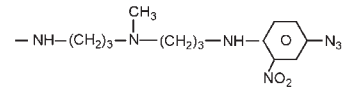
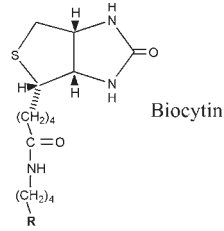
R	Name	Reactivity	Available attached to
	SE	Amines	B,I,D
	X-SE	Amines	B,D
	XX-SE	Amines	B
	X-SSE	Amines	B
	XX-SSE	Amines	B
	X-hydrazide	Aldehydes	B,D
	XX-hydrazide	Aldehydes	B
	Iodoacetyl	Thiols	B
	HPDP	Thiols	B
	Maleimide	Thiols	B
	Photoactivatable	Nonspecific	B

Table 2
Structures of Reactive Biocytin Derivatives



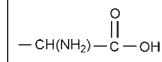
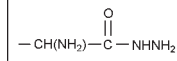
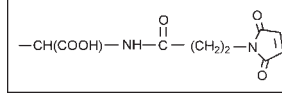
R	Name	Reactivity
	Biocytin	
	Hydrazide	Aldehydes
	Maleimide	Thiols

Table 3
Summary of Recommended Conditions for Reactive Biotin Derivatives

Reactive group	MW[a]	Reaction pH	MR for IgG (at 10 mg/mL)
SE	341.38	8.0–8.3	10–20[b]
X-SE	454.54	8.0–8.3	10–20[b]
XX-SE	567.70	8.0–8.3	10–20[b]
X-SSE	556.58	8.0–8.3	10–20[b]
XX-SSE	669.74	8.0–8.3	10–20[b]
X-hydrazide	371.50	6.0–7.2	20–25
XX-hydrazide	484.66	6.0–7.2	20–25
Iodoacetamide	510.42	7.5–8.0	16–20
HPDP	539.77	6.0–9.0	16–20
Maleimide	533.69	6.5–7.2	16–20
Photoactivatable	533.65[c]	7.0–7.5	5

[a]When the reactive group is attached to biotin.
[b]For IgG at <1 mg/mL (30–40), 1–3 mg/mL (20–25), 4–10 mg/mL (10–20).
[c]As sold by Pierce Chemical Company (Rockford, IL).

c. Molecular weight-selective, centrifugable concentration devices (*see* **Note 5**).
d. Anhydrous DMSO or DMF (*see* **Note 6**).

5. Optional materials:
   a. Sodium cyanoborohydride, available from Sigma/Aldrich and other vendors.
   b. HABA (4'-hydroxyazobenzene-2-carboxylic acid), available from Pierce Chemical Co., Sigma/Aldrich, and other vendors.
   c. Avidin or streptavidin, available from Molecular Probes, Inc. (A-887, S-888), Pierce Chemical Co., Sigma/Aldrich, and other vendors.
   d. D-biotin, available from Molecular Probes, Inc. (cat. nos. B-1595, B-20656), Pierce Chemical Co., Sigma/Aldrich, and other vendors.

3. Methods

A summary of reactive biotin reagent-specific recommendations for labeling antibodies at 10 mg/mL is presented in **Table 3**. Detailed protocols are described in **Subheadings 3.1.** to **3.4.**

3.1. Biotinylation Using Amine-Reactive Biotin Derivatives

3.1.1. Reaction Setup

After the antibody starting material has been prepared in the amine-free reaction buffer of choice (*see* **Note 3**), the researcher should then calculate the amount of amine-reactive biotin needed.

1. Select the molar ratio of labeling reagent to antibody (MR). The MR represents the number of moles of amine-reactive biotin that are added to each mole of antibody in the reaction mixture. The recommended MR to use depends on the antibody concentration. For IgG antibodies at <1 mg/mL, we recommend a MR of 30–40. Antibodies at 1–3 mg/mL and 4–10 mg/mL should be labeled at a MR of 20–25 and 10–20, respectively.
2. Calculate how much labeling reagent is required to achieve the selected MR. It is convenient to calculate this value based on the quantity of antibody to be labeled:

mg of labeling reagent per mg antibody =
(MW of label × MR)/MW of antibody or fragment (**Eq. 1**)

3. Calculate the total amount of labeling reagent required:

total mg of labeling reagent = answer from **Eq. 1** × total mg of antibody (**Eq. 2**)

4. Calculate the µL of labeling reagent stock solution to add based on a 10 mg/mL stock solution:

volume of labeling reagent = answer from **Eq. 2** × 100 (**Eq. 3**)

5. Prepare the reactive biotin stock solution. Weigh out slightly more labeling reagent than is actually needed. Dissolve it in the appropriate volume of anhydrous dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) to obtain a concentration of 10 mg/mL. Vortex mixing or brief sonication can be used to facilitate dissolution, if necessary. This solution may deteriorate on storage (*see* **Note 6**). Note that **Eqs. 1–3** can be used to calculate the appropriate amounts to use of all of the other labeling reagents discussed in this chapter. Additional variations in these procedures are discussed in **Note 7**. Molecular weights of the commonly used reactive biotin derivatives are listed in **Table 3**.

3.1.2. Addition of Reactive Biotin

1. Stir the antibody solution gently at room temperature prior to adding the labeling reagent. If the antibody is already in a buffer at pH 8.0–8.3, slowly add the volume of labeling reagent solution determined using **Eq. 3** drop-wise while stirring. If the buffer pH is not in this range, it should first be adjusted as discussed in **Note 3** before adding the labeling reagent. (*See* **Note 6** for information about reusing labeling reagent stock solutions.)
2. Incubate the reaction mixture with stirring for 60–90 min at room temperature.
3. Remove the unconjugated biotinylation reagent by dialysis or gel filtration (*see* **Note 4**).

3.2. Biotinylation Using Thiol-Reactive Biotin Derivatives

3.2.1. Reduction of Antibody Disulfide Bonds

1. Prepare the antibody for reduction. For labeling IgG antibodies with thiol-reactive reagents, the antibody should be at 5–10 mg of protein/mL in 20 m*M* sodium or potassium phosphate buffer, pH 7.5–8.0 containing 150 m*M* NaCl or 10 m*M* phosphate-buffered saline ethylene diamine tetraacetic acid (PBS-EDTA). When biotin maleimide or biocytin maleimide derivatives are used, the buffer used for the reduction and subsequent steps should be at pH 6.5–7.2.
2. Reduce the antibody starting material. Add 2-mercaptoethylamine HCl (2-MEA, cysteamine HCl) to the antibody solution to a final concentration of 6–7 mg/mL (approx 53–62 m*M*) while stirring. Dithiothreitol (DTT) at a final concentration of 50 m*M* also can be used for reduction.
3. If 2-MEA is used, incubate the reaction mixture at 37°C for 90 min with stirring or gentle shaking. Incubate the mixture for 30 min at room temperature if DTT is used for reduction.
4. Remove excess 2-MEA (or DTT) from the reduced antibody (desalting). This step is easily performed on a gel filtration column equilibrated with the appropriate PBS (*see* **step 1**) containing 5 m*M* EDTA (*also see* **Note 4**). The presence of the reduced antibody in the column effluent should be monitored by A_{280}. Because any residual 2-MEA (or DTT) will inhibit the subsequent labeling reaction, it is important to pool only the most concentrated protein fractions eluting earliest from the desalting column. Desalting by dialysis versus the appropriate PBS containing 5 m*M* EDTA may be performed. In this case, the dialysis buffer should be changed frequently to assure that the 2-MEA (or DTT) has been completely removed.
5. Concentrate the desalted antibody, if necessary, to 10–20 mg/mL (*see* **Note 5**). Biotinylate the reduced antibody immediately to avoid reoxidation of the thiols.

3.2.2. Biotinylation Reaction

1. Biotinylate the reduced antibody. Thiol-reactive biotinylation reagents are usually used at labeling reagent: antibody MR of 16–20. Use **Eqs. 1–3** in **Subheading 3.1.1.** to determine the appropriate amount of labeling reagent to add to the reduced antibody. Slowly

add the labeling reagent solution drop-wise to the reduced antibody while stirring. (*See* **Note 6** for information about reusing biotinylation reagent stock solutions.)
2. Incubate the reaction mixture for 90 min at room temperature. If the labeling reagent is iodoacetyl-LC-biotin (Pierce), both the reagent and the reaction mixture should be protected from light.
3. Remove unreacted labeling reagent from the biotinylated antibody by dialysis, gel filtration, or with a spin column.

3.3. Biotinylation of Antibody Carbohydrates With Biotin Hydrazide Derivatives

Biotin hydrazides will react with antibody carbohydrate moieties after oxidation with sodium metaperiodate. This step opens the sugar rings between vicinal hydroxides and converts them to aldehydes that will react with hydrazides.

3.3.1. Periodate Oxidation of the Antibody

1. Prepare the antibody for periodate oxidation. Dialyze the glycosylated antibody at 2–10 mg/mL overnight at 4°C vs 100 m*M* acetate buffer (pH 6.0) and keep the solution on ice during the oxidation step. Because this reaction is light sensitive, amber or opaque containers should be used for all reagents.
2. Oxidize the antibody. Prepare a 20-m*M* solution of sodium metaperiodate in ice-cold acetate buffer (pH 6.0) and add an equal volume drop-wise to the antibody while stirring. Incubate the mixture for 120 min on ice.
3. Desalt the oxidized antibody. To remove iodate and formaldehyde byproducts, dialyze the oxidized antibody overnight at 4°C in the dark versus an amine-free buffer at pH 6.0–7.2 or use gel filtration. For optimum biotinylation, the oxidized antibody should be concentrated to approx 10 mg protein/mL (*see* **Note 5**).

3.3.2. Biotinylation of the Oxidized Antibody

1. Prepare the labeling reagent stock solution. Biotin, biocytin, and desthiobiotin hydrazide derivatives are typically used at a labeling reagent: antibody MR of 20–25. Biotin and desthiobiotin hydrazide stock solutions should be prepared at 10–20 mg/mL in DMSO or DMF while biocytin hydrazide can be dissolved in the reaction

buffer. Use **Eqs. 1–3** in **Subheading 3.1.1.** to calculate the amount of labeling reagent needed. (*See* **Note 6** for information about reusing labeling reagent stock solutions.)
2. Add the labeling reagent to the oxidized antibody. The appropriate volume of hydrazide reagent should be added drop-wise to the oxidized antibody while stirring.
3. Incubate the stirring reaction mixture for 120 min at room temperature.
4. Desalt the biotinylated antibody by dialysis or gel filtration. Note that after hydrazide-based biotinylations, many researchers feel that it is necessary to stabilize the linkages between the hydrazides and aldehydes by treating the conjugate with sodium cyanoborohydride.

3.3.3. Stabilize Hydrazide-Aldehyde Bonds (Optional)

1. Prepare a 100 m*M* solution of sodium cyanoborohydride in reaction buffer (pH 6.0).
2. Add 1/20 vol of this stock drop-wise to the antibody while stirring.
3. Incubate the reaction mixture for 120 min at 4°C. Sodium cyanoborohydride is extremely toxic and should only be handled in a fume hood while wearing appropriate personal protective equipment. Cyanoborohydride-containing solutions also contain cyanide and must be disposed of according to appropriate local regulations.
4. Desalt the antibody conjugate by dialysis or gel filtration.

3.4. Biotinylation of Antibodies With Photoactivatable Biotin

Because photoactivatable biotin is not commonly used to biotinylate antibodies, the following protocol is a general one ***(2)***. Researchers should optimize the method for their particular antibodies.

3.4.1. Preparing the Antibody

1. Dissolve or buffer-exchange the antibody at 4 mg protein/mL in PBS devoid of amine-, thiol-, and carbonyl-containing additives, since these components will also react with the labeling reagent. Do not cover or cap the container.

3.4.2. Preparing the Photoactivatable Biotin Stock Solution

1. In subdued light, prepare a photoactivatable biotin stock solution in deionized H_2O or buffer at 1–5 mg/mL and protect it from light. This solution is stable for at least a year if it is stored in the dark at –20°C.

3.4.3. Biotinylation of the Antibody

1. Determine the appropriate amount of photobiotin to use with **Eqs. 1–3** in **Subheading 3.1.1.** Use a labeling reagent: antibody MR of 5.
2. Add the labeling reagent to the antibody and mix well. This should also be performed in subdued light.
3. Chill the reaction mixture on ice and irradiate it from above with visible light at a wavelength of 350 nm. The sample should be irradiated upright at a distance of 10–20 cm from the light source for 15–20 min. The optimum wavelength of 350 nm can be obtained from a 250-W high-intensity mercury lamp. Sunlamps such as a Philips Ultrapnil (MLU300W), a National Self-Ballasted (BHRF 240-250V, 250W W-P), or a General Electric Sunlamp (RSM275W) can also be used. Appropriate eye and skin protection should be worn during the irradiation step.
4. Desalt the biotinylated antibody by dialysis or gel filtration.

3.5. Determining the Degree of Biotinylation

Use of amine-reactive biotin or desthiobiotin derivatives at the recommended MR typically leads to a degree of labeling (DOL) of three to eight biotins (or desthiobiotins) per intact IgG molecule, which is optimal for most detection paradigms. Degrees of labeling in this range have no adverse effects on antigen binding by the antibody or any of its physicochemical properties. Thus, it is not absolutely necessary to measure the DOL before using the biotinylated antibody. As long as each antibody molecule contains at least one biotin (or desthiobiotin), the former can be detected with reporter group-labeled biotin-binding proteins. However, multiple biotins (or desthiobiotins) on the antibody do enable more than one streptavidin or avidin to bind to it and will increase the detected signal intensity.

Degrees of antibody biotinylation beyond those discussed previously may result in increased background in the researchers' experiments. However, determining the DOL obtained at different biotinylation reagent: antibody MR is crucial for optimizing the reaction conditions that result in reproducible antibody labeling. A detailed discussion of the numerous methods available for determining the DOL is beyond the present scope. The reader is referred to **refs. *9–11*** for more detail. We describe here only the most popular method ***(12)***. In **Subheadings 3.5.1.** to **3.5.3.**, the terms "biotin" and "desthiobiotin" are used interchangeably.

3.5.1. The HABA Assay

The easiest and most commonly used method for measuring the average DOL is based on displacement of the deep-orange dye HABA (4'-hydroxyazobenzene-2-carboxylic acid) by biotin from avidin or streptavidin ***(12)***. When HABA binds to these proteins, a dye absorbance peak at 500 nm (A_{500}) appears. As increasing amounts of biotinylated protein are added to the complex, the biotin displaces the weaker-binding HABA from the protein. This results in a decrease in A_{500}, which is proportional to the amount of biotin added. In one variation of the assay, the biotinylated antibody is first digested to completion with the protease pronase to generate many small protein fragments, some of which are biotinylated. This procedure permits one to determine the average total number of biotins conjugated to the antibody. However, using the intact, biotinylated antibody in the HABA assay gives an estimate of the average number of accessible biotins, which is usually lower. The latter more realistically reflects how readily the labeled antibody can be recognized by reporter group-labeled biotin-binding proteins.

3.5.2. HABA Assay Setup

1. Prepare the assay buffer. The recommended buffer is 50 m*M* phosphate buffer (pH 6.0) containing 150 m*M* NaCl.
2. Dissolve avidin or streptavidin in this buffer at 0.5 mg/mL (final volume of 10 mL).

3. Prepare a biotin solution in assay buffer at a final concentration of 0.25 m*M*. Biotin is poorly soluble in aqueous solutions, so we typically dissolve the biotin powder first in a small volume of DMSO and then rapidly dilute it with buffer (pH 6.0) to the desired final volume. The solution should be stirred vigorously during this step.
4. Dissolve HABA in 10 m*M* NaOH at a concentration of 10 m*M* (2.42 mg/mL).
5. Prepare a biotin standard curve. Add 250 µL of the HABA solution to 10 mL of the avidin or streptavidin solution. Incubate the mixture at room temperature for 10 min and then withdraw two 0.9-mL aliquots. Add 100 µL of the phosphate buffer (pH 6.0) to each aliquot and determine the A_{500} of the samples. The average of these values represents the maximum A_{500} that the other samples will be compared to. Distribute 12–16 aliquots of 0.9 mL each of the HABA-avidin or streptavidin into reaction tubes. In duplicate, add increasing amounts of the biotin solution in a total volume of 100 µL to the tubes to achieve final biotin concentrations ranging from 1.2 to 23 µ*M* (1.2 to 23 nmol of biotin per tube). The linear range of the assay extends beyond 23 nmol of biotin, but it is usually not necessary to include additional points. Stir the reactions for 5–10 min at room temperature and record the A_{500} of each sample. Average the duplicate readings at each biotin concentration and subtract these values from the average starting A_{500} value. Plot the differences on the ordinate versus the nmol of biotin on the abscissa. Unweighted linear regression should be used to construct the best straight line though the points to generate the biotin standard curve.

3.5.3. Determine the DOL of a Biotinylated Antibody Sample

1. Dilute the sample in assay buffer (pH 6.0) to 1, 0.5, and 0.25 mg protein/mL. Note that **Subheadings 3.5.2.** and **3.5.3.** can be performed simultaneously.
2. Add 100-µL aliquots of the antibody dilutions in duplicate to separate 0.9 mL aliquots of the HABA-avidin or streptavidin solution and stir at room temperature for 5–10 min. If the DOL of some of the samples is very high, a precipitate may form during the incubation that should be removed by centrifugation prior to spectrophotometric analysis. The decrease in A_{500} for each sample should then be recorded. The average value from each pair of samples is used to determine the nmol of biotin in the various aliquots of antibody

tested. These values are derived from the biotin standard curve. The ratio of the nmol of biotin and the nmol of antibody assayed represents the DOL.

3. Determine the DOL. **Eq. 4** should be used to obtain this value:

$$[(\text{nmol biotin} \times \text{MW of antibody or antibody fragment} \times 10^{-6}/(\text{mg/mL of antibody or fragment} \times 0.1\ \text{mL})] = \text{mol biotin/mol antibody} = \text{DOL} \quad (\textbf{Eq. 4})$$

A brief discussion of the limitations of the HABA assay is found in **Note 8**.

4. Notes

1. The final concentration of the antibody protein in the biotinylation reaction is an important variable. Preferably, the antibody should be at 3–10 mg protein/mL. Antibodies at lower concentrations can still be labeled, but adjustments in the MR of the reactive label must then be made. Since it is often necessary to biotinylate small quantities of antibodies, the FluoReporter® Mini-Biotin-XX Protein Labeling Kit (Molecular Probes, Inc.) mentioned in 2.2 is available for researchers who want to directly biotinylate 0.1–3 mg antibody. Researchers should determine whether their antibody samples contain bovine serum albumin or gelatin, which are often added by vendors as preservatives. These protein additives are generally present at high concentrations relative to the antibody (especially for monoclonal antibodies), making them inconvenient to remove. Consequently, the biotinylation of an antibody containing a protein stabilizer requires a higher MR of labeling reagent: antibody in the reaction. The resulting biotinylated mixture of antibody and stabilizer is likely to generate high background in imaging and other applications. If researchers want to remove stabilizer proteins from antibodies, they can use various affinity chromatographic methods (e.g., protein A- or G-agarose columns). Descriptions of these affinity-based methods are beyond the present scope. The reader is referred to Chapter 7 (this volume) and to **refs. *13–15*** for protocols. Submicrogram amounts of mouse, rabbit, and human antibodies also can be indirectly biotinylated or labeled with other tags using Zenon Technology (Molecular Probes, Inc.). This mode of immunolabeling is ideal for antibodies containing stabilizer proteins or those found in ascites fluids.

2. Many reactive biotin derivatives are available and we have not described all of them. The structures of the reactive biotin derivatives discussed in this chapter are shown in **Table 1**. Reactive biotins based on biocytin (ε-*N*-[D-biotinyl]-L-lysine) are shown in **Table 2**. Typical intact IgG and IgM molecules contain approx 90 and 350 lysine residues, respectively ***(4)***. Many of these lysines are exposed on the protein surface and are available for labeling with amine-reactive biotin derivatives. For labeling amines, we recommend using succinimidyl esters of biotin (biotin-NHS esters) because of their superior amine reactivity and their ease of use. Biotin SE can be used, but we recommend biotin-X-SE or biotin-XX-SE instead. The X and XX represent 1 or 2 aminohexanoic acid moieties that are attached to the carboxyl group of biotin in order to provide a 7- or 14-atom spacer between the biotin and the reactive ester (*see* **Table 1**). After biotinylation, these spacers increase the distance between the biotins and the antibody molecules to which they are attached. This makes the biotins more accessible to reporter group-labeled biotin-binding proteins. Biotin sulfosuccinimidyl esters, which have enhanced H_2O solubility compared to SE derivatives, can also be used. Desthiobiotin-X-biotin SE or 2-iminobiotin SE are available for those who want to biotinylate antibodies on amines with biotin analogs whose binding to biotin-binding proteins is reversible ***(5,8)***. For information on other amine-reactive biotin and desthiobiotin derivatives, the reader is referred to **refs. *1–3***. For further information on biotinylation kits based on amine-reactive biotins and desthiobiotin, the reader should visit www.probes.com on the Internet. Although typical intact IgG and IgM proteins contain approx 30–40 and approx 180 cysteine residues, respectively, these are linked in inter- and intrachain disulfide bonds that contribute to the formation and maintenance of the classical Y-shaped immunoglobulin structure ***(4)***. Consequently, one or more of these disulfides must be reduced to free thiol groups before they will react with thiol-reactive biotin derivatives. This procedure is described in **Subheading 3.2.1.** It is worth noting that many researchers prefer not to reduce their antibodies because loss of biological activity may occur. However, biotinylation of antibody $F(ab')_2$ and F(ab') fragments with thiol-reactive biotins is commonly performed. The $F(ab')_2$ fragments are typically generated by pepsin digestion of whole immunoglobulins using well-established protocols ***(16)***. The free thiol-containing

F(ab') fragments are generated by treating the $F(ab')_2$ fragments with reducing agents in the presence of EDTA ***(16)***. F(ab) fragments can also be generated by digestion of antibodies with papain ***(16)***.

Biotin derivatives containing thiol-reactive groups include biotin iodoacetamide, biocytin maleimides, and biotin-HPDP (*see* **Tables 1–3**). Biotinylation with biotin-HPDP is reversible because the biotin is attached to the protein by a pyridyl disulfide bond that is cleavable with strong reducing agents like β-mercaptoethanol or sodium borohydride ***(2)***. Researchers should be aware that exposure to reducing agents may destroy the antibody's biological activity and can dissociate $F(ab')_2$ or F(ab') fragments into smaller components. A variety of biotin derivatives for labeling the carbohydrate moieties found on antibodies are available (*see* **Tables 1–3**). Carbohydrates are usually attached to the Fc portion of intact antibodies, although glycosylated F(ab) fragments have been reported ***(4)***. Biotinylation of antibodies on their carbohydrates is often preferable because the carbohydrates are at the opposite end of the antibody molecule from the antigen-binding sites. However, some monoclonal antibodies are not glycosylated and researchers should verify the presence of carbohydrate residues before attempting biotinylation by this approach. Before labeling antibody carbohydrates, however, they must be oxidized to aldehydes with periodate ion (*see* **Subheading 3.3.1.**). Biotin-XX-hydrazide, desthiobiotin-X-hydrazide, and biocytin hydrazide (enhanced H_2O solubility) are typical reactive labeling reagents for aldehydes (*see* **Tables 1–3**). Some researchers believe that the biotin hydrazone-antibody bonds formed in these reactions are relatively unstable. Thus, it is advisable to reduce the hydrazone bonds to more stable substituted hydrazide bonds by treating the conjugate with sodium cyanoborohydride (*see* **Subheading 3.3.2.**). Photoreactive biotin (photobiotin) derivatives are not commonly used to biotinylate antibodies because they are nonspecific labels that react with a variety of protein functional groups (*see* **Subheading 3.4.2.**). However, photobiotins, which typically contain azide moieties on aromatic rings, are highly reactive and they can be used under physiological conditions ***(2)***. It has been reported that the degree of biotinylation achieved with photobiotins is lower than other reactive biotins because the reactive nitrenes generated during irradiation have a short lifetime ***(2,17)***.

3. Many antibodies are available commercially as solutions in PBS (pH 7.2–7.5). This buffer is easily adjusted to pH 7.5–8.3, the optimum

for amine labeling, by adding sodium bicarbonate. Because the reactivity of lysine amines increases at basic pH, we typically adjust the antibody solution to pH 8.0–8.3 by adding to the sample one-tenth volume of a 1 *M* sodium bicarbonate solution in deionized H_2O. This yields a final bicarbonate concentration of 100 m*M*. For convenience, the 1 *M* sodium bicarbonate can be prepared in bulk, divided into convenient aliquots, and stored at –20°C for later use. Once defrosted, bicarbonate solutions can be stored at 4°C for up to 2 wk, if desired. Other buffers such as HEPES and EPPS containing tertiary amines are acceptable. However, buffers containing Tris-HCl, glycine, ethanolamine, triethylamine or other amine-containing components should not be used because these substances will react with the biotinylation reagents. Buffers containing ≤0.1% (w/v) sodium azide or thimerosal as preservatives are acceptable, but biocide concentrations higher than this should be reduced by dialysis or dilution. Antibodies containing up to 50% (v/v) glycerol can be biotinylated as described in this chapter, but the high viscosity of such solutions makes manipulating them very inconvenient. It is advisable to remove high concentrations of glycerol by dialysis before labeling. Researchers should consider always dialyzing their antibody starting materials extensively to remove unwanted buffer components and to equilibrate the protein in their buffer of choice. Lyophilized antibodies can be dissolved directly in 100 m*M* sodium bicarbonate buffer (pH 8.0–8.3) or another amine-free buffer in this pH range. Immunoglobulin M is unstable at basic pH, so buffers like PBS at pH 7.2–7.4 should be used when biotinylating these antibodies. Increasing the labeling reagent: protein MR can compensate for the lower reactivity of biotin SE derivatives in this pH range.

4. Gel filtration media such as Biogel P-30 (fine or medium) or Biogel P-6 (fine or medium; Bio-Rad Laboratories; Hercules, CA); Sephadex G-10, G-15, or G-25 (Amersham Biosciences; Piscataway, NJ); or equivalent matrices made by many other manufacturers are typically used. For optimum separation of the biotinylated antibody from unattached label, researchers should choose a gel matrix with an exclusion limit smaller than the molecular weight of the protein sample. Thus, the protein will elute in the void volume of the column while smaller unwanted components will be strongly retarded by the matrix. Neither biotin nor antibody molecules are colored, so the progress of the gel filtration column must be monitored by absorbance at 280 nm. Small quantities of biotinylated antibodies can be

separated from excess reactants by centrifugation through a variety of spin columns packed with one of the gel filtration matrices previously mentioned.

5. It is often necessary to concentrate the antibody starting material and/or the biotinylated antibody after the unreacted biotinylation reagent has been removed. A variety of centrifugable concentrators containing molecular weight-selective, low protein-binding permeable membranes are available and are preferred because they save time and minimize product losses. Researchers should use devices containing membranes with MWCO ≤50,000 to concentrate intact antibodies. Devices with lower MWCO membranes (e.g., 10,000) should be used for $F(ab)_2$ or F(ab') fragments, in particular. Other methods for concentrating protein solutions such as dialysis versus solid polyethylene glycol can be used, but these are time consuming and inconvenient and are not recommended.
6. Most reactive biotin derivatives are typically dissolved in anhydrous DMSO or DMF. Organic solvents are required to dissolve biotin SE derivatives because they are poorly soluble or insoluble in aqueous solutions. The H_2O-soluble biotin sulfosuccinimidyl esters or biocytin derivatives are preferred if researchers do not wish to expose their antibodies to organic solvents. To reduce the amount of solvent added to the antibody, working solutions of reactive biotins can be prepared at concentrations >10 mg/mL. However, the volume of label added to the antibody must be decreased accordingly to avoid changing the MR. Once the label is dissolved, it should be used as soon as possible to minimize hydrolysis of the reactive esters. Any remaining label solution should be discarded. If researchers must reuse a reactive labeling reagent solution prepared in DMSO or DMF, it can be stored at –20°C for short periods. However, the reactivity of the stored reagent will decrease over time owing to hydrolysis. Upon reuse, the MR should be increased to compensate for decreased reactivity. How much to increase the MR must be determined empirically. If H_2O-soluble biotin SE labels are used, the labeling reagent solution should be prepared immediately before use and then discarded. Stock solutions of biotin hydrazides can be stored at –20°C and reused several times.
7. Most researchers have only a small amount of antibody available. The FluoReporter® Mini-Biotin-XX Protein Labeling Kit from Molecular Probes is designed to label as little as 100 µg of antibody and

is recommended for direct biotinylation of such small quantities. Sometimes researchers have antibodies whose protein concentration is <1 mg/mL. If concentrating the protein is not an option, the labeling reagent: antibody MR should be increased to 30–40. If the antibody has a protein concentration >10 mg/mL, the MR ratio should be lowered accordingly. The antibody can also be diluted to ≤10 mg/mL.

8. Although it is a useful tool, the HABA assay is not ideal for determining the DOL for several reasons. First, it is not particularly sensitive, so researchers should consider more rigorous methods for determining the DOL if higher levels of sensitivity are desired ***(9–11)***. Second, the HABA assay is subject to considerable variability from experiment to experiment. Thus, it is essential to run the biotin standard curve each time the assay is used and to test multiple aliquots of the sample to obtain an average DOL value. However, the HABA assay is easy and inexpensive to perform, it requires no sophisticated equipment other than a spectrophotometer, and it is sensitive enough for most situations researchers are likely to encounter using the protocols described herein.

References

1. Haugland, R. P. (2002) *Handbook of Fluorescent Probes and Research Products*, 9th ed. Molecular Probes, Inc., Eugene, OR, pp. 119–134.
2. Savage, M. D., Mattson, G., Desai, S., Nielander, G. W., Morgensen, S., and Conklin, E. J. (1992) *Avidin-Biotin Chemistry: A Handbook* Pierce Chemical Company, Rockford, IL.
3. Hermanson, G. T. (1996) Avidin-Biotin Systems, in Bioconjugate Techniques, Academic Press, San Diego, CA, pp. 570–592.
4. Aslam, M. and Dent, A. (1998) *Bioconjugation. Protein Coupling Techniques for the Biomedical Sciences*. Grove's Dictionaries Inc., New York, NY.
5. Hirsch, J. D., Eslamizar, L., Filanoski, B. J., Malekzadeh, N., Haugland, R. P., Beechem, J. M., and Haugland, R. P. (2002) Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection and isolation. *Anal. Biochem.* **308,** 343–357.

6. Haugland, R. P. and You, W. W. (1996) Coupling of antibodies with biotin, in *The Protein Protocols Handbook*. (Walker, J. M., ed.), Humana Press, Totowa, NJ, pp. 293–301.
7. Haugland, R. (2000) Antibody conjugates for cell biology, in *Current Protocols in Cell Biology*, vol. 2 (Bonifacino, J., Dasso, M., Harford, J., Lippincott-Schwartz, J., and Yamada, K., eds.) John Wiley & Sons, New York, NY, pp. 16.5.1–16.5.22.
8. Fudem-Goldin, B. and Orr, G. (1990) 2-iminobiotin-containing reagent and affinity columns, in *Methods in Enzymology*, vol. 184 (Wilchek, M. and Bayer, E., eds.), Academic Press, New York, NY, pp. 167–173.
9. Der-Balian, G. P., Gomez, B., Masino, R. S., and Parce, J. W. (1990) A fluorometric method for determining the degree of biotinylation of proteins. *J. Immunol. Methods.* **126,** 281–285.
10. Rao, S. V., Anderson, K. W., and Bachas, L. G. (1997) Determination of the extent of protein biotinylations by fluorescence binding assay. *Bioconjugate Chem.* **8,** 94–98.
11. Livianiou, E., Costopoulou, D., Vassiliadou, I., Leondios, L., Nyalala, J. O., Ithakissios, D. S., et al. (2000) Analytical techniques for determining biotin. *J. Chromatog. A.* **881,** 331–343.
12. Green, N. (1965) A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. *Biochem. J.* **94,** 23c–24c.
13. Page, M. and Thorpe, R. (1996) Purification of IgG using protein A and protein G, in *The Protein Protocols Handbook*. (Walker, J. M., ed.) Humana Press, Totowa, NJ, pp. 733–734.
14. Page, M. and Thorpe. R. (1996) Purification of IgG using affinity chromatography on antigen-ligand columns, in *The Protein Protocols Handbook*. (Walker, J. M., ed.) Humana Press, Totowa, NJ, pp. 739–741.
15. Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992) *Immobilized Affinity Ligand Techniques*. Academic Press, San Diego, CA.
16. Andrew, S. M. and Titus, J. A. (2000) Fragmentation of immunoglobulin G, in *Current Protocols in Cell Biology*, vol. 2) (Bonifacino, J., Dasso, M., Harford, J., Lippincott-Schwartz, J., and Yamada, K., eds.) John Wiley & Sons, New York, NY, pp. 16.4.1–16.4.10.
17. Lacey, E. and Grant, W. N. (1987) Photobiotin as a sensitive probe for protein labeling. *Anal. Biochem.* **163,** 151–158.

12

Preparation of Colloidal Gold Probes

David Hughes

Summary

This chapter describes the production of immunogold probes for use in immunocytochemistry at the light and electron microscope levels. The protocols provide sufficient detail to enable workers to produce immunogold probes in their own laboratories. Reagents and equipment required should be readily accessible in most modern immunochemistry facilities. The protocols include descriptions of gold sol production and subsequent gold labeling of probes such as antisera. The chapter notes give suggestions for the quality control of particle production, approaches for optimizing gold probe use, and details of the latest technological advances, such ultrasmall gold particle technology.

Key Words: Colloidal gold labeling; immunochemistry; *in situ* hybridization; immunogold; nanogold; undecagold; cluster; GOLDFISH.

1. Introduction

The use of colloidal gold particles is now well established in the field of biological affinity labeling. Although the application of colloidal gold probes in microscopy has developed rapidly during the

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

last 30 yr, scientists have been interested in colloidal metal phenomena for much longer. As far back as 1857 ***(1)***, Michael Faraday, in an address to the Royal Society, recognized the reactive properties of colloidal metal particles (in particular gold) with light. Indeed, the literature shows that recognition of the potential for use of colloidal gold in microscopy goes back to the 17th century ***(2)***. Since 1933 ***(3)***, a range of methods has been devised for producing gold sols. It was not until 1971, however, that Faulk and Taylor ***(4)*** published a method for binding proteins to gold particles for use in immunocytochemistry at the electron microscope level. Nowadays, colloidal gold is incorporated into probes that are widely used in the biological sciences both for light and electron microscopy. A gold probe is an electron-dense sphere of gold coated with an immunologically active protein. The properties of gold-labeled probes that make them so suitable for light and electron microscopy studies are summarized in **Table 1**. Although high-quality colloidal gold reagents are available from a range of international manufacturers, the preparation of gold-labeled probes is a relatively straightforward procedure for workers to perform in their own laboratories. Two aspects are to be considered when making the probe, namely, gold sol production and binding the gold to the probe molecule. Such probes may be readily prepared in most well-equipped immunochemistry laboratories using combinations of the following approaches (*see* **Notes 1–4**).

1.1. Production of Gold Sol

A suspension of gold (Au) spheres is produced by the chemical reduction of yellow gold chloride (chloroauric acid) in solution. This happens in a sequence of stages ***(5)***. Initially, the reduction of Au^{3+} produces a supersaturated molecular Au solution. Nucleation is initiated when the concentration of Au increases and the gold atoms cluster and form nuclei. Particle growth proceeds with the deposition of molecular gold on the nuclei. The theoretical size of the gold is inversely proportional to the cube root of the number of nuclei formed if it is assumed that the conversion of Au^{3+} to Au is complete and that the concentration of gold remains constant ***(6)***.

Table 1
Properties and Features of Colloidal Gold as a Marker in Microscopy

- Gold probes are widely used in light microscopy transmission electron microscopy, scanning electron microscopy, freeze fracture, *in situ* hybridization, negative staining, and enzyme cytochemistry as are easy and inexpensive to prepare.
- They are readily adsorbed to a variety of macromolecules including antibodies, lectins, enzymes, lipids, high-energy phosphate compounds such as ATP, nucleic acids, and reporter probes such as streptavidin.
- Unbound gold sols and gold-labeled probes both have a long shelf life.
- Gold particles are electron dense and do not generally decay in an electron beam.
- Covalent linking of newer, smaller gold particles to macromolecules is even more stable than conventional adsorption.
- Probes offer high labeling intensity and sensitivity. The smaller the gold particle, the more sites that are labelled.
- There is no diffusion of reaction products as is often experienced in immunoenzyme labeling.
- The smaller the particle, the less size variation occurs; gold clusters in particular have a very specific size.
- Clusters show negligible tendency to aggregate compared with other gold preparations.
- Smaller gold particles in particular have high-resolution properties.
- High resolution of smaller gold clusters can be used for labeling specific sites on single biomolecules.
- The particulate nature of gold markers is especially useful in the subtle delineation of cell structures.
- Gold particles show high penetration into cells and tissues. This is especially so with the newer, smaller probes.
- Gold particles form an efficient nucleus for metallic silver enhancement—silver enhancement may be used to render gold labeling visible by EM, LM and, in some novel applications, even directly by eye.
- Gold probe labeling can be used with other affinity labeling procedures for colocalization experiments.
- Gold particles do not interfere with the functional integrity of biological compounds after attachment
- Because of the particulate nature of gold particles, they can provide quantitative data at the molecular level.
- Gold labels can be used to provide dynamic information about molecular movement and membrane conformational changes in cells.

The use of different reducing agents allows us to produce particles of a prechosen size range. The speed of reduction of the gold chloride determines how many gold nuclei are formed. In a closed system, this will determine the final size of the gold. The aim has always been to work towards monodispersed suspensions, avoiding particle aggregates, and to encourage as little variance as possible in the mean particle diameter of samples. Experience has shown that the desired routine sizes are 5, 10, and 15 nm. All these are useful for transmission electron microscope studies, and the 5 nm of gold is recommended for light microscopy (*see* **Note 5**). Gold spheres of 30 nm are occasionally used for scanning electron microscope immunocytochemistry. A 1-nm probe is now readily available commercially (*see* **Notes 6** and **7**) and is proving useful as a more efficient nucleus for silver enhancement (*see* **Notes 8–12**). The following procedures have been described in the literature as being useful for colloidal gold production:

1. White phosphorus reduction: white phosphorus, and indeed sodium or potassium thiocyanate, are fast reducers. Chloroauric acid is boiled under reflux with white phosphorus ***(7)*** dissolved in diethylether. Boiling is continued until the solution turns from a brownish shade to red—this usually takes approx 5 min. This method produces gold particles in the smaller size range (2–12 nm). The accurate production of the particle size range is not easy and, as the procedure is potentially highly dangerous, it is not commonly used.
2. Ascorbate reduction: this method ***(8)*** entails the fairly rapid addition of sodium ascorbate to chloroauric acid while stirring. If the reaction is conducted on ice, the gold particle size range is between 6 and 8 nm. Higher temperatures tend to increase the particle size. The method is also really of academic interest currently because it is not easy to control the final sphere diameter.
3. Citrate reduction: chloroauric acid is boiled under reflux with sodium citrate ***(9)*** until the solution turns red. Particle sizes achieved are in the range of 15–150 nm, and a particular diameter is chosen for a batch by adjusting the amount of citrate added to the boiling flask. This method has been used, therefore, for producing probes in the larger particle size range.
4. Combined tannic acid–citrate reduction: in 1985, Slot and Geuze ***(5)*** described a method of using two reducing agents, tannic acid and

sodium citrate, in combination to accurately control the diameter of gold particles yielded. Adjusting the amount of tannic acid in the mixture will control the sphere diameter very precisely at the point within the size range of 3–17 nm. The method produces sols with very little variance in mean particle diameter. Centrifugation to purify the end product is not usually needed as gold particle aggregates are infrequently found.

1.2. Making Gold–Protein Complexes

To be of use as immunocytochemical markers, the gold spheres in the sol have to be bound to specific ligands such as proteins (e.g., immunoglobulins) and lectins. The binding is by straightforward electrostatic absorption relying on the negative charge of the gold interacting with the positive charge of the protein and the complex obtained is highly stable. Moreover, the molecules bound to the gold retain their biological properties, an indispensable feature for immunocytochemistry. Unbound gold sol is unstable and changes color from red to blue in the presence of salts (e.g., sodium chloride). This can be used as a test for finding the amount of protein required to saturate (i.e., stabilize) a given quantity of gold sol. The protein–gold complex is isolated from excess protein and gold particle aggregates by density centrifugation. The complexes thus retrieved are stable for at least a year when refrigerated. Samples may be stored for even longer periods in 50% glycerol at –20°C.

2. Materials

1. Gold chloride crystals.
2. 1% Aqueous trisodium citrate dihydrate.
3. 25 m*M* and 0.2 *M* Potassium carbonate.
4. 0.1 *M* Hydrochloric acid.
5. 10% Aqueous sodium chloride.
6. 1% Aqueous tannic acid.
7. The protein to be complexed with the gold: it is essential that the isoelectric point of the protein is known.
8. Ultracentrifuge and 10-mL ultracentrifuge tubes.

9. All distilled water to be used should be double distilled and filtered through a 0.45-µm Millipore filter.
10. All glassware should be thoroughly cleaned and siliconized. If these precautions are not taken, the gold spheres will adhere to the side vessel walls, which is evidenced by a red coloration of the glass.
11. Electron microscope facilities with Formvar/carbon-coated grids. For complete quality control, access to a monochrome image analyser is also required.
12. 1% Aqueous polyethylene glycol (Carbowax 20).
13. Phosphate-buffered saline (PBS): 0.1 *M* phosphate, 0.15 *M* sodium chloride, pH 7.4.
14. PBS containing 0.2 mg/mL of polyethylene glycol.
15. Sodium azide (**Caution:** highly toxic).

3. Methods

There are three stages in the production of gold probes: 1) production of gold spheres, 2) estimation of the amount of protein to be added to the gold, and 3) making the required amount of probe.

3.1. Production of the Gold Spheres (5)

1. Freshly prepare a gold solution from an ampoule of gold chloride crystals by adding 1 mL of a 1% aqueous gold chloride solution to 79 mL of distilled water.
2. Prepare the reducing mixture with 4 mL of 1% trisodium citrate dihydrate, 2 mL of 1% tannic acid, 2 mL of 25 m*M* potassium carbonate and distilled water to make 20 mL.
3. Warm the solutions to 60°C and quickly add the reducing mixture to the gold solution while stirring. The temperature is critical at this stage. Evidence of sol formation is the red color of the mixture.
4. After the sol has formed, heat the mixture to boiling, and then cool. According to Slot and Geuze ***(4)***, the quantities stated here should produce 4 nm (±11.7%) particles. For 6 nm (±7.3%) particles, add 0.5 mL of potassium carbonate to the sodium citrate. The potassium carbonate counteracts the pH effect of the tannic acid. At less than 0.5 mL, the tannic acid has no effect on the pH and may be omitted. Therefore, for 8.2nm (±6.9%) particles, add 0.125 mL of tannic acid to the sodium citrate, and for 11.5 nm (±6.3%) particles, add 0.03 mL

Fig. 1. Formvar grid coated with a colloidal suspension of 14-nm gold particles prepared using the method of Slot and Geuze ***(5)***. Most gold particles are spherical singlets, doublets, or triplets (g). Occasionally, the particles are tetrahedral (t) or in clusters (c). Electron micrograph ×125,000.

of tannic acid. The sol forms within seconds if a high amount of tannic acid has been added or will take up to 60 min if the tannic acid has been omitted. A calibration curve can be constructed to enable a rapid identification of the amounts of reducing agent required to produce a given gold particle size. The electron micrograph in **Fig. 1** shows a Formvar grid preparation of 14-nm gold particles routinely prepared in the author's laboratory using the method of Slot and Geuze ***(5)***.

5. Dry a small aliquot of the gold onto a Formvar/carbon-coated 400-mesh copper grid.
6. Wash away any salts in the preparation by floating the grid, specimen-side down, on distilled water.

7. View the grid at 125,000× under the transmission electron microscope. Electron micrographs should be taken and scanned into either a monochrome or full-color image analyser for morphometric analysis. It is also possible to attach the image analysis system directly to the electron microscope and so eliminate the intermediary time-consuming photographic stage. Even the simplest of image analysis programs available nowadays will be able to give a rapid evaluation of the gold particle size, shape, distribution, and the level of aggregation. In a good preparation, at least 80–85% of gold particles should be unclustered singlets. If the size distribution is unacceptably high, the preparation can be purified by centrifugation over a continuous sucrose or glycerol density gradient ***(5)***.

3.2. Titration to Determine the Minimum Amount of Protein to Stabilize the Gold Sol (8)

1. Adjust the pH of the gold sol to 0.5 pH units above the isoelectric point of the protein to be complexed. Care should be taken when adjusting the pH because nonstabilized colloidal gold will plug the pore of the pH electrode. Take an aliquot of a few milliliters of the gold and add five drops of 1% aqueous polyethylene glycol before measuring the pH. Make the necessary adjustments to the pH and repeat until the required pH is obtained. Do not return these aliquots to the remaining colloidal gold sol. Add 0.1 *M* HCl to lower the pH or add 0.2 *M* potassium carbonate to raise the pH, which each of these performed with vortexing.
2. Measure five aliquots of 0.5 mL of gold sol.
3. Prepare five aliquots of serially diluted protein in distilled water and add one of these, while shaking, to each of the 0.5-mL gold sol aliquots.
4. After 1 min, add 0.1 mL of 10% aqueous sodium chloride to each tube. Where there is excess protein in the tubes, the sol will not change color, but in those tubes where there is insufficient protein to stabilize the gold, flocculation will have occurred and the liquid will be blue. The correct concentration of protein is the minimal amount that will inhibit flocculation. Horisberger ***(10)*** suggests that for accurate determination of color change, a spectrophotometric assay should be used.

3.3. Production of the Colloidal Gold Probe

Once the minimum amount of protein necessary to stabilize a given quantity of gold is known, any quantity of gold probe can be produced.

1. Dissolve the required amount of protein in 0.1–0.2 mL of distilled water in a centrifuge tube and add 10 mL of the gold sol.
2. After 2 min, add 1 mL of 1% aqueous polyethylene glycol solution to stabilize the gold probe ***(8)***.
3. Centrifuge the mixture at a speed depending on the size of the gold complex: 15 nm at 60,000*g* for 1 h at 4°C ***(8)***, 12 nm at 50,000*g* for 45 min at 4°C ***(4)***, 5–12 nm at 105,000*g* for 1.5 h at 4°C ***(8)***, 5 nm at 125,000*g* for 45 min at 4°C ***(5)***, and 2–3 nm at 105,000*g* for 1.5 h at 4°C ***(8)***. The pellet formed consists of two phases ***(8)***. There is a large loose part, which is the protein–gold complex. In addition, there is a tight, dense pellet on the side of the tube, which contains aggregated gold particles and gold particles that have not been fully stabilized.
4. Resuspend the loose part of the pellet in 1.5 mL of PBS containing 0.2 mg/mL of polyethylene glycol. This can be stored for up to 1 yr at 4°C. If necessary, 0.5 mg/mL of sodium azide may be added to prevent small organisms from growing in the probe. It should then be suitably diluted before use.

The previously described technique is the basis for the preparation of colloidal gold probes and the principles of probe production are identical for each type of probe. Those who are interested should refer to Slot and Geuze ***(5)***, for preparation of protein A-gold probes, Roth ***(8)*** for production of antibody–gold complexes, Tolsen et al. ***(11)*** for production of the avidin–gold complex, Horisberger ***(10)*** for production of the lectin–gold complex, and Bendayan ***(12)*** for production of the enzyme–gold complex. New applications for gold probes are regularly appearing in the literature (e.g., *see* **Notes 13** and **14**) and a range of websites are now available for keeping researchers abreast of latest technological advances (*see* **Note 15**).

4. Notes

1. There are many ways of testing the probe, but the most convincing is by using a known positive sample. Therefore, this could be a histological section, an electron micrograph specimen, or a dot-blot. Estimation of the concentration of the probe by optical density measurements is a good method to standardize the concentration of probes from one batch to another, but in addition, it is always preferable to test the performance of the probes on known positive samples.
2. It is reasonably easy to make good-quality probes in the laboratory, but the investigator has to consider nonscientific factors, such as time and cost. Many gold conjugates are available commercially, in particular the commonly used secondary antibodies used in indirect immunocytochemistry. A good compromise is to purchase the reagents that will be in constant demand and make the required quantity of those that are not readily available. When purchasing probes, it is important to seek information on the properties of the probe. Commercial gold probes are usually sold with certain variables closely controlled. The degree of particle aggregation is stated and the optical density of the solution given as a guideline to the concentration of the probe. Particle size variance will have been calculated by one of two methods. A laser diffraction particle sizer ***(13)*** might have been used or, alternatively, electron micrographs may have been made from Formvar grid preparations of the gold sol, particle sizes being measured by image analysis of the micrograph. In addition, especially when using double or triple labeling in transmission electron microscopy, the distribution of gold particle sizes and the variance should be noted—a 5-nm probe may comprise a particle size range of 3–6 nm, of which a large number of particles may potentially overlap with those from 1-nm or 10-nm samples with wide distributions.
3. Use of metallic gold probes in electron microscopy: immunogold labeling is a well-established procedure in both transmission and scanning electron microscopy. The ability to bind a range of proteins to gold probes of different but well-defined size offers great scope for single, double or even triple labeling at the electron microscopy level. The different applications for gold probes in electron microscopy have been reviewed in detail ***(14)***.
4. The use of metallic gold probes in light microscopy: using larger (e.g., 30-nm) gold labels, it is sometimes possible to build-up enough

gold label in frozen or paraffin sections to enable antigen localization at the light microscope level. This technique is no more sensitive, however, than immuno-enzyme methods. Gold particles as small as 1 μm may be located by nanometer particle video microscopy (Nanovid; **ref. *15***), but the ideal way to increase the sensitivity of immunogold labeling is by silver enhancement (immunogold–silver staining; **ref. *16***).

5. Specialist microscopy for viewing gold labeling. Because of the light reflecting properties of gold ***(17)*** and silver ***(18)*** particles, it is possible to use either darkground or epipolarization microscopy ***(19,20)*** for the enhanced visualization of both types of labeling at the light microscope level. The technique is especially useful when used in conjunction with transmitted ordinary light as other tissue structures may be examined at the same time. Epipolarization-bright field double illumination may also be used for image analysis of multilabeling immunocytochemistry ***(21)***. Immunogold visualization in highly sensitive capping experiments with leucocyte surface proteins has been demonstrated using confocal scanning microscopy ***(22)***. Such methodology has helped to bridge the gap between light and electron microscopical studies.
6. It has been reported that some commercial preparations of colloidal gold–antibody complexes may contain free active antibody. Such free antibody will compete with antibody–colloidal gold particles for antigen binding sites and may reduce labeling intensity. The presence of free protein may be identified using a simple test procedure ***(23)***.
7. The use of probes prepared by a covalent attachment procedure of gold particles and proteins has been recently described as offering a number of advantages over conventional gold probes, including better resolution, stability, uniformity, sensitivity and complete absence of aggregation ***(24)***.
8. Recent advances in colloidal gold technology: there continue to be new frontiers in gold labeling that merit exploration. These include ultrasmall colloidal gold probes, new metal clusters, clusters with novel binding functionalities, new metal cluster conjugates with other molecules, and fluorescent metal cluster probes.
9. Ultrasmall colloidal gold probes: enhanced labeling efficiency and possibly greater penetration into samples have been associated with small gold particle sizes ***(25–27)***. Colloidal gold particles in the size range of 1 to 3 nm have been generated for immunolabeling. The use

of 2–3 nm "thiocyanate-gold" probes was first described in 1986 and refinements of guidelines for preparing ultrasmall gold particles published in 1998 ***(28,29)***. Despite the potential advantages associated with enhanced labeling and penetration, ultrasmall probes have in practice been used less frequently than larger colloidal gold particles (>5 nm) mainly because the smaller particles require sophisticated imaging equipment for detection in routine cell or tissue preparations. Ultrasmall colloidal gold has several disadvantages, namely, the size range of the particles is very large and aggregation with antibodies leads to large impenetrable particles, and probe attachment is less stable.

10. Gold Clusters: an alternative approach to colloidal gold labeling of immunoprobes has been the development and use of metal cluster compounds (e.g., gold clusters; **refs. *30–32***) that are in the 0.8–1.4 nm range. Gold clusters have a core of multiple gold atoms. Undecagold has a core of 11 gold atoms with a diameter of 0.82 nm ***(33,34)***. The cluster is made water-soluble by altering covalently attached organic groups on the surface of the cluster, followed by linking to various proteins. A larger gold cluster is the 1.4-nm nanogold particle, which contains in the region of 67 gold atoms in its core and is covalently linkable to proteins ***(35)***. Silver enhancement is often needed to make the probes more visible, the smallest being undetectable even under electron microscopy. Indeed, the rationale behind many applications involves labeling with the tiny, highly penetrative clusters and then employing silver enhancement for visualization. However, enhancement is variable so that quantitative electron microscopy work is unreliable. Selected deposition of atomic gold onto the probes, instead of silver, has been suggested as a more controllable method of enhancement ***(36)***. If osmium tetroxide post fixation is required after silver enhancement, the silver shell should be stabilized by gold chloride toning ***(37,38)***.
11. Greengold: it is a chromatographically distinct component of Nanogold having a green color which, by gel filtration, runs ahead of a the similar-sized (1.4 nm) brown cluster; greengold accounts for about 50% of a nanoprobe preparation. Greengold is a highly stable, heavy atom label that has found to be most generally useful in STEM. The particle size, uniformity, scattering properties, and S/N ratio of these clusters was described by Wall in 1999 ***(39)***.

12. Fluoronanoprobes: antibody (whole immunoglobin G or fragment antigen binding fragment) can be conjugated to the 1.4-nm cluster nanogold and a fluorochrome to produce a probe that permits the correlative microscopical observation of the same cell profiles labeled in a single procedure using two imaging techniques ***(40)***. For example, high-resolution correlation between fluorescence microscopy and electron microscopy images may be performed in the same ultrathin sections and the procedure is especially useful when employed with antiphotobleaching methods ***(41–43)***.
13. Gold-facilitated autometallographic *in situ* hybridization (GOLD-FISH) has been introduced as a bright-field alternative to established FISH for direct visualization of gene amplification using conventional microscopy ***(44,45)***. Streptavidin–nanogold is used to generate bright-field gene copy signals using gold-based autometallography, catalyzed reported deposition, and a biotin-labeled probe.
14. Gold labeling may be used in the recently introduced catalyzed reporter deposition-immunogold technique where biotinylated tyramide molecules are attached the antibody-antigen complex site; the biotinylated sites are visualized by interaction with streptavidin-gold ***(46,47)***.
15. Various websites provide extensive information on gold probe preparation and use. Of particular value are the regular Nanoprobe newsletters (http://www.nanoprobes.com) and immunogold newsletters (http://www.british-biocell.co.uk).

References

1. Faraday, M. (1857) Experimental relations of gold (and other metals) to light. *Phil. Trans. Roy. Soc.* **147,** 145–181.
2. Handley, D. A. (1989) The development and application of colloidal gold as a microscopic probes, in *Colloidal Gold: Principles, Methods and Applications*, Vol 1 (Hayat, M. A., ed.), Academic Press, San Diego, pp. 1–32.
3. Weiser, H. B. (1933) *Inorganic Colloid Chemistry*, Vol 1, Wiley, New York.
4. Faulk, W. and Taylor, G. (1971) An immunogold method for the electron microscope. *Immunocytochemistry* **8,** 1081–1083.

5. Slot, J. W. and Geuze, H. J. (1985) A new method of preparing gold probes for multiple labelling cytochemistry. *Eur. J. Cell. Biol.* **90,** 533–536.
6. Frens, G. (1973) Preparation of gold dispersions of varying size: controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature* **241,** 20–22.
7. Slot, S. W. and Geuze, H. J. (1981) Sizing of protein A—colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* **90,** 533–536.
8. Roth, J. (1982) The protein A gold (pAg) technique—a qualitative and quantitative approach for antigen localisation on thin sections, in *Techniques in Immunocytochemistry*, Vol. 1 (Bullock, G. R. and Petrusz. P., eds.), Academic, London, pp. 107–133.
9. Frens, G. (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nat. Phys. Sci.* **241,** 20–22.
10. Horisberger, M. (1985) The gold method as applied to lectin cytochemistry in transmission and scanning electron microscopy, in *Techniques in Immunocytochemistry*, Vol. 3 (Bullock, G. R. and Petrusz. P., eds.), Academic, London, pp. 155–178.
11. Tolsen, N. D., Boothroyd, B., and Hopkins, C. R. (1981) Cell surface labelling with gold colloidal particles: the use of avidin and staphylococcal protein A-coated gold in conjunction with biotin and Fc-bearing ligands. *J. Microsc.* **123,** 215–226.
12. Bendayan, M. (1985) The enzyme-gold technique: a new cytochemical approach for the ultrastructural localisation of macromolecules, in *Techniques in Immunocytochemistry*,. Vol. 3 (Bullock, G. R. and Petrusz. P., eds.), Academic, London, pp. 179–201.
13. Malvern Instruments, Malvern, Worcestershire.
14. Beesley, J. E. (1985) Colloidal gold: a new revolution in marking immunocytochemistry. *Proc. RMS.* **20,** 187–196.
15. De Branbender, M., Nuydens, R., Geuens, G., Moeremans, M., and De Mey, J. (1986) The use of sub-microscopic gold particles combined with video contrast enhancement as a simple molecular probe for the living cell. *Cell Motil. Cytoskeleton.* **6,** 105–113.
16. Holgate, C. S., Jackson, P., Cowen, P., and Bird, C. C. (1983) Immunogold-silver staining: a new method of immunostaining with enhanced sensitivity. *J. Histochem. Cytochem.* **31,** 938–944.
17. De Mey, J. (1983) Colloidal gold probes in immunocytochemistry, in *Immunocytochemistry: Practical Applications in Pathology and*

Biology (Polak, J. M. and Van Noorden, S., eds.), Wright-SPG, Bristol, pp. 82–112.

18. De Waele, M., De Mey, J., Renmans, W., Labeur, C., Jochmans, K., and Van Camp, B. (1986) Potential of immunogold-silver staining for the study of leucocyte sub-populations as defined by monoclonal antibodies. *J. Histochem. Cytochem.* **34,** 1257–1263.
19. Hughes, D. (1987) Developments in IGSS: a review. *Lab. Pract.* **36,** 62–69.
20. Ellis, I. O., Bell, J., and Bancroft, J. D. (1989) Polarized incident light microscopical enhancement of immunogold and immunogold-silver preparations: Its role in immunohistochemistry. *J.Pathol.* **159,** 13–16.
21. Hughes, D. (1990) Immunocytochemistry—selecting an approach to the identification of aspirated human renal allograft endothelium and epithelium. *Trans. R. M.S.* **1,** 701–706.
22. Shotton, D. M. (1989) Confocal scanning optical microscopy and its applications for biological specimens. *J. Cell Sci.* **94,** 175–206.
23. Kramarcy, N. R. and Sealock, R. (1991) Commercial preparations of colloidal gold–antibody complexes frequently contain free active antibody. *J. Histochem. Cytochem.* **39,** 37–39.
24. Hainfield, J. F. and Furuya, F. R. (1992) A 1.4-nm gold cluster covalently attached to antibodies improves immunolabelling. *J. Histochem. Cytochem.* **40,** 177–184.
25. Robinson, J. M., Takizawa, T., and Vandré, D. D. (1998) Ultrasmall gold particles: important probes for immunocytochemistry. *Microsc. Res. Tech.* **42,** 13–23.
26. Robinson, J. M., Takizawa, T., and Vandré, D. D. (2000) Enhanced labeling efficiency using ultrasmall immunogold probes: immunocytochemistry. *J. Histochem. Cytochem.* **48,** 487–492.
27. De Valck, V., Renmans, W., Segers, E., Leunissen, J., and De Waele, M. (1991) Light microscopical detection of leukocyte cell surface antigens with a one-nanometer gold probe. *Histochemistry* **95,** 483–490.
28. Baschong, W., Lucocq, J. J., and Roth, J. (1985) 'Thiocyanate gold': small (2–3 nm) colloidal gold for affinity cytochemical labelling in electron microscopy. *Histochemistry* **83,** 409–411.
29. Baschong, W. and Stierhof, Y.-D. (1998) Preparation, use, and enlargement of ultrasmall gold particles in immunoelectron microscopy. *Microsc. Res. Techn.* **42,** 66–79.

30. Robinson, J. M., Takizawa, T., and Vandré, D. D. (2000) Applications of gold cluster compounds in immunocytochemistry and correlative microscopy: comparison with colloidal gold. *J. Microsc.* **199,** 163–179.
31. Hainfeld, J. F. and Powell, R. D. (2000) New frontiers in gold labeling. *J. Histochem. Cytochem.* **48,** 471–480.
32. Jahn, W. (1999) Review: chemical aspects of the use of gold clusters in structural biology. *J. Struct. Biol.* **127,** 106–112.
33. Frey, P. A. and Frey, T. G. (1999) Synthesis of undecagold labeling compounds and their applications in electron microscopic analysis of multiprotein complexes. *J. Struct. Biol.* **127,** 94–100.
34. Safer, D. (1999) Undecacluster labeling of proteins at reactive cysteine residues. *J. Struct. Biol.* **127,** 101–105.
35. Hainfeld, J. F. and Furuya, F. R. (1992) A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J. Histochem. Cytochem.* **40,** 177–184.
36. Weipoltshammer, K., Schofer, C., Almeder, M., and Wachtler, F. (2000) Signal enhancement at the electron microscopic level using Nanogold and gold-based autometallography. *Histochem. Cell. Biol.* **114,** 489–495.
37. Sawada, H. and Esaki, M. (2000) A practical technique to postfix nanogold-immunolabeled specimens with osmium and to embed them in Epon for electron microscopy. *J. Histochem. Cytochem.* **48,** 493–498.
38. Leitinger, G., Pabst, M. A., and Kral, K. (2000) Gold toning preserves integrity of silver enhanced immunogold particles during osmium tetroxide treatment for demonstration of a biogenic amine. *Brain Res. Brain Protoc.* **5,** 30–38.
39. Wall, J. S. (1999) Visualizing 'greengold' clusters in the STEM. *J. Struct. Biol.* **127,** 161–168.
40. Takizawa, T., Suzuki, K., and Robinson, J. M. (1998) Correlative microscopy using FluoroNanogold on ultrathin cryosections: proof of principle. *J. Histochem. Cytochem.* **46,** 1097–1102.
41. Takizawa, T. and Robinson, J. M. (2000) FluoroNanogold is a bifunctional immunoprobe for correlative fluorescence and electron microscopy. *J. Histochem. Cytochem.* **48,** 481–486.
42. Takizawa, T. and Robinson, J. M. (2000) Analysis of antiphotobleaching reagents for use with FluoroNanogold in correlative microscopy. *J. Histochem. Cytochem.* **48,** 433–436.

43. Powell, R. D., Halsey, C. M. R., and Hainfeld, J. F. (1998) Combined fluorescent and gold immunoprobes: reagents and methods for correlative light and electron microscopy. *Microsc. Res. Techn.* **42,** 2–12.
44. Tubbs, R., Pettay, J., Skacel, M., Powell, R., Stoler, M., Roche P., et al. (2002) Gold-facilitated in situ hybridization: a bright-field autometallographic alternative to fluorescence in situ hybridization for detection of Her-2/neu gene amplification. *Am. J. Pathol.* **160,** 1589–1595.
45. Tubbs, R., Skacel, M., Pettay, J., Powell, R., Myles, J., Hicks, D., et al. (2002) Inter-observer interpretative reproducibility of GOLD-FISH, a first generation gold-facilitated autometallographic bright field in situ hybridization assay for HER-2/neu amplification in invasive mammary carcinoma. *Am. J. Surg. Pathol.* **26,** 908–913.
46. Bendayan, M. (2001) Worth its weight in gold. *Science* **291,** 1363–1365.
47. Mayer, G. and Bendayan, M. (2001) Amplification methods for the immunolocalization of rare molecules in cells and tissues. *Prog. Histochem. Cytochem.* **36,** 3–85.

13

Immunogold Probes in Light Microscopy

David Hughes

Summary

Since their introduction 60 yr ago, immunolabeling procures for use in light microscopy have become increasingly sophisticated, with greater levels of sensitivity and versatility. This chapter reviews the range of immunocytochemical procedures now available, discussing the attributes of each method that must be considered when choosing the most appropriate approach for the tissue target concerned. The immunogold silver staining technique is described in detail because the method has many advantages over rival labeling procedures, including its dual applicability at the light and electron microscope levels.

Key Words: Immunocytochemistry; immunogold; silver enhancement; immunofluorescence; immunoenzyme; avidin biotin; IGSS; PAP; APAAP; epipolarization.

1. Introduction

All immunocytochemical techniques are based on a similar principle of incubating the target antigen with an appropriate antibody solution, but they may incorporate one or more from a range of dif-

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

ferent microscopically dense markers for visualizing the sites of binding. Light microscope immunocytochemistry was initiated by the classical work of Coons and co-workers in 1950, who developed the immunofluorescence technique for antigen localization ***(1,2)***. This was followed some time later in 1966 with the introduction by Nakane and Pierce of the immunoperoxidase procedure ***(3)***, after which there have been various attempts to increase the sensitivity of techniques for localizing tissue antigens, including peroxidase antiperoxidase ***(4)***, avidin biotin complex ***(5)***, and alkaline phosphatase anti-alkaline-phosphatase ***(6)***. These techniques are all still routinely used for immunocytochemistry, and the various advantages and disadvantages of each procedure that must be considered when choosing an appropriate procedure are addressed in **Table 1**. A recent review has discussed the major advances that have been made more recently in immunocytochemistry, which include increasing in label diversity, improving method sensitivity, and multiple labeling ***(10)***.

During the past two decades, it has been possible to use colloidal gold probes for immunolabeling at the light microscope level. Gold spheres, usually between 1 and 15 nm in diameter and in colloidal suspension, are coated with an immunological protein. As in the immunoenzyme techniques, the immunological protein could be one of a wide range of proteins. It could be a primary antibody, for use in a one-step system, or a secondary or tertiary antibody, for indirect labeling. It could be protein A, protein G, or, if an avidin–biotin system is used, streptavidin or an antibiotin monoclonal antibody could be coupled to the gold. Immunolabeling is conducted by incubating the antigen with the primary antibody–gold complex in the direct technique or primary antibody followed by the gold conjugate in the indirect technique. Strong immunogold labeling may sometimes be observed as its natural red/pink color under the light microscope, but frequently the labeling is too weak to detect unless a further enhancement stage is used. In 1983, Holgate et al. ***(11)*** described a novel technique that is applied in essentially the same manner as immunoenzyme staining, but involves the use of colloi-

Table 1
Relative Merits of the Various Available Immunolabeling Procedures

Technique	Advantages	Disadvantages
Direct immunofluorescence.	Rapid; simple procedure. Multiple labeling is possible using different filter combinations. Confocal microscopy may be used.	Extravagant when more than just a few target antigens are to be studied.
Indirect immunofluorescence.	Reasonably rapid. Bright signal against a dark background is very sensitive. Many primary antibodies may be incorporated into the common procedure. Multiple labeling is possible using different filter combinations. Confocal microscopy may be used.	Fading of fluorochromes. General tissue architecture is not visible. Preparations not permanent. Fluorescence microscope required. However, newer, brighter fluorochromes are more resistant to fading, especially when used with anti-fade mounting media.
Direct immunoenzyme.	Rapid. Permanent preparations. Simple nuclear counterstains may be used for studying tissue architecture in relation to immunolabeling.	Use of toxic chromogens is involved. Need to block endogenous enzyme activity. Extravagant when more than just a few target antigens are studied.
Indirect immunoenzyme procedures.	Reasonably rapid. Permanent preparations. Simple nuclear counterstains may be used for studying tissue architecture in relation to immunolabeling.	Use of toxic chromogens is involved. Need to block endogenous enzyme activity.
New build-up immunoenzyme mogens. approach based on older method *(7,8)*.	Highly sensitive. Useful for retrieving technical failures in immunocytochemistry.	Extremely laborious. Uses toxic chromogens. Need to block endogenous enzyme activity.
Immunoenzyme sandwich procedures such as peroxidase anti-peroxidase and alakaline phosphatase anti-alkaline phosphatase.	Sensitive.	Laborious. Uses toxic chromogens. Need to block endogenous enzyme activity.
Avidin/streptavidin biotin.	Sensitive.	Laborious. Toxic chromogens. Need to block endogenous biotin activity.
Colloidal gold labeling.	Relatively rapid. Sharp, black, high-resolution reaction product. Sensitive. Permanent preparations. Non-toxic reagents. Can be studied using various forms of microscopy including dark ground, epipolarization, confocal laser scanning and electron microscopy.	Prone to fading. Capricious in inexperienced hands. Temperature-dependant.
Enzyme polymer procedures. Increased sensitivity second antibody procedures *(9)*	Rapid. Sensitive.	Kits are relatively expensive.

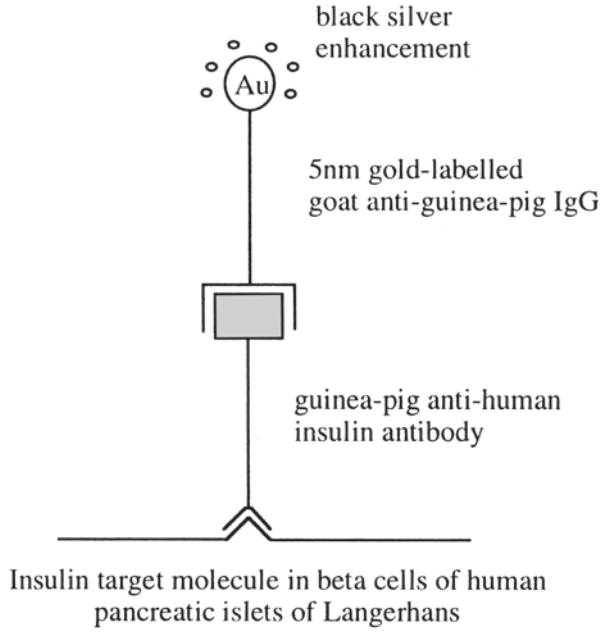


Fig. 1. Schematic representation of the indirect IGSS procedure as applied to the demonstration of insulin in human pancreatic islets of Langerhans.

dal gold-labeled antisera that are strongly visualized for light microscopy by silver enhancement (**Fig. 1**). This method is called immunogold–silver staining (IGSS) and makes use of the Danscher physical developing solution ***(12)*** to create a layer of black, metallic silver over the gold-labeled binding sites. **Table 2** describes the historical progression of the IGSS method. Danscher's reagent is a mixture of silver lactate (the silver ion source), hydroquinone (the silver reducer), and gum acacia (a colloid that prevents rapid, autoreduction of the silver lactate in solution). A range of easy-to-use, commercial alternatives to this mixture are also now available because IGSS is now an established procedure with a role both at the light microscopy and the electron microscopy level (*see* **Note 1**).

Table 2
Historical Development of IGSS

Authors	Technique
Faraday ***(13)***	First mention of gold–protein complexing and interaction with light.
Faulk and Taylor ***(14)***	Electron microscopy of gold probes established.
Danscher ***(12)***	Silver probe for gold detection in tissue sections.
Holgate et al. ***(11)***	Adaptation of the Danscher procedure for IGSS.

2. Materials

1. An antibody of known specificity and species. It does not matter whether the antibody is monoclonal or polyclonal.
2. A gold probe, preferably 5 nm in diameter or less, coated with an immunological protein specific to the primary antibody. In principle, small gold particles produce a higher labeling intensity of the target antigen because of reduced steric hindrance. If the primary antibody is from a rabbit, gold coated with protein A or anti-rabbit antiserum may be used. If the primary antibody is from mouse, gold coated with protein G or an antibody raised against the correct isotype of immunoglobin G is used. If the antibody is biotinylated, gold complexed to either streptavidin or a monoclonal antibody to biotin is used. These will be referred to as the gold probe. In the context of the final result, it does not matter which probe is used as long as it reacts with the primary antibody.
3. Lugol's iodine.
4. 5% Sodium thiosulfate.
5. Phosphate-buffered saline (PBS: 0.01 *M* sodium phosphate, 0.5 *M* NaCl, pH 7.2, as suggested by Slot and Geuze ***(15)***.
6. Heat-inactivated serum from the second antibody species (not for use with the protein A-gold technique).

7. 1% Bovine serum albumin in PBS (BSA-PBS).
8. Commercial silver enhancement kit.
9. Double-distilled water.
10. Gill's hematoxylin.
11. Industrial methylated spirit (IMS).
12. Xylene.
13. Gum acacia (500 g/L in distilled water).
14. Trisodium citrate dihydrate.
15. Citric acid.
16. Hydroquinone (0.85 g/15 mL distilled water, freshly prepared).
17. Silver lactate (0.11 g/15 mL distilled water, freshly prepared).
18. Tris-buffered saline (TBS): 0.05 *M* Tris-HCl in isotonic (0.9%) saline, pH 7.6.
19. Trypsin: 0.1% in TBS.
20. Calcium chloride.
21. Neutral buffered formalin.
22. 1% Glutaraldehyde.
23. 1% Eosin in distilled water.
24 Silver enhancement solution: the silver solution for enhancing the gold probes is prepared by mixing the following reagents in the order given and using immediately. All solutions are made in distilled water. Keep the silver lactate and the final mixture containing the silver lactate dark by wrapping the containers within foil and use in a darkroom. At the acid pH, silver lactate provides the source of silver ions necessary for deposition on the gold particles. The reducing agent for the reaction is hydroquinone and the gum acacia is included to prevent the silver from being reduced too rapidly ("autoreduction") which would lead to high background levels ("autonucleation").
25. Gum acacia (7.5 mL or 50 g/L) prepared by stirring overnight and filtering through gauze. Stock solutions may be kept in frozen aliquots.
26. Citrate buffer (10 mL) at pH 3.0. This consists of 23.5 g of trisodium citrate dihydrate and 2.5 g of citric acid monohydrate dissolved in 100 mL of distilled water.
27. Freshly prepared hydroquinone (15 mL).
28. Freshly prepared silver lactate (15 mL).

3. Methods

3.1. Paraffin Sections (Fig. 1)

1. Fix the specimen in neutral buffered formalin and embed in wax. Cut into 3- to 5-µm sections.
2. Dewax the sections in xylene (two changes, 5 min each) and rehydrate through three changes of IMS to tap water.
3. Lugol's iodine/Hypo sequence (in the author's experience, this is only required when using Danscher's reagent, and not if a commercial silver enhancement kit is used). Immerse in Lugol's iodine (5 min) and remove the iodine by rinsing thoroughly in 5% sodium thiosulfate, followed by washing in PBS.
4. Wipe excess liquid from the area around the sections and apply sufficient 5% heat-inactivated normal serum from the second antibody species to cover each entire section (15 min). If protein A-gold probes are used, flood the sections with BSA-PBS rather than normal serum. It is important to keep the sections covered and in a humid environment throughout all incubations, preferably using a purpose-made humidity chamber. Depending on the size of the section, approx 50–200 µL of reagent is sufficient to cover each preparation.
5. Tip the slides, wipe away the normal serum from around the section and replace, without rinsing, with specific primary antibody for 1 h. The appropriate dilution of the primary antibody should be decided previously either by following the manufacturer's recommendations, or by titration assay—conducting a series of dilutions and determining which gives the best signal to noise ratio.
6. Rinse the slides in BSA-PBS, three changes of 2 min each.
7. Cover the sections with gold probe, again suitably diluted in BSA-PBS following either the manufacturer's recommendations or by titration assay.
8. Rinse the slides in BSA-PBS, three changes of 2 min each.
9. If weak antibody-antigen binding is suspected, the binding may be stabilized by fixation in 1% glutaraldehyde in PBS for 10 min.
10. Rinse sections in high-quality distilled water to remove all traces of chloride ions and other impurities that might contaminate the silver enhancing solution. Some commercial enhancement solutions are

reported to be resistant to contamination; the user should evaluate this carefully, especially if high levels of nonspecific background are encountered.

11. If a commercial silver enhancement kit is used, make up enhancer immediately before use according to the manufacturer's instructions, especially with respect to times, temperatures and lighting conditions. Every 2 min, examine the progress of the enhancement using a bench microscope but be aware that some of the more sensitive enhancers, in particular Danscher's Reagent, may undergo spontaneous reduction when exposed to strong light. It may be useful to place a control slide continuously on the microscope stage; the lamp may then be turned on when required to examine the progress of enhancement (*see* **Notes 2** and **3**).
12. When the reaction has developed sufficiently, according to the investigator's preference, wash the slides first in distilled water and then in running tap water.
13. To prevent the silver intensification from fading, fix the reaction product with 5% sodium thiosulfate for 5 min and wash with tap water.
14. Counterstain as required; either 1 min in Gill's hematoxylin alone, 1% eosin alone, or hematoxylin followed by eosin will give good contrast with the black silver deposition.
15. Wash well in tap water, dehydrate in IMS, clear in xylene, and mount in a synthetic mounting medium.
16. Sites of antibody-antigen localization will appear black when viewed with transmitted white light (**Fig. 2**).
17. There are various ways of adjusting, improving and measuring the IGSS reaction product (*see* **Notes 4–11**).

3.2. Frozen Sections and Cytocentrifuge Preparations

Cryostat sections and cytocentrifuge preparations should be air-dried for at least 1 h but preferably overnight before immunostaining. Before immunolabeling, cryostat sections should be fixed in cold acetone for 10 min and cytospin slides should be fixed for 90 s in a 1:1 mixture of acetone and methanol at room temperature. After fixation, follow the IGSS method for paraffin sections from **step 3**. Cytospin preparations are usually adequately covered by standard

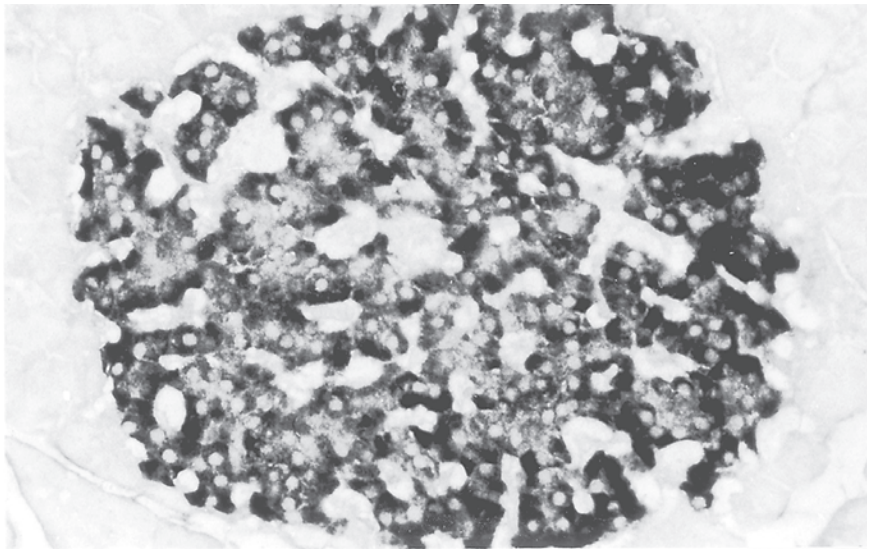


Fig. 2. Photomicrograph of β-cells in a pancreatic islet of Langerhans–IGSS labeling of insulin (magnification ×400).

25-µL aliquots of reagent. If immunolabeling is weak, the sections may be permeabilized for 5 min in 0.1% trypsin in TBS containing 0.1% calcium chloride at 37°C ***(16)***.

3.3. Cell Suspensions

De Waele et al. ***(17)*** recommended the immunolabeling of isolated cells while in suspension. After the final antibody incubation, cells are washed and cytocentrifuged and then silver enhanced and counterstained on the slide as usual. Although labeling may be stronger than in previously cytocentrifuged cells, the procedure involves centrifugation and resuspension of cell pellets after the labeling and washing stages, which may result in cell damage and depletion.

IGSS labeling has found more recent application in the fields of electron microscopy and deoxyribonucleic acid microarray technology (*see* **Notes 12** and **13**).

4. Notes

1. IGSS is still a fairly recent innovation in the fields of research and diagnostic histopathology, but the method is now making advances in some interesting applications ***(18)***. There have been reports describing labeling of leucocytes in hematology ***(19)*** and also in transplantation pathology ***(20)*** and cytology ***(21)***, the latter making use of Romanowsky counterstaining so that cells can be identified both morphologically and phenotypically at the same time. The IGSS method has been used in the monoclonal antibody diagnosis of B-cell lymphomas using paraffin-embedded material ***(22)***, and it has been suggested that cell surface antigens can be better demonstrated with periodate-lysine-paraformaldehyde-dichromate-fixed material ***(23)***. IGSS has been found to give superior results with a whole range of antisera used in routine paraffin histopathology, including regulatory peptides, intermediate filaments, and the calcium binding protein S100 ***(24)***. Using the procedures outlined in this chapter, it can be shown that IGSS offers a number of distinct advantages over other immunolabeling methods:
   - gold sols are easy and cheap to produce
   - gold particle size can be closely regulated; smaller size probes make a better nucleus for silver enhancement
   - antibody/gold conjugation is relatively straight-forward and the resulting probes remain stable for a long time if refrigerated
   - the method is economical as greater sensitivity permits incubation with more highly diluted antisera
   - IGSS preparations are permanent and can be re-examined at later dates
   - gold conjugates are non-hazardous and the procedure does not involve the use of carcinogenic chromogens. No precautions are required for handling or disposal of materials.
2. Problems with immunolabeling have been discussed in detail elsewhere ***(25,26)*** and are usually are attributed to one of the following: 1) Personal; has the operator made an error in the technique? 2) No antigen; the antigen may have been damaged during processing or there may be no antigen in the sample. 3) No antibody; the antibody may have been destroyed during preparation or there may be no specific antibody in the serum. Problems with IGSS labeling usually fall into two categories. Either there is no immunolabeling, or the

labeling is so high that the accompanying background obscures the specific labeled sites. These problems are at first sight daunting but usually the remedy is quite simple. When there is no immunolabeling, it is always advisable to run a positive control with each experiment to confirm that the reagents are functioning properly. If there is no immunolabeling on the experimental sections, the concentration of the antibody may be too low, or indeed too high. In the latter case, there is no room for the antibody to bind, therefore reducing the signal ***(25)***. It is possible that the antigen may be masked by the thickness of the section. Cut thinner sections or permeabilize the sections with a detergent, such as Triton X-100, or conduct proteolysis to unmask the antigen ***(26)***. A common problem is the fixation regime. If the fixation is too harsh, the antigenicity of the specimen will be lost, whereas if the fixation regimen is too light antigens and other material may leach from the tissue. Too much labeling is often accompanied by an unacceptably high background, and this may be caused by a very poor antibody. It is always wise to ascertain the titer and specificity of an antibody before immunolabeling and estimate the expected results. If a suitable antibody has been chosen, the concentration of immunological reagents may be too high, or there may be a nonspecific attachment of reagents to the tissue. The former is remedied by conducting a series of dilutions of the reagents and silver development times and selecting concentrations that produce high signal to low noise. High background caused by the nonspecific attachment of reagents to the specimen is reduced by including 0.5% Triton X-100 in the buffers used for diluting the reagents. A preincubation of the specimen with 1% BSA and 1% gelatin in PBS ***(26)*** will block some of these nonspecific sites and will also, if necessary, block reactive electrostatic sites on the gold probes ***(27)***. Beltz and Burd ***(25)*** recommend that the addition of up to 0.05 *M* sodium chloride should reduce nonspecific labeling by preventing ionic interactions between the sera and the tissue. They warn also that high salt can interfere with low affinity antigen-antibody binding, and this technique should be used with care. Beltz and Burd ***(25)*** also recommend that if there is trouble with background immunolabeling, the antiserum can be cross adsorbed with fresh tissue from the same host that does not contain the antigen in question. If these fail, they suggest the selection of another antibody against the same antigen. Although immunocytochemistry

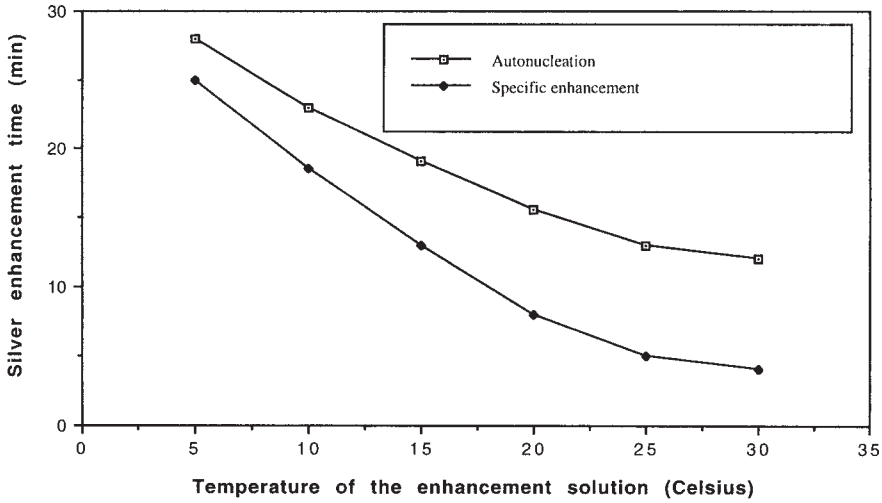


Fig. 3. Temperature-time curve for standardizing silver enhancement. Silver enhancement time in IGSS increases with lower operating temperature. The use of a calibration curve assists in optimizing the enhancement procedure.

is now a routine technique, care must still be taken to obtain optimum results. Pay particular attention to freshness of reagents, especially buffers, which will become contaminated with bacteria that may interfere with immunolabeling. During incubations, care should be taken to prevent the specimens from drying as evaporation will concentrate the antibody solutions leading to increased background deposition.

3. Problems with nonspecific background silver deposition: background silver deposition may result from the use of poor-quality antibodies, incorrectly diluted antibodies, old silver-enhancing solutions, poor-quality distilled water, or incorrect silver enhancement times. The silver enhancement procedure is temperature dependent and where laboratory temperatures vary a lot, especially at different times of the year, it is useful to construct a standardized temperature-enhancement time graph and keep it readily available at the bench (**Fig. 3**). As an example, adequate silver enhancement may take only 5 min at 25°C whereas the same result may take up to 15 min to obtain at 15°C. Enhancement times may be controlled more precisely by storing the enhancer components in the refrigerator at

Table 3
Historical Development of Specialist Microscopy Procedures for Viewing IGSS

Authors/dates	Technique
Jenkins and White (1950) ***(28)***	Reflection contrast microscopy
Ploem (1975) ***(29)***	Viewing of living cells attached to slides
De Mey (1983) ***(30)***	Epipolarization of immunogold alone
De Waele (1986) ***(31)***	Epipolarization of IGSS
Kazama et al. (1994) ***(32)***	Confocal microscopy of IGSS. Use of reflection mode especially in double labeling.
Uriel et al. (1995) ***(33)***	
Neri et al. (1997) ***(34)***	

4°C. Although autonucleation should not be a problem when using modern commercial enhancement kits, silver deposition may be avoided by performing the reaction in a darkroom with a Safelight 5902 or F904.

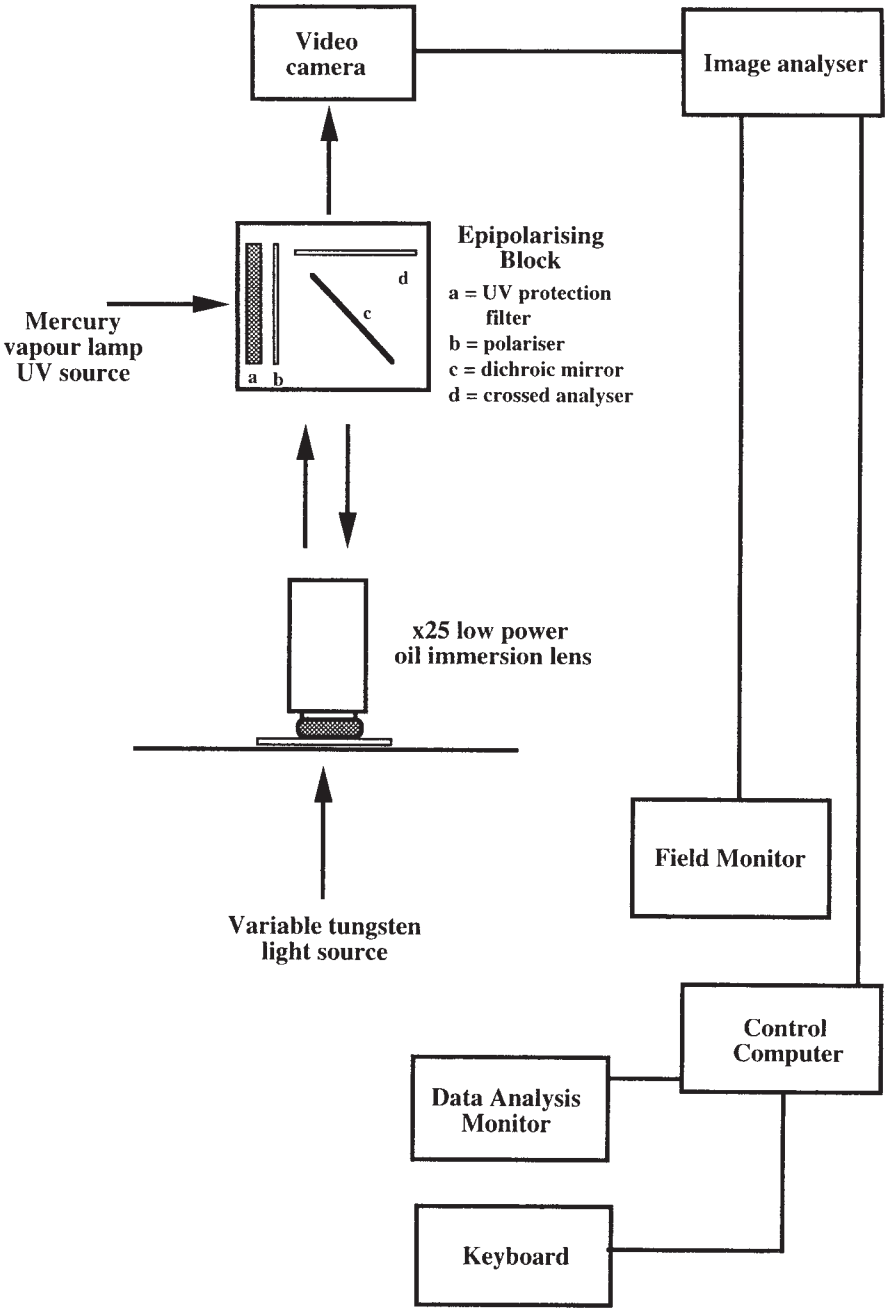
4. Epipolarization Microscopy: an interesting extension of the IGSS techniques lies with some of the optical properties of silver grains. Sites of silver deposition are strikingly demonstrated under dark ground illumination, although it is not possible to see the surrounding tissue morphology at the same time. Most fluorescence microscopes may now be adapted to permit the study of IGSS under epipolarization, a procedure that has evolved from reflection contrast microscopy (*see* **Table 3**). Powerful episcopic illumination from a mercury vapor light source passes through interchangeable filters, an adjustable diaphragm and into the special epipolarization block. This block contains a ultraviolet protection filter, a dichroic half-mirror, and an analyzer. The only light that can pass through the polarizer and return from the sample to the analyzer is the intense, back-scattered light from silver labeling (**ref. *35***; **Fig. 4**). The epipolarized light appears as a bright turquoise signal. The image is usually strong enough to allow ordinary diascopic light to be used at the same time for visualizing other tissue structures. Epipolarization is especially useful when silver enhancement is faint, in automated image analysis where the most clear, precise labeling is desirable

and also when a heavy counterstain has been used and the silver deposit is unclear. The use of water or oil, medium-power, objective lenses is recommended because problems with glare may be encountered at low power magnification when a dry objective is used. Also, as epipolarization microscopy greatly increases the sensitivity of IGSS detection, any background silver deposition will also be illuminated more sharply. Epipolarization will often still give a clear result with IGSS preparations that have either faded or turned brown after incomplete stabilization with sodium thiosulfate.

5. Two aspects of IGSS make the technique highly suitable for subsequent study using image analysis. Firstly, the intense, sharp, black reaction product is easily discriminated by either monochrome or full-color image analysis systems. Second, epipolarization may be used to provide even more discrimination between the IGSS signal and the background, which is particularly useful when a heavy counterstain has been employed for identifying other features of the preparation. A procedure has been described ***(36)*** whereby lymphocyte subpopulations labeled with IGSS may be enumerated by examining the total, hematoxylin-counterstained cell population under diascopic white light and then changing to epipolarizing illumination to count the proportion of IGSS-labeled cells that are present (**Fig. 4**). Occasionally, caution is required with the interpretation of computer-analyzed IGSS data, for example, when other black tissue components are present such as carbon particles within lung sections ***(37)***.
6. Whereas most immunohistochemical procedures may only successfully use a delicate nuclear counterstain, a wide variety of counterstains may be used after IGSS labeling, including trichromes and more selective techniques such as those used in the identification of micro-organisms. The Romanowsky procedure has been used as a counterstain for the morphological examination of lymphocyte populations previously labeled with IGSS incorporating lymphocyte subset markers ***(21,36)***. Only silver impregnation procedures and

---

Fig. 4. *(continued on opposite page)* Flow diagram illustrating the application of image analysis to IGSS using a conventional diascopic white light –1 episcopic polarized light double-illumination light microscope system. Adapted from **ref. *35*** with permission.


Video camera
Image analyser
Epipolarising Block
a = UV protection filter
b = polariser
c = dichroic mirror
d = crossed analyser
Mercury vapour lamp UV source
a
b
c
d
x25 low power oil immersion lens
Field Monitor
Variable tungsten light source
Control Computer
Data Analysis Monitor
Keyboard

techniques giving a black or near-black coloration should be avoided; epipolarization may be helpful in the latter situation but silver impregnation preparations behave variably under epipolarized light.

7. The color development of silver grains in IGSS has been used to convert the black silver signal to red, yellow, or blue–green ***(38)***. The method is a histochemical application of the chemical reactions more traditionally used in color photography.
8. The combination of IGSS with a variety of different immunoenzyme and immunofluorescence procedures has been evaluated ***(39)***, and the application of IGSS followed by immunoalkaline phosphatase seems to provide the most successful results.
9. IGSS is now being used for locating DNA probes with *in situ* hybridization, especially incorporating the new 1-nm gold labels ***(40)***.
10. Work has shown that IGSS labeling procedures can be performed more rapidly using microwave stimulation ***(41)***.
11. IGSS may also be performed on cryostat sections, which may then be plastic embedded for semithin sectioning ***(42)***.
12. Although the IGSS technique was originally introduced for use in light microscopy, the procedure has also found application in combined light microscope and electron microscope studies ***(43)*** as well as in the electron microscope field alone ***(44)***.
13. The use of silver enhancement of colloidal gold is not restricted to the microscopical study of cells. The development of microarrays has revolutionized gene expression analysis and molecular diagnosis through miniaturization and the multiparametric features. Microarrays have traditionally been detected in fluorescence, but recently a method using nanogold particles has been developed. The signal generates results from the precipitation of silver onto nanogold particles bound to streptavidin, the latter being used for detecting biotyinylated DNA ***(45)***.

References

1. Coons, A. H., Creech, H. J., and Jones, R. N. (1941) Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* **47,** 200–202.
2. Coons, A. H. and Kaplan, M. H. (1950) Localisation of antigen in tissue cells. *J. Exp. Med.* **91,** 1–13.

3. Nakane, P. K. and Pierce, G. B. (1966) Enzyme-linked antibodies: preparation and application for localisation of antigens. *J. Histochem. Cytochem.* **14,** 929–931.
4. Sternberger, L. A. (1970) The unlabelled antibody-enzyme method of histochemistry. *J. Histochem. Cytochem.* **18,** 315–333.
5. Hsu, S-M., Raine, L., and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex in immunoperoxidase techniques. *J. Histochem. Cytochem.* **29,** 577–580.
6. Cordell, J. L., Falini, B., Erber, W. N., Ghosh, A. K., Abdulaziz, Z., MacDonald, S., et al. (1984) Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP Complexes). *J. Histochem. Cytochem.* **32,** 219–229.
7. Petrusz, P., Dimeo, P., Ordronneua, P., Weaver, C., and Keefer, D. A. (1975) Improved immunoglobulin enzyme bridge method for light microscopical demonstration of hormone-containing cells of rat adenohypophysis. *Histochemie* **46,** 9–26.
8. Mangham, D. C. and Isaacson P. G. (1999) A novel immunohistochemical detection system using mirror image complementary antibodies (MICA). *Histopathology* **35,** 129–133.
9. Shi, S-R., Guo, J., Cote, R. J., Young, L. L., Hawes, D., Shi, Y., et al. (1999) Sensitivity and detection efficiency of a novel two-step detection system. (PowerVision) for immunohistochemistry. *Appl. Immunohistochem. Mol. Morphol.* **7,** 201–208.
10. Van Noorden, S. (2002) Advances in immunocytochemistry. *Folia Histochem. Cytobiol.* **40,** 12–124.
11. Holgate, C., Jackson, P., Cowen, P., and Bird, C. (1983) Immunogold-silver staining: a new method of immunostaining with enhanced sensitivity. *J. Histochem. Cytochem.* **31,** 938–944.
12. Danscher, G. (1981) Localisation of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry* **71,** 81–88.
13. Faraday, M. (1857) Experimental relations of gold (and other metals) to light. *Phil. Trans. Roy. Soc.* **147,** 145–181.
14. Faulk, W. and Taylor, G. (1971) Immunogold method for the electron microscope. *Immunocytochemistry* **8,** 1081–1083.
15. Slot, J. W. and Geuze, H. J. (1985) A new method of preparing gold probes for multiple labelling cytochemistry. *Eur. J. Cell. Biol.* **90,** 533–536.

16. Finley, J. C. W. and Perutz, P. (1982) The use of proteolytic enzymes for improved localisation™ of tissue antigens with immunocytochemistry, in *Techniques in Immunocytochemistry*, vol. 1 (Bullock, G. R., and Petrusz, P., eds.), Academic, New York, pp. 239–250.
17. De Waele, M., De Mey, K., Moeremans, M., De Brabender, M., and Van Camp, B. (1983) Immunogold staining for the light microscopic detection of leucocyte cell surface antigens with monoclonal antibodies. *J. Histochem. Cytochem.* **31,** 938–944.
18. Hughes, D. (1987) Developments in IGSS: a review. *Lab. Pract.* **36,** 62–69.
19. Romasko, F., Rosenburg, J., and Wybran, J. (1985) An immunogold silver method for the light microscopic analysis of blood lymphocyte subsets with monoclonal antibodies. *Am. J. Clin. Pathol.* **84,** 307–316.
20. Sako, H., Nakane, Y., Okino, K., Nishihara, K., Kodama, M., Paku, K., et al. (1987) Immunocytochemical study of the cells infiltrating human renal allografts by the ABC and the IGSS methods using monoclonal antibodies. *Transplantation* **44,** 43–50.
21. Hughes, D. A., Kempson, M. G., Carter, N. P., and Morris, P. J. (1988) Immunogold-silver/Romanowsky staining: simultaneous immunocytochemical and morphological analysis of fine-needle aspirate biopsies. *Transplant. Proc.* **20,** 575–576.
22. Holgate, C. S., Jackson, P., Lauder, I., Cowen, P. N., and Bird, C. C. (1983) Surface membrane staining of immunoglobulins in paraffin sections of non-Hodgkins lymphomas using the immunogold-silver technique. *J. Clin. Pathol.* **36,** 742–746.
23. Holgate, C. S., Jackson, P., Pollard, K., Lunny, D., and Bird, C. C. (1986) Effects of fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin-processed tissues. *J. Pathol.* **149,** 293–300.
24. Hacker, G. W., Springall, D. R., Van Noorden, S., Bishop, A. E., Grimelius, L., and Polak, J. M. (1985) The immunogold-silver technique: a powerful tool in histopathology. *Virchows Arch.* **406,** 449–461.
25. Beltz, B. S. and Burd, G. D. (1989) *Immunocytochemical Techniques: Principles and Practice*, Blackwell, Oxford, UK.
26. Beesley, J. E. (1989) *Colloidal gold: A new perspective for cytochemical marking. Royal Microscopical Society Handbook*, 17. Oxford Scientific Publications, Oxford, UK.

27. Behnke, O., Ammitzboll, T., Jessen, H., Klokker, M., Nilhausen, K., Tranum-Jensen, H., et al. (1986) Non-specific binding of protein-stabilised gold sols as a source of error in immunocytochemistry. *Eur. J. Cell Biol.* **41,** 326–338.
28. Jenkins, E. W. and White, H. E. (1950) *Fundamentals in Optics*, McGraw-Hill, New York.
29. Ploem, J. S. (1975) Reflection contrast microscopy as a tool for the investigation of the attachment of living cells to glass surface, in *Mononuclear Phagocytes in Immunity, Infection and Pathology* (Von Furth, R., ed.), Blackwell, Oxford, UK, pp. 405–421.
30. De Mey, J. (1983) Colloidal gold probes in immunocytochemistry, in *Immunocytochemistry: Practical Applications in Pathology and Biology* (Polak, J. M., and Van Noorden, S., eds), Wright-SPG, Bristo,l pp. 82–112.
31. De Waele, M., De Mey, J., Rewmans, W., Labeur, C., Rochmans, K., and Van Kamp, B. (1986) Potential of immunogold-silver staining for the study of leucocyte sub-populations as defined by monoclonal antibodies. *J. Histochem. Cytochem.* **34,** 1257–1263.
32. Kazama, J. J., Aikata, T., Arakawa, M., and Ozawa, H. (1994) A new histochemical double stain method using three-dimensional analysis with confocal laser scanning microscopy. *Biotech. Histochem.* **69,** 324–328.
33. Uriel, J., Lorenzo, H. K., Macho, A., de Feijter, A. W., and Mishal, Z. (1995) Visualization of silver-enhanced reaction products from protein and immuno-colloidal gold probes by laser scanning confocal microscopy in reflection mode. *Histochem. Cell. Biol.* **103,** 355–361.
34. Neri, L. M., Cinti, C., Santi, S., Marchisio, M., Capatini, S., and Maraldi, N. M. (1997) Enhanced resolution of specific chromosome and nuclear regions by reflectance laser scanning confocal microscopy. *Histochem. Cell. Biol.* **107,** 97–104.
35. Ellis, I. O., Bell, J., and Bancroft, J. D. (1989) Polarised incident light microscopical enhancement of immunogold and immunogold-silver preparations: its role in immunohistology. *J. Pathol.* **159,** 13–16.
36. Hughes, D. (1990) Immunocytochemistry—selecting a procedure for the identification of vascular endothelial and tubular epithelial cells aspirated from human renal allografts. *Trans. R. M. S.* **1,** 701–706.
37. Hughes, D. A., Fowler, S., Chaplin, A. J., and Morris, P. J. (1991) mmunogold-silver staining of leucocyte populations in lung sections containing carbon particles requires cautious interpretation. *Histochem. J.* **23,** 196–199.

38. Fritz, P., Hoenes, J., Schenk, J., Mischelinski, A., Grau, A., Saal, J. G., et al. (1986) Colour development of immunogold-labelled antibodies for light microscopy. *Histochemistry* **85,** 209–214.
39. Gillitzer, R., Berger, R., and Moll, H. (1990) A reliable method for simultaneous demonstration of two antigens using a novel combination of immunogold-silver staining and immunoenzymatic labelling. *J. Histochem. Cytochem.* **38,** 307–313.
40. Jackson, P., Lalani, E. N., and Boutsen, J. (1988) Microwave stimulated immunogold-silver staining. *Histochem. J.* **20,** 353–358.
41. Jackson. P., Dockey, D. A. Lewis, F. A., and Wells, M. (1990) Applications of 1nm gold probes on paraffin sections for in situ hybridisation histochemistry. *J. Clin. Pathol.* **43,** 810–812.
42. Gao, K, Giffin, B. F., Morris, R. E., Cardell, E. L., and Cardell, R. R. (1994) Optimal visualisation of immunogold-silver staining of hepatic PEPCK with epipolarised light microscopy. *J. Histochem. Cytochem.* **42,** 823–826.
43. Otsuki, Y., Maxwell, L. E., Magari, S., and Kubo, H. (1990) Immunogold-silver staining method for light and electron microscopic detection of lymphocyte cell surface antigens with monoclonal antibodies. *J. Histochem. Cytochem.* **38,** 1215–1221.
44. Lackie, P. M., Hennessy, R. J., Hacker, G. W., and Polak, J. M. (1985) Investigation of immunogold-silver staining by electron microscopy. *Histochemistry* **83,** 545–550.
45. Alexandre, I., Hamels, S., Dufour, S., Collet, J., Zammatteo, N., De Longueville, F., et al. (2001) Colorimetric silver detection of DNA microarrays. *Anal. Biochem.* **295,** 1–8.

14

Immunoelectron Microscope Techniques in Plant Virus Diagnosis

Daphne M. Wright

Summary

The detection of virus in samples using electron microscopy can be enhanced by the use of specific antibodies to trap particles. Antibodies can further be used to label immobilized particles on grids to aid their identification.

Key Words: Electron microscopy; virus; grid; decoration; trapping; labeling, protein A.

1. Introduction

Immunoelectron microscopy (IEM) is the term generally used for techniques that detect the specific binding of antibody to antigen that can be visualized by electron microscopy ***(1)***. The use of these techniques results in a 2- to 10,000-fold increase in particle numbers in comparison with methods not using antisera ***(1)*** and allows the transmission electron microscope (TEM) to become a sensitive tool, which the plant virus diagnostician or researcher can use to

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

visualize, specifically detect, and confirm the presence and identity of plant viruses. Only small amounts of even crude antisera and virus extract are necessary with short incubation times for all steps. Carbon-coated electron microscope grids are treated with specific antibody that is used to "trap" virus particles (antigen) that are present in a sap extract, rather like a biological "sticky tape." After trapping, the treated grids are washed thoroughly, negatively stained, and can be visualized in the TEM. Virus particles present can be specifically "labeled" by further incubation with a more concentrated antibody preparation, which has the effect of "decorating" the virus particles with an excess of antibody molecules ***(2)***. This can be further enhanced by labeling the decorated virus particles with colloidal gold particles complexed with protein A ***(3)***.

2. Materials

1. Infected material.
2. Electron microscope grids of 400 mesh or higher (i.e., 40 µm or less) previously coated with carbon.
3. Either fine watchmakers' forceps fitted with a suitable sized "o" ring enabling the forceps to be held closed when holding an electron microscope grid or reverse action forceps.
4. Glass slides.
5. Pestle and mortar.
6. Pasteur pipets and bulbs.
7. Glass rod with flattened end.
8. 2% Uranyl acetate in distilled water.
9. Filter paper (cut into 2.5-cm squares).
10. Grid box for storage.
11. Petri dish lined with Parafilm.
12. Wax pencil for labeling the Petri dish.
13. Distilled water.
14. Variable volume pipets to cover ranges: 0.5–10 µL, 5–50 µL, 50–200 µL, 200–1000 µL, and 1–5 mL.
15. Pipet tips of appropriate size.
16. Antisera (appropriate to the virus/es required).
17. Small beaker for washings.

18. Small microfuge tubes, sizes 0.5 and 1.5 mL.
19. Small hand grinder.
20. Grinding bags.
21. Buffer, for example, 0.06 *M* phosphate buffer, pH 6.5 (for others, *see* relevant methods).
22. Disposable gloves.
23. Clock timer (0–60 min).

3. Methods

The methods described are as follows:

1. Immunosorbent electron microscopy (ISEM; **ref. *4***).
2. Antiserum/virus mixture ***(1,4–6)***.
3. The use of protein A in IEM ***(3,7–9)***.

*3.1.1. ISEM (*see ***Note 1; Fig. 1****)*

This involves coating or "activating" a filmed grid with a specific antiserum, loading with an extract of virus-infected material and, finally, staining. The antiserum concentrates the virus particles and markedly increases the amount visible on the treated grid ***(10)***. This technique is especially useful for the detection of viruses that may be low in concentration, or restricted to limited areas, within the plant.

1. Using a variable volume pipet dilute required specific antiserum (*see* **Note 2**) to 1:500 (*see* **Note 3**) in 0.06 *M* phosphate buffer pH 6.5 (*see* **Note 4**) in a 0.5-mL microfuge tube.
2. In a Parafilm lined Petri-dish place 20-µL drops of the diluted antiserum (*see* **Note 5**) and, using the watchmaker's forceps, float carbon-coated grids (*see* **Note 6**), carbon-side down, on them. Incubate at room temperature for 15 min (*see* **Notes 7** and **8**).
3. Grind infected material (*see* **Note 9**) in a muslin-lined bag in phosphate buffer, pH 6.5, with a hand grinder (*see* **Note 10**) to a dilution of 1:10.
4. Place 20-µL drops of sap extract on Parafilm in a Petri dish
5. Hold the antiserum coated grids in the forceps and wash with 20 drops of phosphate buffer, in a constant stream using a Pasteur pipet, on the treated side (*see* **Note 11**). Drain excess liquid with filter paper and place on sap for 15 min at room temperature (*see* **Note 7**).

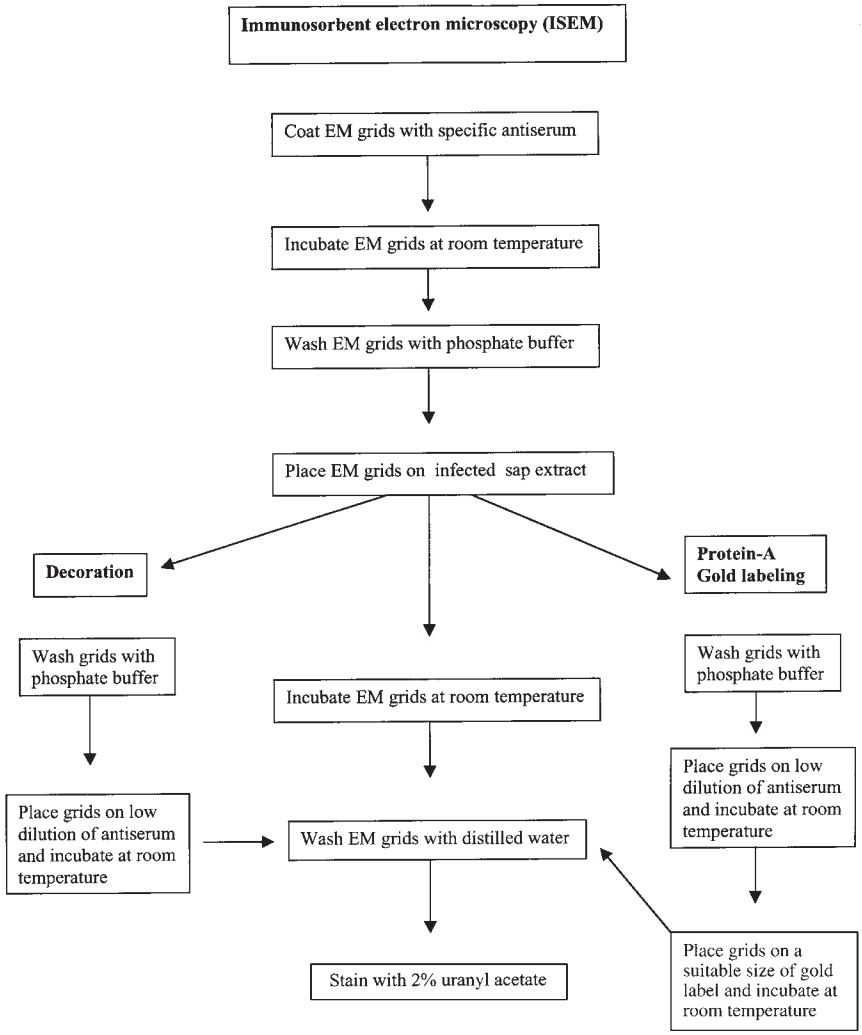


Fig. 1. Techniques involved in IEM.

6. Wash the grids with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate (*see* **Note 12**), draining excess liquid as before (*see* **Notes 13** and **14**).

*3.1.2. ISEM Combined With Antibody "Decoration" (**Fig. 1**)*

Virus particles that have been already adsorbed or "trapped" on the grid by ISEM are further incubated with the same specific anti-

body but at a lower dilution. This has the effect of "decorating" or coating the virus particles individually, thus specifically identifying them (***[2]***; *see* **Note 15**). Such flexibility is extremely valuable especially in diagnostic situations for the identification of unknown viruses or for differentiating morphologically identical viruses in mixed infections ***(10)***.

1. Using a variable volume pipet dilute required specific antiserum (*see* **Note 2**) to 1:500 in phosphate buffer pH 6.5 (*see* **Note 4**) in a 0.5-mL microfuge tube.
2. In a Parafilm lined Petri dish, place 20-µL drops of the diluted antiserum (*see* **Note 5**) and, using the watchmaker's forceps, float carbon-coated grids (*see* **Note 6**), carbon-side down, on them. Incubate at room temperature for 15 min (*see* **Note 7**).
3. Grind infected material in a muslin-lined bag in 0.06 *M* phosphate buffer, pH 6.5, with a hand grinder (*see* **Note 10**) to a dilution of 1:10.
4. Place 20-µL drops of sap extract on Parafilm in a Petri dish
5. Hold the antiserum coated grids in the forceps and wash with 20 drops of phosphate buffer ph 6.5, in a constant stream using a Pasteur pipet, on the treated side (*see* **Note 11**). Drain excess liquid with filter paper and place on sap for 15 min at room temperature (*see* **Note 7**)
6. Dilute specific antiserum to 1:100 (*see* **Note 3**) in a 0.5-mL microfuge tube and place 20-µL drops on Parafilm in a Petri dish.
7. Wash the grids with 20 drops of buffer and drain as before. Place them on the diluted antibody and incubate for 15 min at room temperature (*see* **Note 7**).
8. Wash the grids with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate (*see* **Note 12**), draining as before (*see* **Note 14**).

3.1.3. Decoration Using Mixed Antisera

This technique can be used to distinguish two (or more) morphologically similar viruses within the same sap extract ***(2,10)***.

1. Using a variable volume pipet dilute 2 (or more) specific antisera (*see* **Notes 2** and **16**) to 1:500 (*see* **Note 3**) in phosphate buffer, pH 6.5 (*see* **Note 4**), in 0.5-mL microfuge tubes.

2. In a Parafilm lined Petri dish place 20-µL drops of the diluted antiserum (*see* **Note 5**) and, using the watchmaker's forceps, float carbon-coated grids (*see* **Note 6**) carbon-side down, on them. Incubate at room temperature for 15 min (*see* **Notes 7** and **8**).
3. Grind infected material (*see* **Note 9**) in a muslin-lined bag in 0.06 *M* phosphate buffer, pH 6.5, with a hand grinder (*see* **Note 10**) to a dilution of 1:10.
4. Place 20-µL drops of sap extract on Parafilm in a Petri dish.
5. Hold the antiserum coated grids in the forceps and wash with 20 drops of phosphate buffer, pH 6.5, in a constant stream using a Pasteur pipet, on the treated side (*see* **Note 11**). Drain excess liquid with filter paper and place on sap for 15 min at room temperature (*see* **Note 7**).
6. Dilute one of the specific antisera to 1:100 (*see* **Note 3**) in a 0.5-mL Microfuge tube and place 20 µL drops on Parafilm in a Petri dish.
7. Wash the grids with 20 drops of buffer as before and drain as before. Place them on the diluted antibody and incubate for 15 min at room temperature (*see* **Note 16**).
8. Wash the grids with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate (*see* **Note 12**), draining as before (*see* **Note 14**).

3.1.4. Decoration Using Crude Sap Extracts **(6)**

1. Crush a small amount of virus infected material (*see* **Note 9**) in 10–20 µL of distilled water on a glass slide.
2. Touch a carbon-coated grid (*see* **Note 6**) on to the preparation and remove after a few seconds.
3. Wash with 30 consecutive drops of phosphate buffer pH 6.5 (*see* **Note 11**), draining with filter paper but do not dry.
4. Dilute specific antiserum to 1:100 (*see* **Note 3**) in a 0.5-mL microfuge tube and place 20-µL drops on Parafilm in a Petri dish.
5. Wash the grid with 20 drops of buffer as before and drain as before. Place it on the diluted antibody and incubate for 15 min at room temperature (*see* **Note 7**).
6. Wash the grid with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate (*see* **Note 12**), draining as before (*see* **Note 14**).

3.2. Antiserum/Virus Mixture (1,4–6)

This involves combining the antigen with the antibody and incubating the mixture. This has the effect of "clumping" the virus particles, sometimes when none have been seen by conventional electron microscopy (*see* **Note 17**), because of low concentration. If an excess of antibody is present each virus particle is surrounded by a halo of antibody molecules called "antibody coating" or "decoration" ***(1,6)***.

1. Using a variable volume, pipet dilute required specific antiserum to one-tenth of its normal precipitin titre (i.e., 1:100 for antiserum with a titre of 1:1000) in 0.06 *M* phosphate buffer, pH 6.5. Antiserum dilutions should be made fresh each time of use.
2. Squash approx 2-mm square of infected material (*see* **Note 18**) in 10–15 µL of diluted antiserum on a glass slide and incubate in a humid container at room temperature for 15 min.
3. Hold a carbon-coated grid in forceps and touch to the mixed drop, wash with 20 drops of phosphate buffer, pH 6.5, 30 drops of distilled water, and three to five drops of 2% uranyl acetate, before draining with filter paper and drying.

3.3. Use of Protein A in IEM

Protein A, obtainable as a purified product, is a *Staphylococcus aureus* bacterial cell wall protein that has the property of binding specifically to the Fc portion of IgG molecules ***(2)*** and can be used for precoating the grids prior to the ISEM procedure ***(7–9)***. This has the effect of increasing the amount of virus trapped to the grid by a modest amount but is useful when using antisera of low titre and can be used to detect distant serological relationships that would not be detected by normal ISEM ***(2)***.

3.3.1. PreCoating Electron Microscope Grids Prior to ISEM (2,8,9)

1. In a Parafilm-lined Petri dish, place carbon-coated grids, carbon film-side down, onto 15-µL drops of 0.01 mg/mL protein A in distilled water for 5 min at room temperature.

2. Wash the grids with 15 drops of phosphate buffer, pH 6.5, and transfer to 20-µL drops of antiserum diluted 1:50 in phosphate buffer, pH 6.5. Incubate at room temperature for 10 min (*see* **Note 19**).
3. Wash grids with 15 drops of phosphate buffer, pH 6.5, drain and transfer to 15-µL drops of sap extract containing virus, diluted in phosphate buffer, pH 6.5, to 1:500. Incubate at room temperature for 30 min.
4. Wash the grids with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate.
5. To decorate virus particles follow **steps 5–7** of **Subheading 3.1.2.** before staining.

3.3.2. ISEM in Combination With Antibody Decoration and Protein A Gold Labeling (PAG; **[3]***; **Fig. 1**)*

By using different sizes of colloidal gold particles, conjugated using protein A (*see* **Note 20**) to an antibody, it is possible to differentiate different virus particles by labeling with different size gold particles, that is, 5 nm, 10 nm, 20 nm, and so on. Thus it is possible to distinguishing two (or more) different viruses within a sap extract on the same grid clearly and unequivocally.

1. Using a variable volume pipet dilute required specific antiserum to 1:500 in phosphate buffer pH 6.5 in a 0.5-mL microfuge tube.
2. In a Parafilm lined Petri dish, place 20-µL drops of the diluted antiserum and, using the watchmaker's forceps, float carbon coated grids, carbon side down, on them. Incubate at room temperature for 15 min.
3. Grind infected material in a muslin-lined bag in phosphate buffer, pH 6.5, with a hand grinder, to a dilution of 1:10.
4. Place 20 µL of sap extract on Parafilm in a Petri dish.
5. Hold the antiserum-coated grids in the forceps and wash with 20 drops of phosphate buffer, pH 6.5, in a constant stream using a Pasteur pipet, on the treated side. Drain excess liquid with filter paper and place on sap for 15 min at room temperature.
6. Dilute specific antiserum to 1:100 in a 0.5-mL microfuge tube and place 20-µL drops on Parafilm in a Petri dish.
7. Wash the grids with 20 drops of buffer as before and drain as before. Place them on the diluted antibody and incubate at room temperature for 15 min.

8. Select a suitable size of PAG (*see* **Note 20**) to 1:50 in phosphate buffer pH 6.5 on the Parafilm. Wash grids as before and place them on the PAG and incubate at room temperature for 15 min.
9. Wash the grids with 20 drops of distilled water and stain with three to five drops of 2% uranyl acetate, drain as before.

3.3.3. PAG Labeling Using Mixed Virus/Antisera

1. Using a variable volume pipet, dilute two (or more) specific antisera to 1:500 in phosphate buffer, pH 6.5, in 0.5-mL microfuge tubes.
2. In a Parafilm-lined Petri dish, place 20-µL drops of the diluted antiserum and, using the watchmaker's forceps, float carbon coated grids, carbon side down, on them. Incubate at room temperature for 15 min.
3. Grind infected material of two (or more) antigens on a glass slide in phosphate buffer, pH 6.5, to a dilution of 1:10.
4. Place 20-µL drops of sap on Parafilm. Wash grids as before and place carbon-coated grids on the sap, carbon side down, replace the Petri dish lid, and incubate at room temperature for 15 min.
5. Select the first specific antiserum and dilute in phosphate buffer pH 6.5 to 1:500 in a centrifuge tube. Place 20-µL drops on the Parafilm.
6. Wash grids as before and place them on the antiserum for 15 min.
7. Dilute a small size of PAG particle, for example, 5 nm, in phosphate buffer, pH 6.5, to 1:50.
8. Wash grids as before. Drain excess liquid with filter paper and place on the PAG for 15 min.
9. Select a second specific antiserum and dilute as before.
10. Wash grids as before and place them on the second antiserum for 15 min.
11. Dilute a larger size of PAG particle, for example, 20 nm, wash grids as before, and place them on the PAG and incubate for 15 min as before.
12. Wash with 20 drops of buffer, five drops of distilled water, and stain with three drops of 2% uranyl acetate, draining excess liquid with filter paper.

3.3.4. PAG Labeling Without ISEM

1. Grind infected material on a glass slide in phosphate buffer, pH 6.5, to a dilution of 1:10.

2. Place 20-µL drops of sap on Parafilm in a Petri dish.
3. Place carbon-coated grids on the sap, carbon side down, cover with the Petri dish lid, and incubate at room temperature for 15 min.
4. Dilute specific antiserum in buffer to 1:500 and place 20-µL drops on the Parafilm.
5. Take the grids off the sap and place them on the diluted antiserum. Incubate for 15 min as before.
6. Dilute PAG in phosphate buffer to 1:50.
7. Wash grids by holding in the forceps and dropping 30 drops of phosphate buffer in a constant stream using a Pasteur pipet on the treated side. Drain excess liquid with filter paper and place on PAG for 15 min at room temperature.
8. Wash with 20 drops of buffer, five drops of distilled water, and stain with three drops of 2% uranyl acetate, drain excess liquid with filter paper.

3.4. Control Grids

It is necessary to prepare control grids to compare them with treated grids to ensure that each experiment or test is working properly and will give the information that is required. Ideally both of the following procedures should be used as controls in all experiments or tests.

3.4.1. Untreated Grids

1. Do not treat the control grids with any antibody at any stage of the procedures as described in **Subheadings 3.1.–3.3.**
2. Prepare the untreated grids, using incubation times and temperatures, in exactly the same way as the treated ones (*see* **Note 21**).

3.4.2. Coating the Grids With Normal Serum (see ***Note 22***)

1. Use an equivalent dilution of normal serum as that being used in the experiment or test as described in **Subheadings 3.1.–3.3.**
2. Follow the rest of the procedures as described in **Subheadings 3.1.–3.3.**

4. Notes

1. Originally called serologically specific electron microscopy ***(11)***, but variously known as antiserum coated grids, serum activated grids or "trapping."
2. Personal experience has shown that, although monoclonal antibodies can be used in ISEM, better results are more often obtained by using crude antiserum. Others have had the same experience ***(4)***. In addition antisera preserved in glycerol can be used successfully for detecting viruses by IEM ***(8)***.
3. Good results are obtained using a 1:500 dilution of antiserum but can also be achieved with higher dilutions (e.g., 1:1000) ***(1,2,4,6)***. Similarly although a 1:100 dilution of antiserum is used for "decorating" virus particles in the methods described here, higher or lower dilutions can be used to give the required amount of antibody 'decoration' to enable the user to be convinced that the technique has been successful.
4. Phosphate buffer pH range 6–7, 0.05–0.2 *M* has been found to be generally compatible for extracting most viruses. However, others, including Tris-HCl buffer (0.05–0.1 *M*, pH range 6.5–7.2), should be tried if results are unsatisfactory ***(4,11)***.
5. A wide range of drop sizes are cited in the literature between 5 and 50 µL ***(4)***, but 20-µL drop is a good general size to use for convenience and reproducibility.
6. There are many different support films available but carbon is the most consistently reliable. Grids can be prepared in the laboratory or purchased commercially, already carbon-coated, from specialist suppliers.
7. Good results are obtained by using a 15-min incubation time at room temperature as a general rule. However, for some antiserum/virus combinations a lower temperature, for example, 4°C, and a longer incubation time, for example, 4 h to overnight, may be preferable for viruses that are phloem restricted and/or in low concentration within the plant. This also can increase sensitivity ***(11)***. If unsatisfactory results are experienced then optimum conditions will need to be determined by experiment.

8. Grids can be dried and stored after the antiserum coating step and can be used up to 6 wk later for trapping virus particles ***(2)***.
9. As a general rule leaf material is chosen but roots, petals, pollen, seeds ***(12)***, fruit, or even virus vectors ***(13,14)*** can be suitable. Choice will depend on the concentration of virus particles, which will differ within a plant and can depend on the virus being examined.
10. There are various methods available for extracting sap. For small amounts crush in buffer on a glass slide with a flattened glass rod ***(6)***, for larger samples grind with a pestle and mortar ***(8,11)***. If an abrasive is used, for example, Carborundum or Celite, the extract may need to be centrifuged to ensure that there is a clear supernatant. In this case the recommendation would be no longer than 1 min at 10,000 rpm ***(4)***.
11. Rinsing must be conducted accurately and consistently for good results to be obtained. The general principal is that the more you rinse the better the results ***(2)***.
12. There are always preferences as to which stain to use for any given virus. An aqueous solution of uranyl acetate (UA) at 2% is a good general stain but others should be tried if this does not give the results required ***(2)***. UA is incompatible with some buffers, especially phosphate, which is the buffer of preference in this chapter. To avoid precipitation, treated grids must be washed with distilled water before UA is used ***(1,4)***. In addition UA is toxic, so users should refer to the Hazard Data Sheet produced by the manufacturer before use.
13. Grids already negatively stained in UA can be rinsed with distilled water, then phosphate buffer and then incubated with antiserum to achieve decoration, followed by re-staining with UA ***(2)***. However, personal experience has shown that this is more often than not unsuccessful and it is better to prepare a fresh grid rather than spend time on an unreliable method.
14. Treated grids can be stored in commercially available grid boxes, after staining, for later examination.
15. It has been observed that more virus particles can be found after coating with antiserum than before it ***(1)***, and it is thought that this is because coating the virus particles with the antibody binds them even more strongly to the grid, decreasing the loss of particles during the negative staining stage of the procedure ***(4)***.
16. The various antisera used to coat the grids can be used systematically (and separately) in the decoration process following this method.

17. To prepare a conventional EM grid, squash a piece of leaf material in 2% potassium phosphotungstate brought to pH 6.8 with 0.1 *M* potassium hydroxide on a glass slide using a glass rod with a flattened end. Place one drop of the sap extract on to a carbon-coated grid, dry with filter paper and examine in the TEM.
18. With high concentrations of virus, particles will form clumps before much coating with antibody has occurred. With lower concentrations of particles they become evenly coated before aggregating ***(4)***. For the latter, use the following method: dilute the sap with phosphate buffer, pH 6.5, until less than one rod shaped or filamentous particle or less than five spherical virus particles are seen per field of view at a magnification of 20,000 in a conventional preparation in the electron microscope (*see* **Note 17**). Then, follow the method as described.
19. Protein A plus antiserum coated grids can be stored for 12 mo or more at –20°C and still retain their activity ***(9)***.
20. These preparations can be bought commercially, either direct from the catalogue or using a customized conjugation service for labeling antibodies with gold particles, in a wide range of sizes suitable for different magnifications.
21. This process will represent the numbers of virus particles, which would be found by conventional electron microscopy (*see* **Note 17**) and therefore will contain far fewer compared to the trapped grid.
22. Serum from an animal that has not been used for specific antibody production ***(4)***. Alternatively, an unrelated antiserum can be used or buffer alone ***(4)***.

References

1. Milne, R. G. and Luisoni, E. (1977) Rapid immune electron microscopy of virus preparations, in *Methods in Virology* (Maramorosch, K. and Koprowski, H. eds.), Academic Press, New York, Vol. 6, pp. 265–281.
2. Milne, R. G. and Lesemann, D. E. (1984) Immunosorbent electron microscopy in plant virus studies, in *Methods in Virology* (Maramorosch, K., and Koprowski, H. eds.), Academic Press, New York, Vol. 8, pp. 85–101.
3. Louro, D. and Lesemann, D. E. (1984) Use of protein A-gold complex for specific labelling of antibodies bound to plant viruses. *J. Virol. Methods* **9,** 107–122.

4. Roberts, I. M. (1986) Immunoelectron microscopy of extracts of virus-infected plants, in *Electron Microscopy of Proteins*, Vol 5, *Viral Structure* (Harris, J. R. and Horne, R. W. eds.), Academic Press, London, pp. 293–357.
5. Ball, E. M. and Brakke, M. K. (1968) Leaf-Dip serology for electron microscopic identification of plant viruses. *Virology* **36,** 152–155.
6. Milne, R. G. and Luisoni, E. (1975) Rapid high-resolution immune electron microscopy of plant viruses. *Virology* **68,** 270–274.
7. Shukla, D. D. and Gough, K. H. (1979) The use of protein A, from *Staphylococcus aureus*, in immune electron microscopy for detecting plant virus particles. *J. Gen. Virol.* **45,** 533–536.
8. Shukla, D. D. and Gough, K. H. (1980) Further studies on the use of protein a in immune electron microscopy for detecting virus particles. *J. Gen. Virol.* **51,** 415–419.
9. Shukla, D. D. and Gough, K. H. (1983) Characteristics of the protein A-immunosorbent electron microscope technique (PA-ISEM) for detecting plant virus particles. *Acta Phytopathol. Hung.* **18,** 173–185.
10. Wright, D. M. (1984) Botanical immunocytochemistry, in *Immunolabelling for Electron Microscopy* (Polak, J. M. and Varndell, I. M. eds.), Elsevier, New York, pp. 323–340.
11. Derrick, K. S. (1973) Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* **56,** 652–653
12. Brlansky, R. H. and Derrick, K. S. (1979) Detection of seedborne plant viruses using serologically specific electron microscopy. *Phytopathology* **69,** 96–100.
13. Roberts, I. M. and Harrison, B. D. (1979) Detection of potato leafroll and potato mop-top viruses by immunosorbent electron microscopy. *Ann. Appl. Biol.* **93,** 289–297.
14. Waterhouse, P. M. and Murant, A. F. (1981) Purification of carrot red leaf virus and evidence from four serological tests for its relationship to luteoviruses. *Ann. Appl. Biol.* **97,** 191–204.

15

Dual-Antibody Sandwich Enzyme-Linked Immunosorbent Assay

William Jordan

Summary

Antibodies can be used to measures levels of molecules in solution using the dual-antibody sandwich enzyme-linked immunosorbent assay. Two antibodies are used that become bridged in the presence of the substance of interest, leading to the production of a colored product by a reporter enzyme.

Key Words: ELISA; antibodies; capture antibody; sandwich; detection antibody; substrate; dual antibody.

1. Introduction

The dual-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA; **Fig. 1**) is a sensitive and specific technique for quantifying molecules in solution. The technique is dependent upon the availability of two antibodies recognizing separate epitopes upon the antigen to be measured such that they are able to bind to the molecule simultaneously ***(1,2)***. The "capture" antibody specific to the substance to be measured is first coated onto a high-capacity

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

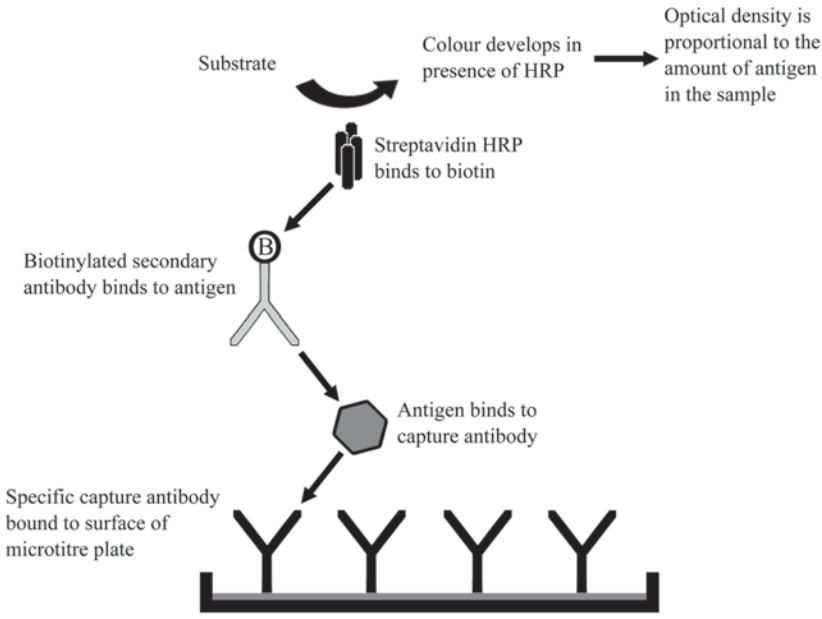


Fig. 1. The dual-antibody sandwich enzyme-linked immunosorbent assay.

protein-binding microtiter plate. Any vacant binding sites upon the plate are then blocked with the use of an irrelevant protein such as bovine serum albumin (BSA). Samples, standards, and controls are then incubated on the plate, allowing the antigen to bind to the capture antibody. The bound sample can be detected using a secondary antibody (recognizing a different epitope on the antigen), thus creating the "sandwich." The detection antibody is sometimes directly conjugated to an enzyme such as horseradish peroxidase (HRP), or, more commonly, biotin-conjugated, allowing an amplification procedure to be conducted with the use of streptavidin-HRP. As streptavidin is a tetrameric protein, binding four biotin molecules, the threshold of detection is greatly enhanced. The addition of a suitable substrate such as 3,3',5,5'-tetramethylbenzidine (TMB) results in a colorimetric reaction to occur in the presence of the HRP. The color can then be measured using a spectrophotometer with the resulting optical density (OD) relating directly to the amount of

antigen present within the sample. Comparison of OD within a sample to a standard curve of known concentrations allows the concentration within that sample to be quantified.

2. Materials

1. Antibodies: antibody "pairs" can often be bought commercially (*see* **Note 1**), comprising a capture antibody and a detection antibody that has been directly biotinylated. Aliquot and freeze the capture antibody at –20°C or lower in small, usable quantities. Most biotinylated antibodies can also be stored frozen for long-term use, although a very slight reduction in the sensitivity of the ELISA may result and it is worth testing a "test freeze" aliquot before aliquoting and freezing an entire batch.
2. Blocking buffer: phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% fatty acid-free BSA.
3. Carbonate coating buffer: For a carbonate coating buffer, use 8.41 g of Na_2HCO_3 in 1 L of water. Dissolve and adjust to desired pH (*see* **Note 2**) with HCl or NaOH. If the buffer has not been recently used check the pH before use. Store at room temperature for no longer than 1 mo or 4°C for up to 3 mo.
4. High-capacity protein binding 96-well microtiter plates: there are a large number of suitable makes including Maxisorp (Nunc), Immunoware (Pierce), Immunlon II (Dynatech), and Costar (*see* **Note 3**).
5. Plate sealers or cling film wrap: used to prevent evaporation from the plate during incubations.
6. Plate-washing apparatus: adequate washing is a vital element of achieving a successful ELISA. Although a number of automatic plate washers are available, they are expensive and the use of a wash bottle with good pressure is perfectly suitable, though a little more time consuming.
7. Samples/standards: standards of known amounts are required for positive controls, and for estimation of levels within samples (through comparison of OD values from the sample to those obtained from the 'standard curve' titration of known amounts). All standards should be diluted in a matrix as near to that of the sample solution as is possible, for example culture medium. Standards are best obtained from a reliable commercial source having been mass calibrated and

should be frozen in small, concentrated aliquots at –20°C or lower. Repeated freeze-thaw cycles must be avoided.

8. Spectrophotometer: any suitable microplate reader able to measure absorbance at the appropriate wavelength. For TMB, this is 450 nm, having stopped the reaction with 0.5 *M* H_2SO_4.
9. Stop solution: 0.5 *M* H_2SO_4.
10. Streptavidin HRP: use in accordance with the manufacturers instructions. Numerous companies sell Streptavidin–HRP, including Sigma, Becton Dickinson, and Biosource.
11. Substrate: one step TMB (Zymed) is a common choice (*see* **Note 4**). Although many other substrates are available for HRP, TMB has high sensitivity with a quick development time. OD can be monitored at 650 nm as the color develops, then at 450 nm, when the reaction is stopped with H_2SO_4. TMB may also be obtained in a lyophilized state and made up fresh with hydrogen peroxidase or as a preprepared one-step solution. TMB can vary considerably between different manufacturers, and this can affect the sensitivity and specificity of the ELISA. With one-step TMB, there is often large batch-to-batch variation as well. Each batch therefore needs to be tested before use.
12. Washing buffer: add 0.5 mL of Tween-20 to 1 L of PBS, pH 7.4. Make up fresh as required.

3. Methods

3.1. DAS ELISA Protocol

1. Dilute the capture antibody to 1 µg/mL in coating buffer pH 9.5. Add 100 µL to each well of a high capacity protein binding 96-well microtiter plate.
2. Seal the plate to avoid evaporation and incubate overnight (12–18 h) at 2–8°C.
3. Wash plate: discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with 300 µL of washing buffer, then discard, ensuring all liquid has been removed by tapping the plate onto clean paper towels. Repeat 3 times.
4. Add 200 µL of blocking buffer to each well. Seal plate and incubate for at least 2 h at room temperature.
5. Discard blocking buffer. Wash plate three times (*see* **step 3**).

6. Prepare a titration series of known standards (for example, in 1.5-mL Eppendorf tubes) diluted in a matrix representing that of the samples (e.g., culture medium). Include a negative control (i.e., culture medium only). Transfer samples and antigen standards to the ELISA plate in duplicate at 100 µL per well. Seal plate and incubate at room temperature for at least 2 h (*see* **Note 5**), or overnight at 4°C for increased sensitivity.
7. Wash plate three times (*see* **step 3**).
8. Dilute biotinylated detection antibody to 1 µg/mL in PBS, pH 7.4. Add 50 µL per well. Incubate at room temperature for 2 h. If problems with non-specific binding of the biotinylated antibody to the plate occur, dilute the antibody in PBS/Tween/1%BSA rather than just PBS.
9. Wash plate three times (*see* **step 3**).
10. Dilute streptavidin–HRP according to manufacturers instructions. Add 100 µL per well. Incubate at room temperature for 30 min.
11. Wash plate three times (*see* **step 3**).
12. Add 100 µL per well of "one-step" TMB. Allow color to develop between 5–20 min. OD may be monitored at this stage at 650 nm as the color develops.
13. Add 100 µL of 0.5 *M* H_2SO_4 to each well to stop the reaction. Read OD at 450 nm.
14. Estimate amount of antigen within samples by comparing ODs to those of known standards (*see* **Note 6**).

3.2. Optimization of ELISA

Optimization of ELISA is often required even when matched sets of antibody reagents are bought. Where a poor signal is seen at the end of the ELISA four parameters may be varied to make improvement. Each one should be varied independently and an optimum dilution factor or value determined for routine use.

1. Vary the pH of carbonate coating buffer between pH 7.0 and 10.0 in 0.5 pH unit steps. Also, try PBS at pH 7.4. Some antibodies, particularly monoclonals will bind better at one specific pH value.
2. Make a dilution of the capture antibody in coating buffer from 0.5 to 10 µg/mL and use to coat ELISA wells. The binding capacity of ELISA wells may be reduced if the concentration of antibody is not

optimal. Overdilution, and paradoxically underdilution may lead to poor well binding.
3. Make a dilution of biotinylated detection antibody from 0.05 to 1.0 µg/mL and use to determine the optimum for the reporter antibody.
4. Make dilutions of streptavidin-HRP conjugate between 1:1000 and 1:40,000 and use to determine the optimum concentration for this reagent.

4. Notes

1. The quality of the antibodies used is perhaps the most important aspect in setting up a good ELISA. Antibodies need to have a high affinity for the sample to be measured, low crossreactivity to other substances and work well together as a pair. Mismatched antibody pairs can lead to poor signals from test substances and high nonspecific background.
2. The pH of the coating buffer affects the amount of antibody, which will bind to the plate. Basically, a higher pH will result in more antibody binding, but may have a detrimental effect upon its immunoactivity. Thus a pH must be found that is suitable for the antibody in question, and this can vary dramatically. When beginning optimization of the assay, test a range of carbonate buffers from pH 7.0 to 10.0 as well as PBS pH 7.4. We usually find a carbonate buffer pH of 9.5 gives good results. In some cases we have found commercially available coating antibodies recommended by the manufacturer to be adsorbed onto the plate at pH 7.4, to be far more effectively adsorbed at higher pH's. This improves the lower detection limit of sensitivity by up to 1000%, and thus allowing the coating concentration to be vastly reduced and creating an extremely cost-effective assay.
3. There can be a significant difference between the protein binding capability of different makes, and even batches of microtiter plate. The only real way to choose a suitable plate is by trial and error or by using a recommended make known to bind the antibody that you intend to coat with.
4. Although the HRP/TMB system is usually a good, reliable, and sensitive combination, HRP has a number of alternative substrates that can be used, such as *o*-phenylene diamine. There are also numbers of options for the enzyme used other than HRP, such as alkaline phos-

phatase, which can be used in combination with the substrate *p*-nitrophenol phosphate. It is important to note that if alkaline phosphate is used, the wash buffer must not contain phosphate. Usually in this case a tris-buffered rather than phosphate buffered wash buffer is used. The choice of enzyme–substrate system depends upon a number of factors including price, sensitivity and whether a spectrophotometer filter is available for the substrate specific wavelength to be measured.

5. Occasionally, an ELISA can give rise to unexpected results in wells close to the edge of the microplate. The phenomenon is often referred to as the "edge effect" whereby unexpectedly low or high OD readings are observed. In some cases this is a result of light or heat sources. For example, if the plate is being incubated near a strong light source such as a window any reaction that is photosensitive (such as the substrate reaction) may give rise to elevated OD levels in those wells closest to the light. More commonly, temperature difference causes the edge effect. This is particularly common when plates are stacked one upon the other or where a solution is taken straight from the fridge and the incubation step is performed at 37°C for a short period of time. In such cases, the outside wells are the first to heat up and thus the reaction rate is greater there, most likely because of the faster rate of movement of molecules at this higher temperature. To avoid these complications, plates should ideally be separated from one another during incubation periods and liquids should be adjusted to the temperature that the incubation step is to be performed at. Overnight incubation steps at 4°C tend to give better results than 37°C with respect to lowering the edge effect. It is also advisable to perform incubation in the dark or in subdued light. Sealing the plates or using a 100% humidified environment can also help.
6. If the major aim of the ELISA is to obtain quantification of substances present in extremely low concentrations there are a number of adaptations to the technique that can be used. For example, Alkaline Phosphatase enzyme systems can be used to lock the colorimetric reaction into a circular redox cycle producing an end product such as red formazan. This results in a hugely amplified signal in comparison to standard amplification methods ***(3)***. Chemiluminescent-amplified ELISA principles have also been used to give very high sensitivity ***(4)***. In fact, this modification of the ELISA development stage can be optimized to measure as little as 1 zeptomole (approx 350 molecules!) of alkaline phosphatase ***(5)***. Although extremely

sensitive, such techniques are extremely time consuming to set up and optimize, and are far more expensive than the simple colorimetric ELISAs described in this chapter.

References

1. Kemeny, D. M (1991) *A Practical Guide to ELISA*. Permagon Press, Oxford, UK.
2. Kemeny, D. M. and Challacombe, S. J. (1988) *ELISA and Other Solid Phase Imunoassays. Theory and Practical Aspects*. John Wiley and Sons, Chicester, UK.
3. Self, C. H. (1985) Enzyme amplification—a general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J. Immunol. Methods* **76,** 389–393.
4. Bronstein, I., Voyta, J. C., Thorpe, G. H., Kricka, L. J., and Armstrong, G. (1989) Chemiluminescent assay of alkaline phosphatase applied in an ultrasensative enzyme immunoassay of thyrotropin. *Clin. Chem.* **35,** 1441–1446.
5. Cook, D. B. and Self, C. H. (1993). Determination of one-thousandth of an attomole (1zeptomole) of alkaline phosphatase: application in an immunoassay of proinsulin. *Clin. Chem.* **39,** 965–971.

16

Competitive Enzyme-Linked Immunosorbent Assay

William Jordan

Summary

Competitive enzyme-linked immunosorbent assay provides an alternative to dual-antibody sandwich enzyme-linked immunosorbent assay and is widely used for the measurement of substances in biological liquids.

Key Words: ELISA; competition; binding; substrate; antibody pairs; standard curve.

1. Introduction

Antibody-based enzyme-linked immunosorbent asays (ELISAs) offer robustness and high sensitivity, making them the assay of choice for many routine diagnostic assays for detecting antigen within a biological fluid. Although the dual-antibody sandwich (DAS) ELISA typically provides the highest level for specificity and sensitivity, this method requires antibody "pairs," which may not be available. In such a situation, a number of alternative ELISA techniques can be used, including the direct ELISA and the competitive

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

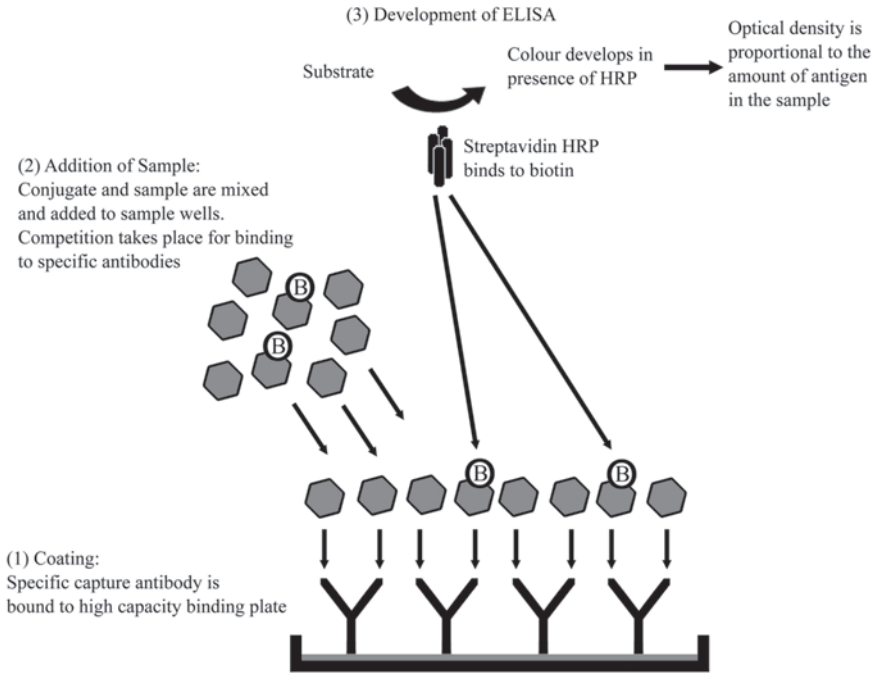


Fig. 1. Principles of a typical competitive ELISA.

ELISA. The focus of this chapter, the competitive ELISA, usually is the preferred choice when a DAS ELISA is not available because it provides greater specificity than the direct ELISA, making it more reliable for the diagnostic procedures it is being used for ***(1**,**2)***.

There are many variations and adaptations of the competitive ELISA, although the general principle for all of these remains the same. A typical representation of this technique is portrayed in **Fig. 1**. The initial stage of this technique involves coating a high capacity protein-binding microtiter plate (ELISA plate) with an antibody that is specific for the antigen to be measured. This allows sample to bind to the plate with a high affinity. The basis of the technique lies in a competition between a preprepared enzyme-conjugated form of the antigen of known concentration with the sample, for binding sites on the ELISA plate. The conjugated form can then be detected using a suitable substrate to develop color in the

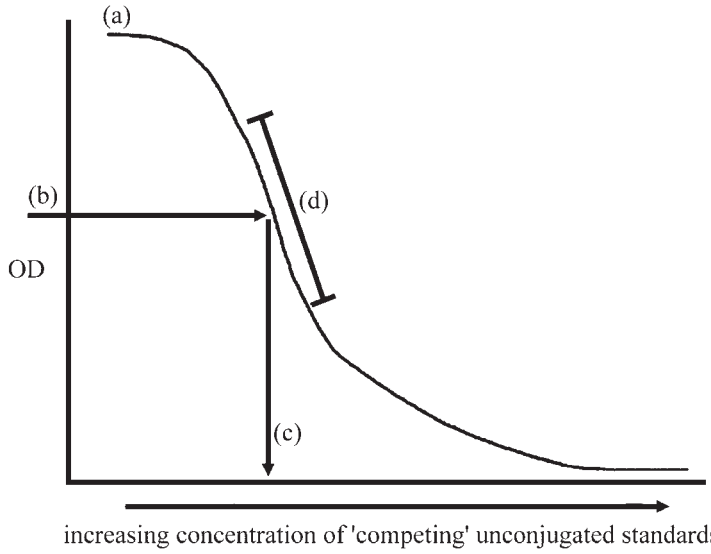


Fig. 2. Estimating antigen concentration using a typical competitive ELISA standard curve.

presence of the enzyme. A reading (OD) can then be gained using a spectrophotometer. When the sample and the conjugated antigen are mixed prior to incubation on the ELISA plate, competition between the (unconjugated) antigen within the sample interferes with the ability of the enzyme-conjugated antigen to bind the capture antibody. Thus, unlike a standard sandwich ELISA, the readout is inversely associated with the amount of antigen (*see* **Fig. 2**).

It is also worth noting the ”blocking ELISA” at this stage. In this variation of the competitive ELISA the sample to be measured is not mixed with the enzyme-conjugated antigen but is preincubated onto the coated plate prior to washing and addition of the conjugated antigen. Thus, the sample “blocks” rather than “competes” for the sites on the plate. This can result in a greater degree of sensitivity, although it is more time consuming as it relies on an additional step. The principle of the assay, however, remains the same as the competitive ELISA. Another common variation of the competitive ELISA is often used to measure levels of antibody in solution. For example in measuring the antibody response in serum to a

pathogen in order to diagnose infection. In this technique, the antigen itself is often coated onto the ELISA plate and an enzyme-conjugated "detection antibody" is used to generate the OD reading. As with the previous example, the mixture of sample (in this case serum) with the detection antibody competes for the antigen coated onto the plates resulting in a reading that can be cross-referenced with a standard curve to gain a quantitative estimate of antibody in the sample.

Irrespective of the variation of the competitive ELISA being used, careful optimization of the technique is essential. In particular, the competitive ELISA relies on careful optimization of the standard curve. During this process a known concentration of conjugated standard antigen is used to give an OD reading that is then competed-out by the antigen within the sample. The amount of conjugated antigen used has a major impact on the sensitivity of the assay. For example, if too much conjugated antigen is used then it takes a larger amount of non-conjugated antigen in the sample to result in a measurable reduction on the OD that can be used to quantify that sample, thus resulting in a low sensitivity of the assay. Using too little of the conjugated antigen results in a low initial OD reading, reducing the potential range of the ELISA. A titration of the conjugated antigen must therefore be performed as the first step in setting up a competitive ELISA. In general, a concentration giving an OD just below the maximal OD obtained is required (approx 90% is usually appropriate). In practice, the amount of conjugated antigen used depends on the sensitivity that is required from the assay.

2. Materials

1. Antibodies: antibodies can often be bought commercially (*see* **Note 1**). The choice of monoclonal or polyclonal antibody can have a significant effect on the success of the technique. In general, if the antibody is to be coated onto the plate, it is preferable to use a monoclonal antibody because the assay should only require optimiz-

ing once (polyclonals can vary tremendously between batches). The sensitivity is also often higher using a monoclonal antibody because of high-level affinity for the antigen (this is of course, antibody-dependent, but in general, binding avidity of a monoclonal is greater than a polyclonal because every antibody will bind to the antigen being measured).

If the technique is to be used for the detection of antibodies in serum for example (which will be polyclonal by nature) a polyclonal antibody may be preferable in order to compete more effectively with the antibody in the serum. Batch to batch variation in polyclonal antibodies, does, however, make the establishment of the ELISA more difficult and each the assay must be optimized every time a new batch is prepared. Antibodies may be aliquoted and frozen at –20°C or lower in small, usable quantities.

2. Blocking buffer: PBS, pH 7.4, supplemented with 1% fatty acid-free bovine serum albumin
3. Coating buffer: for carbonate coating buffer, use 8.41 g of Na_2HCO_3 in 1 L of water. Dissolve and adjust to desired pH (*see* **Note 2**) with HCl or NaOH. If the buffer has not been recently used check the pH before use. Store at room temperature for no longer than 1 mo or 4°C for up to 3 mo.
4. High-capacity protein binding 96-well microtitre plates: there are a large number of suitable makes of plate for adsorbing hydrophilic molecules such as antibodies (*see* **Note 3**). These include Maxisorp (Nunc), Immunoware (Pierce), Immunlon II (Dynatech), and Costar. If coating antigen onto the plate, however, a different plate type may be necessary. For example, hydrophobic molecules such as lipoproteins may require an alternative such as Polysorp (Nunc)
5. Plate sealers or cling film wrap: used to prevent evaporation from the plate during incubations
6. Plate-washing apparatus: adequate washing is a vital element of achieving a successful ELISA. Although a number of automatic plate washers are available, they are expensive and the use of a wash bottle with good pressure is perfectly suitable (although a little more time consuming).
7. Samples/standards: standards (enzyme conjugated) are best obtained from a reliable commercial source having been mass calibrated and

should be stored in small, concentrated aliquots. It is usually recommended that samples are stored frozen at –20°C or lower and to avoid repeated freeze-thaw cycles.

8. Spectrophotometer: any suitable microplate reader able to measure absorbance at the appropriate wavelength. For example 450 nm for the substrate tetramethylbenzidine (TMB).
9. Stop solution: 0.5 *M* H_2SO_4.
10. Streptavidin HRP: use in accordance to manufacturers instructions. Numerous companies sell Streptavidin-HRP, including Sigma, Becton Dickinson, and Biosource.
11. Substrate: one-step TMB (Zymed) is a common choice (*see* **Note 4**). Although many other substrates are available for HRP, TMB has high sensitivity with a quick development time. OD can be monitored at 650 nm as the color develops, then at 450 nm when the reaction is stopped with H_2SO_4. TMB may also be obtained in a lyophilized state and made up fresh with hydrogen peroxidase or as a preprepared solution one-step solution. TMB can vary considerably between different manufacturer's, and this can affect the sensitivity and specificity of the ELISA. With one-step TMB there is often large batch-to-batch variation as well. Each batch therefore needs to be tested before use.
12. Washing buffer: add 0.5 mL of Tween-20 to 1 L of PBS pH 7.4 (*see* **Note 5**).

3. Methods

3.1. Competitive ELISA Protocol

1. Dilute the capture antibody to 1 µg/mL in coating buffer pH 9.0. Add 100 µL to each well of a high-capacity protein binding 96-well microtiter plate.
2. Seal the plate to avoid evaporation and incubate overnight (12–18 h) at 2–8°C (*see* **Note 6**).
3. Wash plate: discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with washing buffer and leave for a couple of seconds before discarding once more. Ensure all liquid has been removed between each wash by repeatedly tapping the plate onto clean paper towels. Repeat the washing process three times to ensure that all unbound antibody is removed from the plate.

4. Add 200 µL of blocking buffer to each well. Seal plate and incubate for 1 h at room temperature.
5. Discard blocking buffer. Wash plate three times (*see* **step 3**). (Optimization of the concentration of the enzyme-conjugated antigen is required for initially establishing the competitive ELISA; *see* **Subheading 3.2.**).
6. Prepare a 2X solution of the conjugated antigen (having previously optimized this antigen concentration to result in a final OD reading approx 90% of the maximal obtained).
7. Prepare a titration series of known unconjugated standards (for example, in 1.5 mL of polypropylene tubes) diluted in a matrix representing that of the samples (e.g., culture medium or human serum). For this example the antigen is conjugated with biotin (*see* **Note 7**) which is then detected using streptavidin–HRP. Include a negative control (i.e., culture medium only). Incubate the samples and standards with the conjugated antigen. Mix well with at a 1:1 ratio with the 2X conjugated antigen. Transfer to the ELISA plate in triplicate at 100 µL per well.
8. Seal plate and incubate at room temperature for 3–4 h or overnight at 4°C for increased sensitivity.
9. Wash plate three times (*see* **step 3**).
10. Dilute streptavidin–HRP according to manufacturer's instructions. Add 100 µL per well. Incubate at room temperature for 30 min.
11. Wash plate four times (*see* **step 3**).
12. Add 100 µL per well of "one-step" TMB. Allow color to develop between 5 and 60 min (10 min is usually sufficient). OD may be monitored at this stage at 650 nm as the color develops.
13. Add 100 µL of 0.5 *M* H_2SO_4 to each well to stop the reaction. Read OD at 450 nm.
14. Estimate amount of antigen within samples by comparing ODs to those of known standards.

3.2. Optimization of ELISA

3.2.1. Optimization of Competitive ELISA

Competitive ELISA protocols require optimization for meaningful results to be obtained.

The most important factor is optimizing the amount of enzyme-conjugated antigen to add to the samples and this is achieved by producing a standard curve.

1. Follow the basic protocol (in **Subheading 3.1.**) until **step 6**.
2. Prepare a set of standards of known amounts of this conjugated antigen.
3. Incubate on the ELISA plate and complete the assay (*see* **steps 7–14**).
4. The resulting OD data are plotted and a curve of standards relating to the OD obtained.
5. The optimum antigen/enzyme conjugate concentration to use is the one giving approx 90% of the maximum value obtained using this curve.

3.2.2. Additional Optimization of Other ELISA Factors

The additional optimization of other ELISA factors may be necessary after determining the best concentration of antigen/enzyme conjugate to use (*see* **Note 8**). Three parameters may be varied to make improvement. Each one should be adjusted independently and an optimum dilution factor or value determined for routine use. Molecules of interest may be masked by components of the test fluid leading to unexpectedly low signals. This may be remedied by mixing the sample with PBS/Tween supplemented with 10% fetal bovine serum (*see* **Note 9**).

1. Vary the pH of carbonate coating buffer between pH 7.0 and 10.0 in 0.5 pH unit steps. Also try PBS at pH 7.4. Some antibodies, particularly monoclonals will bind better at a specific pH.
2. Make a dilution of the capture antibody in coating buffer from 0.5 to 10 µg/mL and use to coat ELISA wells. The binding capacity of ELISA wells may be reduced if the concentration of antibody is not optimal. Overdilution, and paradoxically underdilution may lead to poor well binding. In some competition ELISA assays up to 100 µg/mL may be required.
3. Make dilutions of streptavidin–HRP conjugate between 1:1000 and 1:10,000 and use to determine the optimum concentration for this reagent.

4. Notes

1. The quality of the antibodies used is perhaps the most important aspect in setting up a good ELISA. Antibodies should have a high affinity for the sample to be measured.
2. The pH of the coating buffer can affect the antibody, which will bind to the plate. Basically, a higher pH will result in more antibody binding but may have a detrimental effect upon its immunoactivity. Thus, a pH must be found which is suitable for the antibody in question. When beginning optimization of the assay, test a range of carbonate buffers from pH 7.0 to 10.0 as well as PBS pH 7.4. A carbonate buffer pH of 9.5 gives good results for binding antibody to a plate. In some cases, commercially available coating antibodies recommended by the manufacturer to be adsorbed onto the plate at pH 7.4 can be far more effective at higher pH's - improving the lower detection limit of sensitivity by up to 1000%, and allowing the coating concentration to be reduced.
3. There can be a significant difference between the protein binding capability of different makes, and even batches of microtitre plate. Some appear to be extremely good for binding antibodies, whilst others more useful for other proteins. The only real way to choose a suitable plate is by trial and error, or by using a recommended type known to bind the protein with which you intend to coat.
4. Although the HRP/TMB system is usually a good, reliable, and sensitive combination, HRP has a number of alternative substrates, which can be used such as *o*-phenylene diamine. There are also number of options for the enzyme used other than HRP, such as alkaline phosphatase, which can be used in combination with the substrate *p*-nitrophenyl phosphate. It is important to note that if alkaline phosphatase is used, the wash buffer must not contain phosphate. Usually in this case a Tris-buffered rather than phosphate-buffered wash buffer is used. The choice of enzyme-substrate system depends on a number of factors, including price, sensitivity and whether a spectrophotometer filter is available for the substrate specific wavelength to be measured.
5. In some cases optimal results may be achieved by using the detergent Tween-20 only during the wash after incubation with the final reactant. The reason for this, though, is not well-understood ***(3)***.

Although Tween-20 is suitable for most applications, in some cases a more gentle detergent such as CHAPS may be more suitable. This may be the case for example, when using a surface such as the Nunc Polysorp brand ***(3)***.

6. Occasionally, an ELISA can give rise to unexpected results in wells close to the edge of the microplate. The phenomenon is often referred to as the "edge effect" whereby unexpectedly low or high OD readings are observed. In some cases this is a result of light or heat sources. For example, if the plate is being incubated near a strong light source such as a window any reaction that is photosensitive (such as the substrate reaction) may give rise to elevated OD levels in those wells closest to the light. More commonly, temperature difference causes the edge effect. This is particularly common when plates are stacked one upon the other or where a solution is taken straight from the fridge and the incubation step is performed at 37°C for a short period of time. In such cases, the outside wells are the first to heat up and thus the reaction rate is greater there, most likely owing to the faster rate of movement of molecules at this higher temperature.

   To avoid these complications, plates should ideally be separated from each other during incubation periods and liquids should be adjusted to the temperature that the incubation step is to be performed at. Overnight incubation steps at 4°C tend to give better results than 37°C with respect to lowering the edge effect. It is also advisable to perform incubation in the dark or in subdued light. Sealing the plates or using a 100% humidified environment can also help.
7. The use of biotinylated secondary antibodies in conjunction with enzyme-conjugated streptavidin (or avidin, extravidin) both increases sensitivity and saves time in that a further step is eliminated from the assay and therefore another step of optimization is not required.
8. If the major aim of the ELISA is to obtain quantification of substances present in extremely low concentrations there are a number of adaptations to the technique that can be used. Such techniques often use alkaline phosphatase enzyme systems, which can be used, for example, to lock into a circular redox cycle producing an end product such as red formazan which, is hugely amplified in comparison to standard amplification methods ***(4)***. Chemiluminescent amplified ELISA principles have also been shown to give very high

sensitivity ***(5)*** and can be optimized to measure as little as 1 zeptomole (about 350 molecules!) of alkaline phosphatase ***(6,7)***. Although extremely sensitive, such techniques are extremely time consuming to set up and optimize, and are far more expensive than the simple colormetric ELISAs described in this chapter.

9. In some cases, molecules present in a sample are masked by the solution that they are in. This problem can sometimes be solved by diluting the samples in PBS/Tween/10% fetal bovine serum. If this is performed, remember to make similar adjustments to the solution used for the standards. Possible interference molecules within samples such as soluble receptors for the antigen can also cause a problem.

References

1. Kemeny, D. M. and Challacombe, S. J. (1988) *ELISA and Other Solid Phase Imunoassays. Theory and Practical Aspects*. John Wiley and Sons, Chicester, UK.
2. Kemeny, D. M (1991) *A Practical Guide to ELISA*. Permagon Press, Oxford, UK.
3. Esser, P. (1990) *Detergent in Polystyrene ELISA*. Nunc Bulletin No. 8, second edition 1997, 1–5.
4. Jenny, R. J., Messier, T. L., Ouellette, L. A., and Church, W. R. (1993) Immunochemical techniques for studying coagulation proteins. *Methods Enzymol.* **222,** 400–416.
5. Bronstein, I., Voyta, J. C., Thorpe, G. H., Kricka, L. J., and Armstrong, G. (1989) Chemiluminescent assay of alkaline phosphatase applied in an ultrasensative enzyme immunoassay of thyrotropin. *Clin. Chem.* **35,** 1441–1446.
6. Cook, D. B. and Self, C. H. (1993) Determination of one-thousandth of an attomole (1zeptomole) of alkaline phosphatase: application in an immunoassay of proinsulin. *Clin. Chem.* **39,** 965–971.
7. Self, C. H. (1985) Enzyme amplification—a general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J. Immunological Methods* **76,** 389–393.

17

Immunoblotting Techniques

Barbara Magi and Sabrina Liberatori

Summary

Immunoblotting techniques use antibodies (or other specific ligands in related techniques) to identify target proteins among a number of unrelated protein species. They involve identification of protein target via antigen–antibody (or protein–ligand) specific reactions. Proteins are typically separated by electrophoresis and transferred onto membranes (usually nitrocellulose). The membrane is overlaid with a primary antibody for a specific target and then with a secondary antibody labeled, for example, with enzymes or with radioisotopes. When the ligand is not an antibody, the reaction can be visualized using a ligand that is directly labeled. Dot blot is a simplified procedure in which protein samples are not separated by electrophoresis but are spotted directly onto membrane. Immunoblotting is now widely used in conjunction with two-dimensional polyacrylamide gel electrophoresis, not only for traditional goals, such as the immunoaffinity identification of proteins and analysis of immune responses but also as a genome–proteome interface technique.

Key Words: Western blotting; immunoblotting; electrophoresis; 2D electrophoresis; immunoaffinity identification; immunoblotting techniques.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

1. Introduction

Immunoblotting, also dubbed Western blotting, exploits antibody specificity to identify target proteins among a number of unrelated protein species ***(1,2)***. Traditionally used for mapping known proteins on electrophoretically resolved mixtures, in the proteome era immunoblotting has been combined with two-dimensional (2D) gel electrophoresis for the rapid visualization and identification of target proteins ***(3)***, protein adducts with drugs ***(4)***, and antigenic proteins associated to pathogens, allergens as well as tumors, with patient serum used as primary antibody ***(5–9)***.

In the postgenomic era, gene expression is becoming a focus of attention and it is widely accepted that one gene does not necessarily encode a single protein product because molecular mechanisms generating different isoforms from the same gene have been described in many different organisms. Although the relation between number of genes and number of potentially encoded proteins has yet to be clarified, it is usually recognized that the number of polypeptides expressed by a genome is greater than expected on the basis of calculated gene numbers. In fact, it has been estimated that the average number of alternates spliced from the transcript of a single mammalian gene may be two to three or more ***(10)*** and that many proteins may be subject to co- and posttranslational modifications and proteolytic processing. For example, up to 20% of protein is acetylated in yeast ***(11)***. Several preliminary proteome projects have also shown that prokaryotic organisms can express more than one protein isoform from a single gene and posttranslationally modified gene products have been reported ***(12)***. Moreover, compared with completely sequenced genomes of other organisms, the unexpected low number of potential protein-coding genes in the human genome makes molecular mechanisms generating different isoforms from the same gene particularly interesting. As shown in Scheler et al. ***(13)*** and Janke et al. ***(14)***, simultaneous 2D gel analysis of cross-reactive protein isoforms derived from a single gene may produce very complex isoforms patterns. More than 50 and 80 2D electrophoretic protein spots where observed using specific antibodies for

HSP27 and Tau protein, respectively. 2D immunoblotting combined with mass spectrometry-based identification methods has been widely applied to the characterization of 2D electrophoretic cross-reactive isoforms of the same protein, e.g., resulting from alternative splicing, co- and/or posttranslational modifications and proteolytic cleavages ***(15–19)***.

Western blotting analysis is also the method of choice for simultaneous visualization of 1D- and 2D-separated proteins sharing common epitopes related to specific posttranslationally modified amino acids or to specific functional/structural domains, for example, antiphosphoresidues and anti-*O*-linked *N*-acetylglucosamine antibodies ***(20–22)***. This approach has been widely used to differentially visualize tyrosine-phosphorylation profiles in cells and tissues under different conditions ***(17,20)***, as well as for studying signal transduction pathways following stimulation ***(23)***. The dynamic and reversible nature of several known posttranslational modifications makes their characterization possible only at protein level, as these features characterize mature gene products and cannot be inferred from crude genome-derived amino acid sequences stored in sequence databases. Two-dimensional immunoblotting is therefore a powerful method for rapid visualization of target proteins, sharing a common feature, to be identified and characterized by microchemical analysis, for example, by mass spectrometry.

Immunoblotting techniques involve the identification of a protein target via antigen–antibody-specific reactions. Proteins are typically separated by electrophoresis in polyacrylamide gels, and then transferred ("blotted") onto chemically resilient membranes (e.g., nitrocellulose, polyvinylidene difluoride) where they bind in the pattern they took in the gel. The membrane is overlaid with a primary antibody directed to the specific target, then with a secondary antibody (anti-immunoglobulin) labeled with radioisotopes, enzymes or other marker compounds.

A simplified procedure in which protein samples are not separated electrophoretically but are spotted directly onto the membrane is called "dot blot" and is a good preliminary technique for the detection of an antigen in a sample.

A number of related techniques for probing membranes containing transferred protein with specific ligands has been described:

- In "Far-Western blotting" the membrane is probed with another protein to detect specific protein–protein interactions ***(24)***. The reaction can be revealed using biotinylated or GST-tagged bait or "probe" protein followed by a streptavidin-HRP or an anti-GST-HRP chemiluminescent detection system, respectively.
- Blot overlays include the probing of membrane with various molecules to detect the presence of specific binding domains, for example, with guanine triphosphate ***(25,26)*** or proteoglycans ***(27)***. In the Southern or North Western blotting, the membrane is probed with deoxyribonucleic acid or ribonucleic acid molecules to detect nucleic-acid binding proteins ***(28)***.
- In glycoprotein detection systems the carbohydrate portions of proteins are oxidized with sodium metaperiodate to generate aldehydes that can react with hydrazides. A biotin hydrazide is used to attach biotin onto the oxidized carbohydrates and horseradish peroxidase-conjugated streptavidin is used for chemiluminescence-based detection (Glycoprotein Detection Module, Amersham Biosciences, Uppsala, Sweden).

2. Materials

2.1. Equipment

1. Blotting apparatus: transfer cell, gel holder, magnetic stirrer, refrigerated thermostatic circulator unit.
2. Power supply.
3. Rocking agitator.
4. Computing Densitometer and/or gel and blot image acquisition system.
5. PC with a computer program for 2D gel analysis.

2.2. Reagents

1. Distilled water.
2. Nitrocellulose membrane.
3. Transfer buffer: 25 m*M* Tris-HCl, 192 m*M* glycine, 20% (v/v) methanol. Do not adjust pH; it is approx 8.3.
4. Filter paper for blotting (Whatman 17 Chr).

5. Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid.
6. Coomassie blue solution: Coomassie blue R-250 0.1% in 40% methanol, 1% acetic acid.
7. Destaining solution: 50% methanol.
8. Phosphate-buffered saline (PBS): 0.15 *M* NaCl, 10 m*M* NaH_2PO_4; bring to pH 7.4 with NaOH.
9. Blocking solution: 3% (w/v) non-fat dry milk in PBS, Triton X-100 0.1% (w/v).
10. Primary antibody solution: primary antibody, appropriately diluted in blocking solution.
11. Secondary antibody solution: secondary antibody, appropriately diluted in blocking solution.
12. Washing solution: Triton X-100 0.5% (w/v) in PBS.
13. 0.05 *M* Tris-HCl, pH 6.8.
14. Amersham Biosciences ECL (enhanced chemiluminescence) kit, cat. no. RPN 2106.
15. Saran Wrap® or other cling-films.
16. X-ray films, 18 × 24 cm (Amersham Hyper film ECL; cat. no. RPN 3103).
17. Developer and fixer for X-ray film (Developer replenisher; fixer and replenisher, 3M, cat. nos. XAF 3 and XAD 3; 3M Italia S.p.A., Segrate, Italy).
18. Stripping buffer: 100 m*M* 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS), 62.5 m*M* Tris-HCl, pH 6.8.

3. Methods

3.1. Transfer

To avoid membrane contamination, wear gloves during all the steps of the protocol.

1. Prepare the transfer buffer (*see* **Notes 1–3**) and cool it to 4°C before the end of the electrophoretic run.
2. Cut to the dimension of the gel, two pieces of filter paper and one piece of nitrocellulose/gel (*see* **Note 4**).
3. After electrophoresis (*see* **Note 5**), wash the gel in distilled water and then equilibrate it in transfer buffer. The ideal time for 1.5-mm gels is 10 to 15 min (*see* **Note 6**).

4. Soak the nitrocellulose membrane for 15–20 min in transfer buffer. Also wet two Scotch-Brite® fiber pads, gel and filter papers in transfer buffer.
5. Assemble the "sandwich" for transfer in this order: fiber pad, filter paper, nitrocellulose, gel, filter paper, and fiber pad. Remove all air bubbles between membrane and gel and between paper and gel.
6. Put the blot sandwich in the gel holder and hold it firmly, to ensure a tight contact between gel and membrane.
7. Fill the cell with transfer buffer and place a stirring bar inside the transfer cell, so that the buffer is stirred during electrotransfer and temperature and conductivity are uniform during electrotransfer.
8. Place the gel holder in the transfer cell with the sandwich oriented as follows: ANODE/fiber pad, filter paper, nitrocellulose, gel, filter paper, fiber pad/CATHODE.
9. Carry out blotting at a constant current until it has reached a total of 1.5–2.0 Å (*see* **Note 7**), refrigerating the buffer to 4°C (*see* **Note 8**) for gels of 16 × 18 cm (such as 2D gels) or at constant voltage (100 V) for 1 h for minigels (*see* **Note 9**).
10. After electrotransfer, disassemble the blotting apparatus and remove the nitrocellulose membrane. To mark the orientation of the membrane, cut away the lower right corner, corresponding to low M_r, high pH.

The membrane can be processed immediately for immunoblotting or can be air-dried and stored at –20°C, within parafilm sheets for extended periods ***(29)***.

3.2. Staining of Total Protein Pattern on Membrane

3.2.1. SDS-PAGE and IEF Gels

In the case of a 1D gel (IEF or SDS-polyacrylamide gel electrophoresis [PAGE]), staining of total protein pattern can be done on one lane of the gel in which the sample or MW standard or pI standard have been loaded. Non-reversible stains more sensitive than Ponceau Red can be used (*see* **Note 10**).

1. Cut the nitrocellulose lane to be stained and immerse it in Coomassie blue solution (**Subheading 2.2.**, **step 6**) for 3 min.
2. Destain for 30 min in destaining solution (**Subheading 2.2.**, **step 7**) and air dry.

3.2.2. 2D Gels

1. Before the immunodetection, stain the nitrocellulose membrane in 0.2% w/v Ponceau S in 3% w/v trichloroacetic acid for 3 min ***(30)*** (*see* **Note 11**).
2. Destain with several changes of distilled water to diminish background color. Because the red spots will disappear in the blocking step, save the image with a chemiluminescence image acquisition system or circle with a waterproof pen some spots before the next steps of immunostaining. Spot stained with Ponceau S will be used as landmarks to match total protein pattern on nitrocellulose against immunoreactive pattern and against silver stained polyacrylamide gel pattern (*see* **Note 11**).

3.3. Immunodetection

3.3.1. Incubation With Antibodies

All steps are conducted at room temperature and with gentle agitation on a rocking agitator.

1. Block nonspecific binding sites in the membrane with three washing steps, each 10 min in duration, in blocking solution (*see* **Notes 12–14**).
2. Incubate overnight in the primary antibody solution at the suitable dilution (*see* **Note 15**) in blocking solution.
3. Wash 3 × 10 min in blocking solution.
4. Incubate for 2 h in the secondary antibody solution (*see* **Note 16**).
5 Wash 3 × 10 min in blocking solution.
6. Wash 30 min in washing solution.
7. Wash 2 × 30 min in 0.05 *M* Tris-HCl, pH 6.8.

After this step one can go forward with ECL detection (*see* **Notes 17** and **18**). Alternatively, one can choose detection with the chromogenic substrate (*see* **Note 19**).

3.3.2. Enhanced Chemiluminescent Detection

To detect the immunoreactive spot(s) with a chemiluminescent method it is necessary to wear gloves to prevent hand contact with film. We routinely use Enhanced Chemiluminescet detection (Amersham Biosciences) because it offers a greater sensitivity and

the possibility of quantification of immune reaction (*see* **Notes 17** and **20**).

Mix equal volumes of detection reagent 1 and detection reagent 2 from the Amersham ECL kit (**Subheading 2.2.**, **step 14**) and immerse the membrane in this solution for 1 min, ensuring that the surface of the membrane is completely covered with solution. The chemiluminescent signal can be acquired by a chemiluminescence image acquisition system or by the following procedure:

1. Place the membrane on a glass and cover it with a layer of Saran Wrap®.
2. Cut away a corner from a piece of autoradiography film to define its orientation (*see* **Subheading 3.1.**, **step 10**). Superimpose the autoradiography film on the nitrocellulose membrane beginning from the upper left corners. Nitrocellulose membrane and X-ray film may have different dimensions. Superimposing at the upper left corner for ECL impression will allow subsequent matching of images.
3. Expose the film for a time variable from 5 s to several minutes. It is usual to begin with a short exposure, followed by development of the film, followed by longer exposures, if necessary.
4. Develop the film with the suitable reagents (**Subheading 2.2.**, **step 17**).

3.4. Stripping

At the end of a cycle of immunodetection, it is possible to strip the membrane with stripping buffer and to carry out subsequent cycles incubating with different primary antibodies (*see* **Note 21**).

The procedure for the stripping we use is as follows:

1. Incubate the membrane in stripping buffer at 70°C for 30 min, with occasional shaking.
2. Wash the nitrocellulose 2 × 10 min in large volumes of washing solution (**Subheading 2.2.**, **step 12**) at room temperature.
3. Block the membrane and perform immunodetection as described in **Subheading 3.3.**

3.5. Matching

3.5.1. SDS-PAGE and IEF Gels

For SDS-PAGE gels (or IEF gels), a MW or pI standard is normally run in one lane. The following procedure is used to identify the immunoreactive band and determine its MW or pI.

1. Scan the ECL-developed film, the Coomassie-stained nitrocellulose membrane, or a lane of the Coomassie-stained gel (in which standard proteins or the sample were separated) with a computing densitometer of sufficient resolution.
2. Use a graphics program to make the images the same length (all were originally from the same gel and are therefore of the same size, but nitrocellulose shrinks when stained).
3. When the images are perfectly aligned, the immunoreactive band can be distinguished in the Coomassie stained lane and its MW or pI determined by comparison with the appropriate lane.

3.5.2. 2D Gels

For an accurate matching process, we use a computer program (*see* **Note 22**) that permits the matching of the digitized images using the spots stained with Ponceau S as landmarks (*see* **Note 23**).

To perform this operation we suggest the following procedure:

1. Scan the ECL-developed film, the Ponceau S-stained nitrocellulose membrane, and the silver-stained gel of the same sample with a computing densitometer with a sufficient resolution (*see* **Notes 24** and **25**).
2. Rotate the nitrocellulose membrane left to right, with an appropriate program, to have the three images with the cut lower corner on the right. In fact, the nitrocellulose membrane has spots only on one face and the scanning process generates an image with the cut lower corner placed on the left.
3. Stack together the film and nitrocellulose membrane images, aligning the upper left corners and the two corresponding borders and placing the cut lower right corner in the same orientation for both.
4. Manually add the Ponceau S spots chosen as landmarks onto the image of the ECL film, with an appropriate software tool.

5. Find the spots on the gel corresponding to landmarks on the film (*see* **Note 26**) and modify the size of the silver nitrate image adjusting it to the smaller one of film by the mean of adequate software. Actually the gel is larger than the film owing to silver staining procedure.
6. Stack together the equalized ECL film and gel images, superimpose the landmarks carefully and run the automatic match program. This operation permits automatically highlighting the silver-stained spots paired with the immunoreactive ones present on the ECL film (*see* **Note 27**).

4. Notes

1. We perform electro-transfer from gels to nitrocellulose membrane, using a wet blotter or tank apparatus, where the gel is submerged in a large volume of buffer during the transfer. For two dimensional or large gels, we use a Bio-Rad transfer cell with 3 L of transfer buffer or an ISODALT cell (Hoefer Scientific Instruments) with 20 L of transfer buffer. ISODALT cells allow the simultaneously transfer of 5 gels. For minigels we use Mini Trans-Blot cell (7.5×10 cm blotting area). The transfer buffer can be used several times, if stored at 4°C. "Semi-dry" electro blotters require smaller volumes of buffer, since only the membrane and filter paper have to be wet and the procedure is faster. However, the "wet" method is recommended when antigen is present in small quantities (such as low abundance spots in 2D gels) and/or its molecular weight is high ***(31)***. It offers more options, such as temperature, time and voltage control.
2. The transfer buffer we use was first described by Towbin et al. ***(1)***. Methanol is toxic and it can be omitted ***(32–34)***. Still, we use it to reduce swelling of the gel during transfer and to increase the binding of proteins to nitrocellulose ***(2,32,35)***. When working with high molecular weight proteins, elimination of the methanol results in a significant increase in protein transfer efficiency. Some recipes recommend the addition of low concentration of SDS to the buffer to help the transfer of high molecular weight proteins (30) and to improve the transfer of a variety of proteins ***(32)***. However SDS reduces the amount of protein bound to the membrane (2) and may adversely affect immunoreactivity by inhibiting renaturation of antigenic sites ***(36)***.

For semi-dry blotting it is possible to use discontinuous buffer and/or "elution promoting" buffer on the gel side and a "retention promoting" buffer on the membrane side ***(37)***.

3. Reagent grade methanol must be used because trace impurities in methanol can increase the conductivity of transfer buffer and decrease transfer efficiency.
4. PVDF may also be used ***(38,39)***. Remember that unlike nitrocellulose, PVDF is a hydrophobic membrane and it must to be presoaked in methanol before use with aqueous solution. The buffer generally used to transfer proteins to PVDF is 10 m*M* 3-[cyclohexylamino]-1-propanesulfonic acid, 10% (v/v) methanol, pH 11.0 ***(40)***, although it is possible to use the buffer described in Towbin et al. ***(1)***.
5. Electrotransfer is usually conducted immediately after the electrophoretic run from unstained gels. However, transfer of proteins from polyacrylamide gels after Coomassie blue or silver staining, has also been reported ***(41–43)***. Proteins can also be transferred for immunodetection from gels previously stained in a reverse (negative) way, for example with imidazole-zinc salts ***(44)***. In these procedures, immunoreactivity patterns on the membrane, and total protein patterns can be obtained from the same gel from which spots have been transblotted, facilitating matching even with poorly reproducible 2D separations.
6. Polyacrylamide IEF gels should be pre-equilibrated with transfer buffer containing 1% SDS and 20% glycerol, instead of methanol (to prevent swelling), since the focused proteins are at their isoelectric point and do not transfer well without equilibration. If it is desired to maintain the proteins in their native conformation, the SDS may be omitted, but the pH of the transfer buffer should be increase to pH 8.8 and the transfer may be less efficient ***(45)***.
7. The transfer efficiency is adversely affected by high molecular weight and the basic pIs of some proteins. Therefore, while attempting to transfer these slow proteins it is possible that some faster proteins cross the nitrocellulose membrane and are lost. In cases like this, one can use two stacked membranes, or membranes with smaller pore diameter, which will prevent the loss of small polypeptides during membrane manipulation ***(46,47)***. *See* **ref. *48*** for information on blotting on various membranes.

   Some low molecular weight, basic proteins, such as histones, lysozymes, cytochromes, and so forth, do not transfer well because

they may be near their pI in currently used buffers, as SDS is lost during the transfer in methanol. Transfer of these proteins can be improved, without impairing transfer of other proteins, by introducing a more basic transfer buffer and/or omitting the equilibration (**Subheading 3.1., step 3** ***[49]***). Alternative buffers have also been proposed ***(50)***.

8. When the transfer is conducted at high voltage it is necessary to refrigerate the transfer tank with a thermostatic circulator.
9. If it is only necessary to check the immunoreactivity of an antibody towards a mixture of antigens, without attributing it to a particular protein, the antigen mixture (2–5 µL) can be spotted directly on the membrane. This technique, known as dot blotting ***(51)*** is useful for fast screenings of many antibodies simultaneously, for example in production of monoclonal antibodies.
10. In SDS gels, multicolored proteins can be used to provide a visual display of marker proteins on the transfer membrane. Various companies sell precolored standards and there is in the literature ***(52)*** a procedure for generating multicolored molecular weight proteins using a variety of Remazol-reactive textile dyes.
11. Chemical staining of protein patterns transblotted onto the nitrocellulose or other membrane plays an important role in 2D immunoaffinity identification, since it provides "landmark" spots to match immunoreactivity patterns to silver-staining patterns (*see* **Subheading 3.5.** and **Note 18**). Several staining procedures can be chosen. This step is usually carried out before the incubation of transblotted membranes with antibodies, using dyes (e.g., Ponceau S, Fast Green, Amido black) or metal-chelates, which do not interfere with protein immunoreactivity ***(46,53–56)***. Staining with substances such as Ponceau S, Fast Green, and metal-chelates is reversible, eliminating interference in the immunoreactivity pattern obtained with chromogenic substrates, but it is not very sensitive. A dye-based staining method, using Direct Blue 71 was recently developed. It is reversible, compatible with immunodetection and with a sensitivity that is 10-fold higher than Ponceau S ***(57)***. Permanent staining can also be used if the immunoreactivity pattern is collected from ECL-impressed films, but the stain must not interfere with immunoreaction.

    Fluorescent dyes have also been recently introduced for membrane staining. For example, SYPRO Ruby protein blot stain is a new,

luminescent metal chelate stain composed of ruthenium in an organic complex that interacts non-covalently with proteins. This stain is more sensitive than Ponceau, Coomassie blue, Amido Black or India Ink and is nearly as sensitive as colloidal gold staining. This fluorescent stain is fully compatible with immunoblotting ***(58)***.

When radioactive labeling is possible, the more accurate total protein patterns can be collected from transblotted membranes by phosphor-imaging. The two images will have the same dimensions so that general alignment and recognition of immunoreactive spots in the total protein pattern can be easily achieved. However the requirements for safe handling of radioactive proteins is a huge limitation to this method.

Perfect alignment of immunoreactive spots to total protein pattern can be obtained, at least with PVDF membranes, by the conjunction of colloidal gold staining for total protein detection and ECL for immunoreactivity on the same membrane ***(59)***. This procedure produce an ECL-impressed film with low exposure allowing the detection of immunoreactive spots and an ECL-impressed film with a strong exposure that produces a background pattern. The final result is a single image where immunoreactive spots appears as dark black spots and the general protein pattern appears as light grey spots.

Colloidal gold ***(60,61)*** and India Ink staining ***(62)*** can be applied also after immunodetection. The latter approach is possible if membrane blocking is carried out using Tween-20 only ***(63,64)***.

Finally another method has been proposed by Zeindl-Eberhart ***(65)*** to localize easily imaged antigen on 2D gels. Proteins are transferred to PVDF membrane, immunostained with specific antibodies using Fast Red or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a detection system, and then counterstained with Coomassie brilliant blue. The membrane appears with immunostained spots colored in red or black and the total protein pattern in blue. The blocking proteins are removed during the staining with Coomassie and so do not create background staining.

In all these methods proteins are transferred onto one membrane and both total protein staining and immunostaining are performed on the same membrane. “Double-replica” blotting methods have also been developed to obtain a membrane with all the proteins stained and that is an almost identical copy of the immunostained one. The first of its kind was described by Johansson ***(66)*** who found that by chang-

ing the direction of the blotting current the proteins could be transferred simultaneously from one gel onto two membranes, on either sides of the gel. A second method described by Neumann and Mullner *(67)* combines the usual electroblotting procedure with the generation of a "contact copy" from a gel. Both systems enable one membrane to be immunostained whereas the second membrane is stained using highly sensitive total protein staining methods. Protein identification is then carried out by comparing the signals from both matrices.

Similarly, a fast and simple method to produce print-quality like Ponceau replicas from blots was recently described *(68)*. The positive replicas are the same size as blots and can be stored without loss of intensity. This makes them useful for localizing immunoreactive spots in complex 2D electrophoretograms.

12. For a membrane from a 1D SDS-PAGE or IEF gel, we use 3–5 mL of solution depending on the dimension of the membrane in each washing and incubation step. For a 16- × 18-cm membrane, we use 50 mL of solution. In general, volumes can be proportionally adjusted to other membrane dimension. It is important that the membrane is entirely soaked in solution during washing and incubation step.
13. Our blocking procedure is suitable for routine use. However, special conditions and reagents are required for immunoblotting with some antibodies, such as anti-phosphotyrosine antibodies *(20,69,70)*. Information on different blocking conditions can be found in references *(51,71–73)*. Chemicon have developed a new blocking agent, composed of non animal proteins, that ensures uniform blocking, without non specific-binding, eliminating all crossreactivity between animal antigens and primary and secondary antibodies. Blocking with a non-ionic detergent such as Tween-20, without added protein has also been used with the advantage that after immunodetection the blot can be stained for total protein pattern *(63,74,75)* (*see* **Note 10**). On the other hand, it has been found that blocking with detergent alone may cause loss of transblotted proteins *(75,76)*. Using PVDF membrane it is possible to use a nonblock technique: this method incorporates three cycles of methanol-water hydration of the membrane, allowing multiple erasure and probing of the same blot with little or no loss of signal *(77)*.

    A modified Western blotting protocol has been developed that increases the binding specificity of antigens and antibodies without

increasing the background. The method is based on intermittent microwave irradiation of the blotting membrane during the immunoblotting step, using 5% skim milk as the diluting buffer ***(78)***. A simple method to improve Western blotting and reduce background has been developed by Wu et al. ***(79)***, that consists of a few modifications to the washing steps and buffer conditions.

14. Fixation of proteins to the membrane can be used to prevent their elution during washing and incubation steps. Some methods have been tried ***(35,80)***, but epitopes are sensitive to this treatment and may no longer be detectable by antibody. Another method is to fix the antibody–antigen complexes to the nitrocellulose membrane with glutaraldehyde, after they had been formed ***(81)***.
15. Optimal dilution of the primary and secondary antibody should be determined by immunoblotting of one-dimensional gels. Dot blot analysis can also be used. Working solutions of antibodies can be stored at –20°C and used several times ***(82)***.
16. Secondary antibodies often give problems of crossreactivity, especially when being used to analyze samples containing antibodies (such as immunoprecipitates, immune tissue, plasma), even when antibodies from different species are used. Langstein and Schwarz proposed a method to avoid this problem, that consists of preconjugating the primary and secondary antibodies ***(83)***. Another solution to this problem is "double-blotting." After the membrane has been incubated with the primary antibody, it is blotted a second time under acidic conditions. Antigen and interfering proteins remain bound to the first membrane, and the primary antibodies are transferred to the second one, which can be probed with secondary antibodies, without non specific binding ***(84)***.
17. The secondary antibody we use is labeled with peroxidase. The major drawback of this approach is that the range of protein loading that can be used to give a linear relationship between the amount of target protein and the signal is quite limited. Considerable advantages for quantitative analysis can be gained by the use of a secondary antibody coupled to fluorophores that allow quantification of fluorescent signal, e.g., by means of a phosphor imager device. This approach theoretically gives a linear signal through a broad range of protein loading ***(85,86)***.
18. ECL detects horseradish peroxidase-conjugated antibodies through oxidation of luminol, in the presence of hydrogen peroxide and a phenolic enhancer under alkaline conditions. ECL reagents are

capable of detecting 1–10 pg of protein antigen. An alternative enhancer that extends the duration of light emission is ECL plus (Amersham Biosciences, **ref.** ***87***). These systems are suitable for the use of charge-coupled device cameras that require longer exposure times for good quantification of immunoreactions.

19. If chemiluminescence is too strong or background is too high, one can change the detection system to a chromogenic substrate. We use 4-chloro1-naphthol ***(88)*** as a chromogenic substrate, according to the following protocol.
    a. After ECL detection (or after **step 7** of **Subheading 3.3.1.**), wash the membrane briefly with Tris-HCl 0.05 *M*, pH 6.8.
    b. Soak it in developing solution 20 mL of Tris-HCl 0.05 *M*, pH 6.8, 7 µL of H_2O_2 30% (v/v); and 5 mL of 4-chloro1-naphthol 0.3% (w/v) in methanol) until the color appears. Stop the reaction by washing in distilled water.
    c. Air dry the membrane and photograph it as soon as possible, because the color fades with time.
20. Chemiluminescent probes enable highly sensitive quantitative analysis of proteins blotted from electrophoretic gels onto a supporting matrix. For a quantitative comparison, it is important to be able to correct for introduced variables such as antibody titre, temperature, substrate etc. Comparison of blots completed on different days requires a chemiluminescent standard. The situation is more complex with 2D gels, where only one sample per gel/blot is used. A method has been published for preparing a chemiluminescent standard for quantitative comparison of 2D Western blot ***(89)***.
21. It is also possible to perform stripping with kits as the CHEMICON Re-Blot™ Western blot recycling kit. Stripping of antibodies also elutes antigens from the membrane and signal intensity decreases in successive cycles. It is important therefore to remember that stripping should be used only for qualitative purpose. As alternatives to stripping, one can use:
    a. Alternative chromogenic substrates for peroxidase at each cycle (rainbow blotting, ***[82]***).
    b. ECL followed by inactivation of peroxidase after each cycle ***(82)***.
    c. Different labels and detection methods at each cycle ***(90)***.
22. To perform the matching process we use the software Melanie 4 (Gene Bio).
23. Matching can also be conducted by inspection by eye of the nitrocellulose and ECL film when the sample contains relatively few spots,

all of them detectable by chemical staining of nitrocellulose. In the majority of cases samples are very complex and many low abundance proteins occur. In these cases matching by computer is necessary, in order to identify immunoreactive spots in silver-stained patterns. The following manual procedure is suggested:

a. Match the exposed film with the nitrocellulose membrane, aligning the upper left corner and the two corresponding borders and placing the cut lower right corner in the same orientation for both.
b. Using a waterproof pen, mark the other two borders of the nitrocellulose on the film and transfer the chemically stained spot present on nitrocellulose on the ECL film in order to use them as landmarks for the next matching with the silver nitrate stained gel.
c. Nitrocellulose membrane and film maintain the initial size, but the size of the gel increases after silver staining. Size equalization can be obtained by photographic or photocopy procedures.
d. On a transilluminator match all the landmarks with the corresponding spots on the silver nitrate stained gel to identify the immunoreactive spots.

When the area of the membrane containing the protein of interest is known, another procedure, described by Lindahl ***(91)***, can be used. Only a limited area of the nitrocellulose containing the proteins is cut out and incubated with antibodies. The rest of the membrane is stained with one of the methods described in **Note 10** (a method not compatible with immunodetection, but much more sensitive than Ponceau S, can be used). Thus the protein spots can be located in the 2D electrophoresis pattern, matched with a corresponding silver stained gel and translated into the protein pattern. This method also allows a considerable saving of antibodies.

24. We use a computing densitometer 300 S from Molecular Dynamics with a resolution of 4000 × 5000 pixels, 12 bits/pixel, which generates 40 megabytes images on 16 bits.
25. The silver stained image used for matching can be taken from your file archive or from images available on the Internet, provided that identical electrophoretic procedures have been applied. The possibility of matching images derived from different 2D electrophoretic procedures has been investigated by Lemkin P. ***(92)***.
26. This step may be difficult if the "landmark" spots stained by Red Ponceau on the nitrocellulose membrane are few. To aid recognition

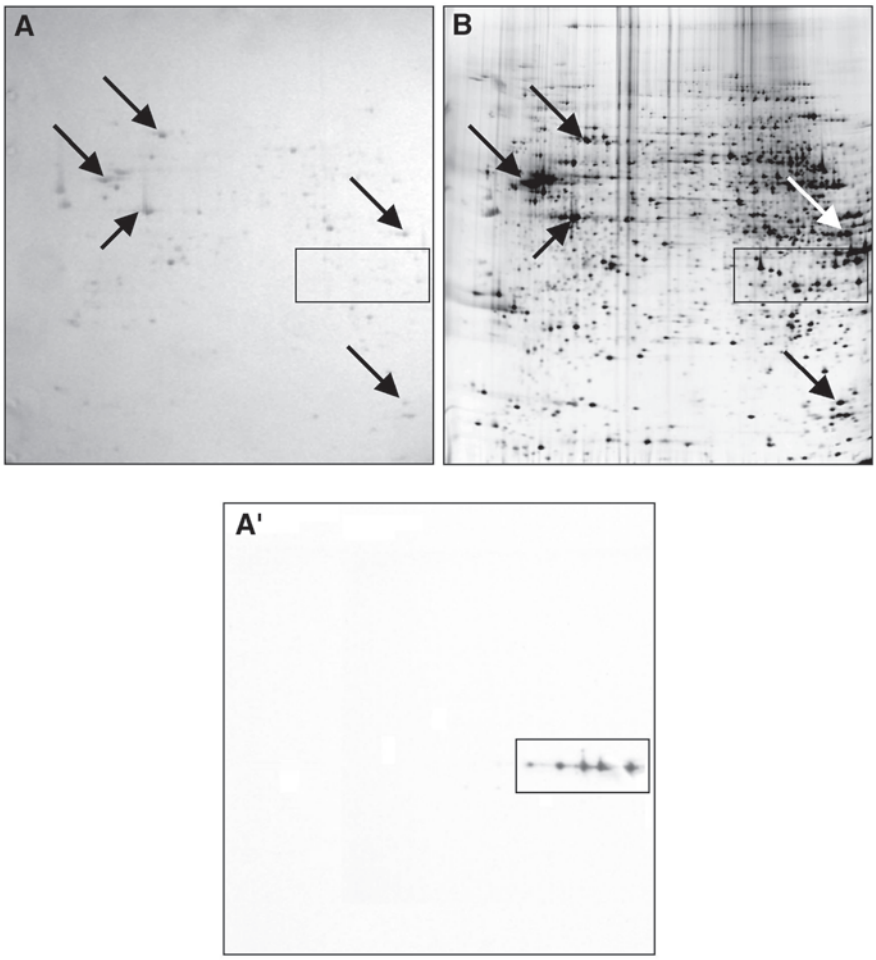


Fig. 1. Mapping of immunoreactive spots on 2-D silver-stained reference image.

A: Blot membrane stained with Ponceau-S digitized image acquired, before immunodetection, with Image Master VDS-CL system (Amersham Biosciences).

B: Digitized image of silver-stained gel. Size is equalized to that of image A on the basis of anchors (arrows).

A' Digitized image of impressed ECL film on blot membrane reported in A.

Recognition of anchor positions (arrows) between A and B and of immunoreactive spots between A and **A'** allows localization of immunoreactive spots on silver-stained gel.

of the spots chosen as landmarks on the silver stained gel, we suggest also staining the gel from which the proteins were transferred with silver. The amount of protein loaded onto this gel must be twice that used for a normal silver-stained gel. Most spots will still be visible on the transferred gel and can be used for an initial matching with the membrane. Using the gel from which the membrane was obtained, the landmarks can be localized correctly. The landmarks are then easily transferred to the silver stained gel by computer matching.

27. The mapping of immunoreactive spots on 2D silver-stained reference images can be improved if the images of the Ponceau and the immuno-stained membranes are scanned using the same system, enabling detection of the total protein pattern directly on to the membrane where the immunoreaction took place. In commercially available gel and blot scanning systems, images generated in different ways can be readily acquired on the same instrument.

References

1. Towbin, H., Staehlin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
2. Burnette, W. N. (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112,** 195–203.
3. Magi, B., Bini, L., Liberatori, S., Marzocchi, B., Raggiaschi, R., Arcuri, F., et al. (1998), Charge heterogeneity of macrophage migration inhibitory factor in human liver and breast tissue. *Electrophoresis* **19,** 2010–2013.
4. Magi, B., Marzocchi, B., Bini, L., Cellesi, C., Rossolini, A., and Pallini, V. (1995) Two-dimensional electrophoresis of human serum proteins modified by ampicillin during therapeutic treatment. *Electrophoresis* **16,** 1190–1192.
5. Sanchez-Campillo, M., Bini, L., Comanducci, M., Raggiaschi, R., Marzocchi, B., Pallini, V., et al. (1999) Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera. *Electrophoresis* **20,** 2269–2279.

6. Geissler, S., Sokolowska-Kohler, W., Bollmann, R., Jungblut, P. R., and Presber, W. (1999) Toxoplasma gondii infection: analysis of serological response by 2-DE immunoblotting. *FEMS Immunol. Med. Microbiol.* **25,** 299–311.
7. Yu, C. J., Lin, Y. F., Chiang, B. L., and Chow, L. P. (2003) Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J. Immunol.* **170,** 445–453.
8. Klade, C. S., Voss, T., Krystek, E., Ahorn, H., Zatloukal, K., Pummer, K., and Adolf, G. R. (2001) Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. *Proteomics* **1,** 890–898
9. Unwin, R. D., Harnden, P., Pappin, D., Rahman, D., Whelan, P., Craven, R. A., et al. (2003) Serological and proteomic evaluation of antibody responses in the identification of tumor antigens in renal cell carcinoma. *Proteomics* **3,** 45–55.
10. Galas, D. J. (2001) Sequence interpretation. Making sense of the sequence. *Science* **291,** 1257–1260
11. Gooley, A. A. and Paker, N. H. (1997) The importance of protein co- and post-translational modifications in proteome projects, in *Proteome research: new frontiers in functional genomics*, Wilkins M. R., Williams K. L., Appel R. D., and Hochstrasser D. F. (eds), Springer-Verlag Berlin Heidelberg .
12. Upreti, R. K., Kumar, M., and Shankar, V. (2003) Bacterial glycoproteins: functions, biosynthesis and applications. *Proteomics* **3,** 363–379.
13. Scheler, C., Muller, E. C., Stahl, J., Muller-Werdan, U, Salnikow J, and Jungblut, P. (1997) Identification and characterization of heat shock protein 27 protein species in human myocardial two-dimensional electrophoresis patterns. Identification and characterization of heat shock protein 27 protein species in human myocardial two-dimensional electrophoresis patterns. *Electrophoresis* **18,** 2823–2831.
14. Janke, C., Holzer, M., Klose, J, and Arendt, T. (1996) Distribution of isoforms of the microtubule-associated protein tau in grey and white matter areas of human brain: a two-dimensional gelelectrophoretic analysis. *FEBS Lett.* **379,** 222–226.
15. Borchert, A., Savaskan, N. E., and Kuhn, H. (2003) Regulation of expression of the phospholipid hydroperoxide/sperm nucleus glutathione peroxidase gene. Tissue-specific expression pattern and identification of functional cis- and trans-regulatory elements. *J. Biol. Chem.* **278,** 2571–2580.

16. Lefebvre, T., Ferreira, S., Dupont-Wallois, L., Bussiere, T., Dupire, M. J., Delacourte, A., et al. (2003) Evidence of a balance between phosphorylation and O-GlcNAc glycosylation of Tau proteins—a role in nuclear localization. *Biochim. Biophys. Acta* **1619,** 167–176.
17. Liberatori, S., Canas, B., Tani, C., Bini, L., Buonocore, G., Godovac-Zimmermann, J., et al. (2004) Proteomic approach to the identification of VDACs protein isoforms in guinea pig brain synaptosomes. *Proteomics* **4,** 1335–1360.
18. Peng, Q., McEuen, A. R., Benyon, R. C., and Walls, A. F. (2003) The heterogeneity of mast cell tryptase from human lung and skin. *Eur. J. Biochem.* **270,** 270–283.
19. Zanusso, G., Righetti, P. G., Ferrari, S., Terrin, L., Farinazzo, A., Cardone, F., et al. (2002) Two-dimensional mapping of three phenotype-associated isoforms of the prion protein in sporadic Creutzfeldt-Jakob disease. *Electrophoresis* **23,** 347–355.
20. Buonocore, G., Liberatori, S., Bini, L., Mishra, O. P., Delivoria-Papadopoulos, M., Pallini, V., et al. (1999) Hypoxic response of synaptosomal proteins in term guinea pig fetuses. *J Neurochem.* **73,** 2139–2148.
21. Kaufmann, H., Bailey, J. E., and Fussenegger, M. (2001) Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. *Proteomics* **1,** 194–199.
22. Gao, Y., Wells, L., Comer, F. I., Parker, G. J., and Hart, G. W. (2001) Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-N-acetylglucosaminidase from human brain. *J. Biol. Chem.* **276,** 9838–9845.
23. Soskic, V., Nyakatura, E., Roos, M., Muller-Esterl, W., and Godovac-Zimmermann, J. (1999) Correlations in palmitoylation and multiple phosphorylation of rat bradykinin B2 receptor in Chinese hamster ovary cells. *J. Biol. Chem.* **26,** 8539–8545.
24. Pasquali, C., Vilbois, F., Curchod, M.-L., van Huijsduijnen, R. H., and Arigoni, F. (2000) Mapping and identification of protein-protein interactions by two-dimensional far-western immunoblotting. *Electrophoresis* **21,** 3357–3368.
25. Huber, L. A., Ullrich, O., Takai, Y., Lutcke, A., Dupree, P., Olkkonen, V., et al. (1994) Mapping of Ras-related GTP-binding proteins by GTP overlay following two-dimensional gel electrophoresis. *Proc. Natl. Acad. Sci. USA* **16,** 7874–7878.

26. Kadono-Okuda, K. and Andres, D. A. (1997) An expression cloning method to identify monomeric GTP-binding proteins by GTP overlay. *Anal. Biochem.* **254,** 187–191.
27. Saito, A., Munakata, H., and Satoh, K. (2002) Glyco-western blotting: biotinylated dermatan sulfate as a probe for the detection of dermatan sulfate binding proteins using western blotting. *Connect Tissue Res* **43,** 1–7.
28. Guillonneau, F., Labas, V., Auvin, C., and Praseuth, D. (2001) A reliable and simple method for two-dimensional electrophoresis and identification of HeLa nuclear alkaline nucleic acid-binding proteins using immobilized pH gradient. *Electrophoresis* **22,** 4391–4403.
29. Bernstein, D. I., Garraty, E., Lovett, M. A., and Bryson, Y. J. (1985) Comparison of western blot analysis to microneutralization for the detection of type-specific antibodies to herpes simplex virus antibodies. *J. Med. Virol.* **15,** 223–230.
30. Sanchez, J. C., Ravier, F., Pasquali, C., Frutiger, S., Paquet, N., Bjellqvist, B., et al. (1992) Improving the detection of proteins after transfer to polyvinylidene difluoride membranes. *Electrophoresis* **13,** 715–717.
31. Okamura, H., Sigal, C. T., Alland, L., and Resh, M. D. (1995) Rapid high-resolution western blotting. *Methods Enzymol.* **254,** 535–550.
32. Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J., and Thomas, G. (1982) The phosphorylation of ribosomal protein S6 in rat tissues following cycloheximide injection, in diabetes, and after denervation of diaphragm. *J. Biol. Chem.* **257,** 12316–12321.
33. Gershoni, J. M. and Palade, G. E. (1983) Protein blotting: principles and applications. *Anal. Biochem.* **131,** 1–15.
34. Gershoni, J. M. (1988) Protein blotting: a manual. *Methods Biochem. Anal.* **33,** 1–58.
35. Gershoni, J. M. and Palade, G. E. (1982) Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem.* **124,** 396–405.
36. Birk, H. W. and Koepsell, H. (1987) Reaction of monoclonal antibodies with plasma membrane proteins after binding on nitrocellulose: renaturation of antigenic sites and reduction of non specific antibody binding. *Anal. Biochem.* **164,** 12–22.
37. Lauriere, M. (1993) A semidry electroblotting system efficiently transfers both high- and low-molecular weight proteins separated by SDS-PAGE. *Anal. Biochem.* **212,** 206–211.

38. Pluskal, M. F., Przekop, M. B., Kavonian, M. R., Vecoli C., and Hicks, D. A. (1986) Immobilon™ PVDF transfer membrane. A new membrane substrate for western blotting of proteins. *Biotechniques* **4,** 272–282.
39. Gultekin, H. and Heermann K. H. (1988) The use of polyvinylidenedifluoride membranes as a general blotting matrix. *Anal. Biochem.* **172,** 320–329
40. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes *J. Biol. Chem.* **262,** 10,035–10,038.
41. Ranganathan, V. and De, P. K. (1995) Western blot of proteins from Coomassie-stained polyacrylamide gels. *Anal. Biochem.* **234,** 102–104.
42. Wise, G. E. and Lin, F. (1991) Transfer of silver-stained proteins from polyacrylamide gels to polyvinylidene difluoride membranes. *J. Biochem. Biophys. Methods* **22,** 223–231.
43. McDonough, J. L., Neverova, I., and Van Eyk, J. E. (2002) Proteomic analysis of human biopsy samples by single two-dimensional electrophoresis: Coomassie, silver, mass spectrometry, and Western blotting. *Proteomics* **2,** 978–987
44. Fernandez-Patron, C., Castellanos-Serra, L., and Rodriguez, P. (1992) Reverse staining of sodium dodecyl sulfate polyacrylamide gels by imidazole-zinc salts: sensitive detection of unmodified proteins. *Biotechniques* **12,** 564–573.
45. Stott, D. I. (2000) Immunoblotting, dot-blotting, and ELISPOT assays: methods and applications. *J. Immunoassay* **21,** 273–296.
46. Lin, W. and Kasamatsu, H. (1983) On the electrotransfer of polypeptides from gels to nitrocellulose membrane. *Anal. Biochem.* **128,** 302–311.
47. Polvino, W. J., Saravis C. A., Sampson, C. E., and Cook, R. B. (1983) Improved protein analysis on nitrocellulose membrane. *Electrophoresis* **4,** 368–369.
48. Eckerskorn, C. and Lottspeich, F. (1993) Structural characterization of blotting membranes and the influence of membrane parameters for electroblotting and subsequent amino acid sequence analysis of proteins. *Electrophoresis* **14,** 831–838.
49. Szewczyk, B. and Kozloff, L. M. (1985) A method for the efficient blotting of strongly basic proteins from sodium dodecyl sulphate-polyacrylamide gels to nitrocellulose. *Anal. Biochem.* **50,** 403–407.

50. Dunn, S. D. (1986) Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. *Anal. Biochem.* **157,** 144–153
51. Stott, D. I. (1989) Immunoblotting and dot blotting. *J. Immunol. Methods* **119,** 153–187.
52. Compton, M. M., Lapp, S. A., and Pedemonte, R. (2002) Generation of multicoloured, prestained molecular weight markers for gel electrophoresis. *Electrophoresis* **23,** 3262–3265.
53. Salinovich, O. and Montelaro, R. C. (1986) Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **156,** 341–347.
54. Reinhart, M. P. and Malamud, D. (1982) Protein transfer from isoelectric focusing gels: the native blot. *Anal. Biochem.* **123,** 229–235
55. Patton, W. F., Lam, L., Su, Q., Lui, M., Erdjument-Bromage, H., and Tempst, P. (1994) Metal chelates as reversible stains for detection of electroblotted proteins: application to protein microsequencing and immunoblotting. *Anal. Biochem.* **220,** 324–335.
56. Root, D. D. and Reisler, E. (1989) Copper iodide staining of protein blots on nitrocellulose membranes. *Anal. Biochem.* **181,** 250–253.
57. Hong, H. Y., Yoo, G. S., and Choi, J. K. (2000) Direct Blue 71 staining of proteins bound to blotting membrane. *Electrophoresis* **21,** 841–845.
58. Berggren, K., Steinberg, T. H., Lauber, W. M., Carroll, J. A., Lopez, M. F., Chernokalskaya, E., et al. (1999) A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports. *Anal. Biochem.* **276,** 129–143.
59. Chevallet, M., Procaccio, V., and Rabilloud, T. (1997) A nonradioactive double detection method for the assignment of spots in two-dimensional blots. *Anal. Biochem.* **251,** 69–72.
60. Moeremans, M., Daneels, G., and De Mey, J. (1985) Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal. Biochem.* **145,** 315–321.
61. Daneels, G., Moeremans, M., De Raeymaeker, M., and De Mey, J. (1986) Sequential immunostaining (gold/silver) and complete protein staining (Aurodye) on western blots. *J. Immunol. Methods* **89,** 89–91.
62. Glenney, J. (1986) Antibody probing of western blots which have been stained with India ink. *Anal. Biochem.* **156,** 315–319

63. Batteiger, B., Newhall, W. J.V., and Jones R. B. (1982) The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membrane. *J. Immunol. Methods* **55,** 297–307.
64. Fultz, C. D. and Witzmann, F. A. (1997) Locating western blotted and immunostained proteins within complex two-dimensional patterns. *Anal. Biochem.* **251,** 288–291.
65. Zeindl-Eberhrt, E., Jungblut, P. R., and Rabes, H. M. (1997) A new method to assign immunodetected spots in the complex two-dimensional electrophoresis pattern. *Electrophoresis* **18,** 799–801.
66. Johansson, K. E. (1986) Double replica electroblotting: a method to produce two replicas from one gel. *J. Biochem. Biophys. Methods* **13,** 197–203.
67. Neumann, H. and Mullner, S. (1998) Two replica blotting methods for fast immunological analysis of common proteins in two-dimensional electrophoresis. *Electrophoresis* **19,** 752–757.
68. Gotzmann, J. and Gerner, C. (2000) A method to produce ponceau replicas from blots: application for western analysis. *Electrophoresis* **21,** 523–525.
69. Kamps, M. P. (1991) Generation of anti-phosphotyrosine antibodies for immunoblotting. *Methods Enzymol.* **201,** 101–110.
70. Michalewski, M. P., Kaczmarski, W., Golabek, A., Kida, E., Kaczmarski, A., Wisniewski, K. E. (1999) Immunoblotting with antiphosphoamino acid antibodies: importance of the blocking solution. *Anal. Biochem.* **276,** 254–257.
71. Towbin, H. and Gordon, J. (1984) Immunoblotting and dot immunobinding. Current status and outlook. *J. Immunol. Methods* **72,** 313–340.
72. Poxton, I. R. (1990) Immunoblotting techniques. *Curr. Opin. Immunol.* **2,** 905–909.
73. Harper, D. R., Kit, M. L., and Kangro, H. O. (1990) Protein blotting: ten years on. *J. Virol. Methods* **30,** 25–40.
74. Mohammad, K. and Esen, A. (1989) A blocking agent and a blocking step are not needed in ELISA, immunostaining dot-blots and western blots. *J. Immunol. Methods* **117,** 141–145.
75. Flanagan, S. D. and Yost, B. (1984) Calmodulin-binding proteins: visualization by ^{125}I-Calmodulin overlay on blots quenched with Tween 20 or bovine serum albumin and poly(ethylene oxide). *Anal. Biochem.* **140,** 510–519.

76. Hoffman, W. L. and Jump, A. A. (1986) Tween 20 removes antibodies and other proteins from nitrocellulose. *J. Immunol. Methods* **94,** 191–197.
77. Sadra, A., Cinek, T., and Imboden, J. B. (2000) Multiple probing of an immunoblot membrane using a non-block technique: advantages in speed and sensitivity. *Anal. Biochem.* **278,** 235–237.
78. Li, W., Murai, Y., Okada, E., Matsui, K., Hayashi S., Horie, M., and Takano, Y. (2002) Modified and simplified western blotting protocol: use of intermittent microwave irradiation (IMWI) and 5% skim milk to improve binding specificity. *Pathol. Int.* **52,** 234–238.
79. Wu, M., Stockleym P. G., and Martinm W. J. 2nd (2002) An improved western blotting technique effectively reduces background. *Electrophoresis* **23,** 2373–2376
80. Kakita, K., O'Connell, K., and Permutt, M. A. (1982) Immunodetection of insulin after transfer from gels to nitrocellulose filters. A method of analysis in tissue extracts. *Diabetes* **31,** 648–652.
81. Ikegaki, N. and Kennett R. H. (1989) Glutaraldehyde fixation of the primary antibody-antigen complex on nitrocellulose paper increases the overall sensitivity of immunoblot assay. *J. Immunol. Methods* **30,** 205–210.
82. Krajewski, S., Zapata, J. M., and Reed J. C. (1996) Detection of multiple antigens on western blots. *Anal. Biochem.* **236,** 221–228.
83. Langstein, J. and Schwarz, H. (1997) Suppression of irrelevant signals in immunoblots by preconjugation of primary antibodies. *Biotechniques* **23,** 1006–1010.
84. Lasne, F. (2001) Double-blotting: a solution to the problem of nonspecific binding of secondary antibodies in immunoblotting procedures. *J. Immunol. Methods* **253,** 125–131.
85. Knepper, M. A. and Masilamani, S. (2001) Targeted proteomics in the kidney using ensembles of antibodies. *Acta. Physiol. Scand.* **173,** 11–21.
86. Murray, J., Gilkerson, R., and Capaldi, R. A. (2002) Quantitative proteomics: the copy number of pyruvate dehydrogenase is more than 10(2)-fold lower than that of complex III in human mitochondria. *FEBS Lett.* **9,** 173–178.
87. Wilkinson, D. (2000) Chemiluminescent techniques for western blot detection let researcher shed their lead aprons. *The Scientist* **14,** 29–32.

88. Hawkes, R. Niday, E., and Gordon, J. (1982) A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* **119,** 142–147.
89. Conrad, C. C., Malakowsky, C. A., Talent, J., Rong, D., Lakdawala, S., and Gracy, R. W. (2001) Chemiluminescent standards for quantitative comparison of two-dimensional electrophoresis western blots. *Proteomics* **1,** 365–369.
90. Steffen, W. and Linck, R. W. (1989) Multiple immunoblot: a sensitive technique to stain proteins and detect multiple antigens on a single two-dimensional replica. *Electrophoresis* **10,** 714–718.
91. Lindahl, M., Stahbom, B., Svartz, J., and Tagesson, C. (1998) Protein patterns of human nasal and bronchoalveolar lavage fluids analyzed with two-dimensional electrophoresis. *Electrophoresis* **19,** 3222–3229.
92. Lemkin, P. (1996) Matching 2-D gels on the Internet. From the Abstract Book of *2nd Siena 2-D Electrophoresis Meeting*, 57; Siena Sept. 16–18.

18

Epitope Mapping

Glenn E. Morris

Summary

Epitope mapping can be used to identify areas of a protein that an antibody recognizes and binds to. Monoclonal antibodies are easier to characterize, but epitope maps can also be produced for polyclonal antisera.

Key Words: Epitope; conformational; sequential; blotting; phage; biopanning.

1. Introduction

Epitope mapping is the process of identifying the region of an antigen that an antibody recognizes ***(1–3)***. It is most frequently applied to protein antigens, though methods for mapping carbohydrate antigens are also being developed ***(4,5)***. Epitopes on proteins are sometimes a simple sequence of amino acids, although conformational epitopes, formed by bringing together amino acid side chains during protein folding, also are common. Monoclonal antibodies (MAbs) are simpler to map because they recognize a single, unique epitope, whereas polyclonal antisera may recognize several epitopes on the same protein. Nevertheless, it is perfectly possible to map at

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

least the major (immunodominant) epitopes recognized by a polyclonal antiserum ***(6)***.

The method of choice for epitope mapping depends on a number of factors. The most useful starting point is to establish whether the antibody recognizes a conformational or a sequential epitope. The simplest way to find out whether an epitope is conformational is by Western blotting after sodium dodecyl sulfate polyacrylamide gel electrophoresis. If the antibody still binds after the protein has been boiled in sodium dodecyl sulfate and 2-mercaptoethanol, the epitope is unlikely to be highly conformational. Antibodies against assembled epitopes often display the high avidities and specificities required for immunoassays, but they are difficult to map. Mapping methods for these are rather vague (e.g., competition enzyme-linked immunosorbent assay [ELISA]), incomplete (e.g., antibody protection of antigen from chemical modification), or long and involved (e.g., X-ray studies or in vitro mutagenesis of recombinant antigens). In contrast, sequential epitopes can be mapped by powerful peptide and fragmentation methods. I have discussed in detail the additional considerations involved in choosing an appropriate mapping method ***(1)***, such as availability of purified antigen or whether the antigen can be expressed from cloned complimentary deoxyribonucleic acid (cDNA). Details of 30 different mapping methods, mainly for B-cell epitopes, can be found in *Epitope Mapping Protocols* ***(1)***. A more recent volume ***(7)*** is especially useful for T-cell epitope mapping.

For the present chapter, I have chosen to present a complete protocol for a phage-displayed random peptide library that is particularly useful for antibodies that work well on Western blots. It has the major advantage that only the antibody and the peptide library are required and, because no antigen is needed, the reader can apply the method to commercially available antibodies or human autoantibodies. In our experience, some MAbs are not amenable to peptide mapping, so we prefer to use a mixture of 5–10 different MAbs (not necessarily against the same antigen) for "biopanning" to ensure a positive result. Selection of phages by biopanning is first carried out using the mixture of antibodies. Colonies of *Escherichia*

coli infected by the selected phage are screened first with the same mixture of MAbs. The positive clones thus identified are then screened with each individual MAb to determine their specificity. MAbs that are able to react with these clones are then removed from the mixture and the biopanning can be repeated with the remaining antibodies ("re-iterative" biopanning; **ref.** ***8***). We recommend testing 30–40 clones for MAb binding after the second biopanning because further rounds of panning are often unnecessary and could, in theory, reduce the diversity of peptides selected in favor of higher affinity sequences. For many years, we have used the excellent phage-displayed random peptide libraries developed by George P. Smith of the University of Missouri at Columbia ***(9–11)***. Most recently, we have applied this method to panels of MAbs against dystrophin–dystroglycan ***(12)*** and the myotonic dystrophy protein kinase ***(13)***. There is a rather similar commercial kit, "Ph.D," marketed by New England Biolabs (Beverly, MA), but the Smith method has some important differences that, in our experience, make it much simpler to use. Both methods use random peptide libraries inserted into coat proteins of filamentous phage and these are progressively enriched for epitope-containing phage by biopanning the phage particles against antibody bound to a solid phase. Typically, mouse MAbs are captured onto a small Petri dish coated with rabbit anti-(mouse Ig). After biopanning, the bound phage are expanded by overnight infection of an *E. coli* suspension culture and recovered from the culture medium for the next round of biopanning. After two or three rounds of biopanning, antibody-positive phage may constitute a high proportion of the total and a cloning step on culture plates is performed in order to identify them. It is at this point that the Smith and the "Ph.D" methods diverge. The Smith method uses phage vectors derived from *fd-tet*, a derivative of M13 carrying a tetracycline-resistance marker, while the "Ph.D" method uses M13 itself. M13 colonies in the "Ph.D" method are produced as "turbid plaques" of slow-growing bacteria in a "lawn" of *E. coli*. These plaques require even more skillful handling than the "clear plaques" produced by lytic phage, such as bacteriophage lambda, and the "Ph.D" protocols recommend an ELISA

method to screen each plaque for antibody-positive phage. This cloning and screening process is greatly simplified in the Smith method by the use of *fd-tet* phages. *E. coli* infected with these vectors grow as colonies, rather than plaques, because uninfected *E. coli* fail to grow on tetracycline plates. These colonies can be picked and replicated as master plates on LB-tet agar and also grown on nitrocellulose sheets for screening by a Western blot method. Phage particles released from the growing colonies attach to the nitrocellulose and the bacteria, which might give high backgrounds in a western blot, can be removed by wiping with a sponge.

Another form of displayed peptide library is the FliTrx Random Peptide Display Library (Invitrogen, Paisley, UK), which uses the bacterial flagellum to display random peptide libraries on the *E. coli* cell surface ***(14)***. This library was constructed in the pFliTrx vector, which positions the random peptides in a flagellin (Fli) thioredoxin (Trx) fusion protein. Biopanning with bacteria works surprisingly well in our experience ***(15)*** and screening on nitrocellulose is similar to the Smith method.

Phage-displayed 6-mer, 12-mer, or 15-mer peptides are of rather limited use for mapping conformational epitopes, although it is possible to create custom libraries for particular antigens by cloning random fragments of the antigen cDNA into phage ***(16)***. These detect conformational epitopes more frequently because the protein fragments expressed are larger. "Mimotopes" (sequences that mimic the shape of the epitope sufficiently to enable antibody binding) can also be isolated from libraries ***(17)*** Peptide mimotopes of carbohydrate antigens can even be identified ***(18)*** and these may have important applications in vaccine design.

2. Materials

2.1. Biopanning and Phage Amplification

1. *E. coli* strain K91Kan. The strain was supplied together with the 15-mer library by Prof. G. P. Smith.
2. TBS: 50 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl. Autoclave.

3. TBST: TBS + 0.5% Tween-20, autoclave.
4. TBS/gelatin: 0.1 g gelatin in 100 mL of TBS. Autoclave to dissolve.
5. 1 *M* Tris-HCl, pH 9.1. Filter-sterilize.
6. Elution buffer: 0.1 *M* glycine-HCl, pH 2.2, 0.1% bovine serum albumin (BSA), 0.01% phenol red.
7. Blocker: 4% BSA in sterile TBS.
8. 80 m*M* NaCl. Autoclave.
9. NAP buffer: 50 m*M* $NH_4H_2PO_4$, pH 7.0 with ammonia, 80 m*M* NaCl.
10. PEG/NaCl: 10 g of PEG 8000, 11.7 g of NaCl, 47.5 mL of water. Autoclave to dissolve.
11. Stock solutions of antibiotics: 10 mg/mL kanamycin in water; filter-sterilize; 5 mg/mL tetracycline in ethanol; store at –20°C.
12. LB broth: 10 g of bactotryptone, 5 g of yeast extract (both from Difco, Detroit, MI), 5 g of NaCl, and water to 100 mL. Autoclave.
13. LB agar plates: LB broth +16 g/L of bactoagar (Difco, Detroit, MI). Autoclave, allow to cool down to 45–50°C and pour into Petri dishes.
14. LB agar plates +100 µg/mL kanamycin.
15. LB agar plates + 40 µg/mL tetracycline + 100 µg/mL kanamycin. Antibiotics are added after autoclaving, just before pouring into Petri dishes.
16. Plasticware: sterile 35-mm and 90-mm Petri dishes, autoclaved yellow and blue pipet tips, 1.5- and 0.5-mL Eppendorf tubes.
17. Autoclaved toothpicks (antibiotic free).

2.2. Colony Screening

1. Nitrocellulose membranes (Schleicher and Schuell, pore size 0.45 µm).
2. Peroxidase-labeled anti-mouse Ig antibodies (DAKO, Carpintiera, CA).
3. Incubation buffer (IB): TBST supplemented with 0.1% BSA, 1% fetal calf serum, and 1% horse serum.
4. Blocking solution: 3% low fat dried milk (from any supermarket) dissolved in TBST
5. Substrate buffer: mix 25.7 mL of 0.2 *M* Na_2HPO_4, 24.3 mL of 0.1 *M* citric acid and 50 mL of water (final pH 5.0).
6. DAB (diaminobenzidine dihydrochloride; Sigma, St Louis, MO) stock solution (80X: 32 mg/mL in water). This should be handled with care because it is a possible carcinogen.
7. Hydrogen peroxide (30% v/v).

2.3. DNA Isolation

1. 25X TBE buffer for agarose gel electrophoresis: Trizma base, 27 g; boric acid, 13.75 g; ethylene diamine tetraacetic acid, 2.33 g. Make up to 100 mL.
2. 2X Agarose gel sample buffer: 2XTBE; 10% sucrose or glycerol; 0.5% Bromophenol blue
3. Agarose (molecular biology grade), ethidium bromide (10 mg/mL in water), DNA size markers (Type VII, Roche Diagnostics, Lewes, UK), and standard equipment for agarose gel electrophoresis (e.g., Horizon 58 apparatus; Gibco BRL, Paisley, UK).
4. UV transilluminator.

3. Method

The procedure for epitope mapping by the phage display method involves: biopanning, amplification, assay for positive colonies, DNA isolation, and sequencing. Some steps of the procedure must be performed at the same time. Therefore, we describe the protocols in **Subheadings 3.1.** and **3.2.** on a day-by-day manner. All steps of biopanning and phage amplification should be carried out under sterile conditions, when possible.

3.1. Biopanning

3.1.1. Day 1

1. Coat 35-mm sterile Petri dishes with 1 mL of rabbit-anti-(mouse Ig) antibodies diluted 1/1000 in TBS (*see* **Note 1**).
2. Incubate overnight at 4°C on rocker.

3.1.2. Day 2

1. Wash plates 1× with TBST.
2. Add blocking solution (4% BSA in sterile TBS) and rock for 1 h at room temperature.
3. Wash plates 6× with TBST. Add 1 mL of MAbs mixture (each MAb is diluted 1/50 in TBST; *see* **Note 2**). Incubate for 1 h at room temperature on rocker.

4. Wash 6× with TBST. Add phage library (10^{11} transducing units in 0.75 mL TBST). Incubate for 4 h at 4°C on rocker.
5. Wash thoroughly 10 times with TBST. Elute bound phage for 5–10 min with 400 µL of elution buffer. Wash the plate surface thoroughly during this elution using a pipet tip to remove all phage. Add 75 µL of 1 *M* Tris-HCl, pH 9.1, and mix (*see* **Notes 3** and **4**).

3.2. Amplification

3.2.1. Day 0

1. Streak out K91Kan from stock on LB/Kan plate. Grow cells overnight at 37°C to obtain single colonies.

3.2.2. Day 1

1. Grow a single colony overnight in a 1.5 mL Eppendorf tube in 1 mL LB/Kan.

3.2.3. Day 2 (Early)

1. Inoculate 20 mL of LB in 100-mL bottle with 20 µL of K91Kan culture. Grow to mid log (3–4 h) with vigorous shaking at 37°C (OD_{600} = 0.45). Leave for 5 min without shaking at 37°C.
2. Pellet the cells at 800*g* for 10 min. Resuspend in 20 mL of 80 m*M* NaCl. Shake gently for 45 min at 37°C.
3. Pellet the cells at 800*g* for 10 min. Resuspend in 1 mL of cold NAP buffer. This bacterial suspension may be stored at 4°C for up to 5 d.

3.2.4. Day 2 (Later)

1. Mix 100 µL of phage eluate with 100 µL of K91Kan cells in NAP in an Eppendorf tube. Rock at room temperature for 10–30 min.
2. Transfer to 20 mL of LB containing 0.2 µg/mL tetracycline. Shake at 37°C for 1 h.
3. Add 80 µL of tetracycline 5 mg/mL stock solution. Continue shaking overnight at 37°C.

3.2.5. Day 3

1. Centrifuge cells at 1000*g* for 15 min.
2. Transfer supernatant into another centrifuge tube and centrifuge at 10,000*g* for 10 min.
3. Transfer supernatant into a Vivaspin 15 (Vivascience) unit. Pour 15 mL of supernatant into the unit. Centrifuge at 800*g* for 15 min. Add the remaining supernatant and centrifuge another 15 min at 800*g*. Collect the concentrate (approx 0.5 mL) and add sterile TBS to 1 mL.
4. Transfer the 1 mL of concentrate to 150 µL of of PEG/NaCl in a second Eppendorf tube. Mix by inverting 100X. Leave on ice for at least 1 h.
5. Microfuge for 10 min at max speed.
6. Remove supernatant, recentrifuge briefly, and remove supernatant.
7. Resuspend pellet in 200 µL of TBS/azide.
8. Use 100–200 µL of this purified phage and 400 µL of TBST for next panning.

This is now ready for the second round of biopanning, so that d 3 now becomes d 2 again (*see* **Note 5**).

3.3. Assay for Positive Colonies

1. Prepare serial dilutions of the "red eluate" in TBSG (*see* **Note 6**).
2. Mix 10 µL of diluted phage and 10 µL of K91Kan cells in NAP (**Subheading 3.2.5.**).
3. Rock for 10 min at room temperature.
4. Add 1 mL of LB containing 0.2 µg/mL tetracycline and shake at 37°C for 30 min.
5. Spread 200 µL on LB/Tet/Kan agar and grow overnight at 37°C (*see* **Note 7**).
6. Cut a nitrocellulose membrane (NCM) to fit a 90-mm Petri dish (*see* **Note 8**).
7. Put NCM onto LB/Tet/Kan agar and let it soak.
8. Using the same toothpicks transfer single colonies onto both a LB/Tet/Kan agar master plate and to a NCM on LB/Tet/Kan agar.
9. Incubate plates overnight at 37°C.
10. Put the master plates in a refrigerator (these can be stored for several weeks).

11. Remove the NCM from agar surface and transfer to another Petri dish with 10 mL of TBST. Using a piece of sponge, remove all the cells from the NCM.
12. Wash six to eight times with TBST. Block NCM for 30 min at room temperature in 3% low fat dried milk in TBST. Incubate for 1 h with MAbs (hybridoma supernatant, 1/50 in IB; *see* **Note 9**) at room temperature on rocker.
13. Wash five times with TBST. Incubate for 1 h with peroxidase-labeled rabbit anti-(mouse IgG) antibodies diluted 1/1000 in IB at room temperature on rocker.
14. Wash five times with TBST and two to three times with distilled water. Add 5 mL of DAB solution in substrate buffer supplemented with 0.012% hydrogen peroxide. Traces of the positive colonies should be bright-brown colored (*see* **Notes 10** and **11**).
15. Make replicas of positive clones with sterile toothpicks on another NCM placed on a LB/Tet/Kan agar surface so, that the replicas form columns that can be cut later into strips for incubation with individual MAbs from the mixture used for biopanning. Grow the cells overnight at 37°C. Repeat **steps 11** to **14** with the exception that incubation with antibodies is performed individually with each MAb.

3.4. DNA Isolation

1. Inoculate 3 mL-portions of LB containing 20 µg/mL tetracycline with positive clones identified in **Subheading 3.4.** Shake the tubes vertically for 16–24 h at 37°C.
2. Microfuge each culture briefly to pellet cells.
3. Pipet supernatant into a vessel containing 450 µL of PEG/NaCl. Mix by 100X invertions and incubate on ice for at least 4 h.
4. Microfuge for 15 min, remove supernatant, recentrifuge briefly, and remove supernatant.
5. Dissolve pellet in 500 µL of TBS by vortexing. These phage are already pure enough for preparing sequencing templates.
6. Put 500 µL of PEG-purified phage suspension into Eppendorf tube. Add equal volume of phenol/chloroform. Vortex vigorously. Microfuge to separate phases. Collect the upper aqueous phase (approx 400 µL) trying to avoid any traces of interphase and lower phase.
7. Transfer aliquot to a second Eppendorf tube with 40 µL of 3 *M* sodium acetate and 1 mL of ethanol. Allow DNA to precipitate for at least 1 h on ice.

8. Microfuge for 15 min. Remove supernatant, recentrifuge briefly, and remove supernatant again.
9. Wash pellet (usually invisible) with 1 mL of 70% ethanol. Remove supernatant, recentrifuge briefly, and remove supernatant.
10. Dissolve pellet in 8 µL of water.
11. Run 1 µL of sample in 1% agarose gel to control purity of DNA. To obtain good sequence results, a sharp band of around 9000 bp should be visible.

Boil 1 g of molecular biology grade agarose in 100 mL of 1X TBE buffer in a glass conical flask until a clear transparent solution is achieved. Cool to 60°C, add 5 µL of ethidium bromide (10 mg/mL), and mix thoroughly. Pour into gel apparatus with sample comb and leave to set for 20 min to allow the gel to set. Add just enough electrophoresis buffer (1X TBE) to cover the gel. Mix phage DNA samples with an equal volume of 2X agarose gel sample buffer and load 10 µL of each into the slots of the submerged gel. Run gel at 200 volts for 11–15 min and examine with a UV transilluminator. The rest of the DNA can be stored at –20°C for a few weeks.

3.5. DNA Sequencing

This step can now be conducted commercially at very low cost by many companies using automated sequencers. DNA can be sent at ambient temperature by overnight mail and sequence results are returned by e-mail. The primers used depend on the vector (fUSE5 or f88-4) and those recommended by G. P. Smith copy the coding strand in reverse; f88-4/15mer, 5'-AGTAGCAGAAGCCTGAAGA; fUSE5, 5'-TGAATTTTCTGTATGAGG. The sequence thus obtained is the reverse complementary strand which must be translated back to the coding strand to get the peptide sequence.

4. Notes

1. The rabbit antibodies used for capturing MAbs may also interact with some phage from the library. This might result in selection of many non-MAb specific clones. Thus, the library should be pre-incubated

with anti-(mouse Ig) antibodies alone in order to prevent non-specific binding of phage by rabbit Ig.

2. Plates might be coated at the same time with MAbs of different specificity—individually or in combination with others. The principle of MAbs combination depends on whether there is some preliminary information about their mutual relationship, for example, competitive assay or peptide binding assay. Afterwards, the plates would be sequentially incubated with the same library preparation.
3. Phenol red is added to elution buffer, and is yellow at pH 2.2. When 1 *M* Tris-HCl, pH 9.1 is added, the elution buffer turns red, hence the name "red eluate."
4. Because the phage containing the desired peptides might be present in the main library as only a few particles, it is recommended to concentrate the first "red eluate" to 100 mL using a membrane concentrator (e.g., Vivascience, VSO 132) before amplifying.
5. On further rounds of panning, the MAbs mixture is reacted only with the corresponding amplified eluate.
6. Tentatively, suitable dilutions of "red eluate" (in TBS/gelatin) appear to be A:1 µL in 1000 µL of TBSG, B: 100 µL of A plus 900 µL of TBSG, C:100 µL of B plus 900 µL of TBSG.
7. It is useful to spread the cells remaining after amplification (**Subheading 3.2.9.**) to obtain single colonies for screening. Add 2 mL of LB broth to the pellet of overnight culture. Make serial dilution (10^{-}, 10^{-5}, 10^{-} should be fine) in LB medium. Use 200 µL to spread on a plate.
8. To fit a 90-mm Petri dish, a NCM might be cut as a rectangle approx 5.0×6.5 cm.
9. Hybridoma SN should preferably be used for colony screening because there is always the possibility of false-positive colonies being selected using ascites. Ascites contain a lot of antibodies with unknown specificity, which could bind phage from the library.
10. For some antibodies, positive clones were found even after the first round of panning. It seems that further enrichment is undesirable in these cases because there is the possibility that this might lead to isolation of fewer clones with higher affinity for the antibodies.
11. If quantitative analysis of antibody-phage binding is desirable, it is possible to perform an ELISA procedure using purified phage as antigen.

Acknowledgments

I thank Prof. George Smith (University of Missouri, Columbia) for supplying his phage-display library kits for many years and the many people in my own lab who have applied them successfully, including Alex Pereboev, Le Thanh Lam, Fiona Wilkinson, Pham Nguyen Y. Chan, and Natalie Randles. This work was supported by grants from the Muscular Dystrophy Campaign, British Heart Foundation, EU Fifth Framework, Muscular Dystrophy Association and Families of SMA.

References

1. Morris, G. E. (1996) Choosing a method for epitope mapping, in *Epitope Mapping Protocols* (G.E. Morris, ed.), Humana Press. Totowa, NJ, pp. 1–9.
2. Morris, G. E. (1998) Epitope Mapping, in *Molecular Biomethods Handbook* (R. Rapley and J.M. Walker, eds.) Humana Press, Totowa, NJ, pp. 619–630.
3. Morris, G. E. (2001) Epitope mapping, in *Encyclopaedia of Life Sciences*, MacMillan Press, London.
4. Liang, R., Yan, L., Loebach, J., Ge, M., Uozumi, Y., Sekanina, K., Horan, N., et al. (1996) Parallel synthesis and screening of a solid phase carbohydrate library. *Science* **274,** 1520–1522.
5. Sanders, R. W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K. O., et al. (2002) The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. *J. Virol.* **76,** 7293–7305.
6. Pereboeva, L. A., Pereboev, A. V., and Morris, G. E. (1998) Identification of antigenic sites on three hepatitis C virus proteins using phage-displayed peptide libraries. *J. Med. Virol.* **56,** 105–111.
7. Westwood, O. M. R. and Hay, F. C. (2001) *Epitope Mapping: a Practical Approach*. Oxford University Press, Oxford.
8. Pereboev, A. V. and Morris, G. E. (1996) Reiterative screening of phage display peptide libraries for epitope mapping, in *Epitope Mapping Protocols* Humana Press, Totowa, NJ, pp. 195–206.
9. Smith, G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228,**1315–1317.

10. Scott, J. K. and Smith, G. P. (1990) Searching for peptide ligands with an epitope library. *Science* **249,** 386–390.
11. Smith, G. P. (1992) Cloning in fUSE vectors available directly from Prof. G. P. Smith, Division of Biological Sciences, University of Missouri.
12. Pereboev, A. V., Ahmed, N., Nguyen thi Man, and Morris, G. E. (2001) Epitopes in the interacting regions of beta-dystroglycan (PPxY motif) and dystrophin (WW domain). *Biochim. Biophys. Acta.* **527,** 54–60.
13. Lam, L. T., Pham, Y. C., Nguyen thi Man, and Morris, G. E. (2000) Characterization of a monoclonal antibody panel shows that the myotonic dystrophy protein kinase, DMPK, is expressed almost exclusively in muscle and heart. *Hum. Mol. Genet.* **9,** 2167–2173.
14. Lu, Z., Murray, K. S., Van Cleave, V., LaVallie, E. R., Stahl, M. L., and McCoy, J. M. (1995) Expression of thioredoxin random peptide libraries on the *Escherichia coli* cell surface as functional fusions to flagellin: a system designed for exploring protein-protein interactions. *Biotechnology* **13,** 366–372.
15. Manilal, S., Sewry, C. A., Pereboev, A. V., Nguyen thi Man, Gobbi, P., Hawkes, S., et al. (1999) Distribution of emerin and lamins in the heart and implications for Emery-Dreifuss muscular dystrophy. *Hum. Mol. Genet.* **8,** 353–359
16. Pereboeva, L. A., Pereboev, A. V., Wang, L. F., and Morris, G. E. (2000) Hepatitis C epitopes from phage-displayed cDNA libraries and their assembly into improved chimeric antigens. *J. Med. Virol.* **60,** 144–151.
17. McConnell, S. J., Kendall, M. L., Reilly, T. M., and Hoess, R. H. (1994) Constrained peptide libraries as a tool for finding mimotopes. *Gene* **151,** 115–118.
18. Hou, Y. and Gu, X. X. (2003) Development of peptide mimotopes of lipo-oligosaccharide from nontypeable Haemophilus influenzae as vaccine candidates. *J. Immunol.* **170,** 4373–4379.

19

Generation and Testing of Immunoaffinity Columns

Weilin L. Shelver

Summary

Immunoaffinity purification is a highly specific, reversible technique that has the potential to be used for the one-step isolation of an analyte from many complex matrices. Application of an immunoaffinity column to isolate and concentrate an analyte may decrease the amount of solvent used, decrease the number of purification steps, shorten analysis time, and simplify sample analysis relative to traditional clean-up techniques. In addition, immunoaffinity techniques may be easily integrated into high throughput systems. In this chapter, an immunoaffinity column generation method is outlined. Techniques and procedures for sample loading, washing, elution, and column storage will be described. In addition, methods used to determine column performance will be discussed. The information provided in this chapter will allow the inexperienced user to quickly become familiar with the generation, testing, and use of immunoaffinity columns.

Key Words: Immunoaffinity chromatography; IAC; isolation; purification; sample preparation; immunoaffinity columns.

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

1. Introduction

Immunoaffinity chromatography can be considered as a subset of affinity chromatography, in which an immunoglobulin is used to bind analyte molecules. Because immunoglobulins show exquisite specificities towards antigens used in their production, immunoaffinity columns are ideal for the purification of the targeted analytes. Either monoclonal or polyclonal antibodies, produced using a variety of techniques ***(1)***, may be used as a source of immunoglobulin for immunoaffinity columns.

Immunoaffinity columns are extremely versatile and have been used for the isolation and concentration of a diverse number of analytes from a wide array of matrices ***(2)***. Analytes may include macromolecules such as proteins and receptors or small molecules such as environmental toxins, antibiotics, or pesticides. Matrices may include animal tissues or excreta, plant extracts, cell culture medium, or virtually any milieu encountered in biological work. Because of its value as a research tool, immunoaffinity chromatography has found extensive use by the pharmaceutical industry to purify therapeutic proteins, the food safety community to purify small amounts of toxins from food and as a general tool for analytical chemists to purify analytes for subsequent instrumental analysis.

The general principle of immunoaffinity chromatography is illustrated in **Fig. 1**. The analyte in the sample matrix is loaded onto the column, the column is washed to remove interfering substances, and the analyte is eluted from the column for subsequent use. The column is the heart of the purification system and must bind the analyte specifically enough to allow other substances to be rinsed off the column, allow the elution of the analyte under conditions that do not elute interferences, and permit the column to be regenerated multiple times for subsequent use.

Important considerations when generating immunoaffinity columns include the conjugation of antibody to the supporting (column) material, packing the column, developing suitable washing and elution protocols, and constructing appropriate tests to evaluate column performance. Of primary importance to the utility of an

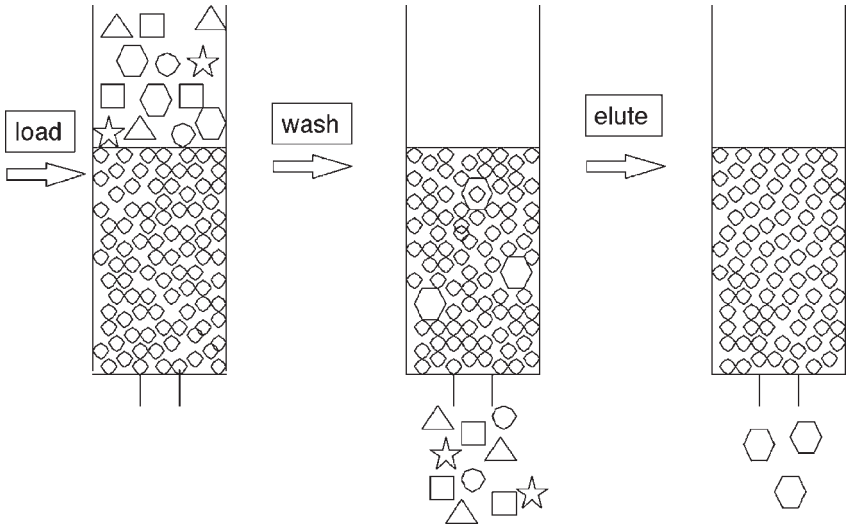


Fig. 1. Diagram of immunoaffinity column.

immunoaffinity column is the suitability of the antibody selected for immobilization onto the column support. The antibody should have adequate affinity toward the analyte so that analyte binding occurs with sufficient selectivity as to not bind interfering matrix components. Solvent conditions under which the analyte is eluted from the affinity column must be of sufficient strength to remove all the analyte, but mild enough so that the antibody is not denatured during elution. The generation of a suitable antibody is not the subject of this chapter, but there is a vast amount of literature on this subject ***(3,4)***.

Conjugation of the antibody to a supporting material should allow for a column with flow properties compatible with low-pressure chromatography and the antibody should retain its essential analyte binding characteristics. The selected chromatographic support must have low levels of nonspecific binding, a high surface area, and good liquid flow properties. In addition, the support must be capable of being modified with a bifunctional molecule; one end of the molecule will react to the support and the other end will react with the antibody. This discussion will focus on the use of cyanogen bro-

mide-activated Sepharose which has been designed to react with free amino groups (i.e., lysine) present in antibodies. A number of alternate supports, listed in **Table 1**, are available (*see* **Note 1**) that bind with different functional groups found on the antibody, most commonly either amino groups or carbohydrates located on the Fc portion of the antibody. The procedure to use these supports may be found in the company literature or on their website.

2. Materials

2.1. General Equipment

The general equipment used can range from very simple columns and test tubes to elaborate pumps, columns, detectors, and fraction collectors automatically controlled by a computer with an appropriate human interface. Minimal equipment is sufficient for exploratory work or one-time development cycles; more sophisticated equipment is beneficial for laboratories engaged in more intensive development. A modest set of useful equipment includes: 1) peristaltic pump; 2) end-over-end tube rotator; 3) fraction collector; and 4) spectrophotometer (UV visible).

2.2. Supporting Beads

We will describe the use of CNBr-activated Sepharose 4B available from Amersham Pharmacia Biotechnology.

2.3. Columns

Depending on the application, immunoaffinity matrices can be packed into open columns, such as screening columns (Fisher Scientific, Pittsburgh, PA), and a variety of glass columns, such as those provided by Amersham Pharmacia Biotechnology, Bio-Rad, or other vendors. The column material must be compatible with your analysis because either plastic or glass can cause problems with low concentrations of some analytes. Column dimensions are dependent

Table 1
Some Examples of Commercially Available Gels Used for the Immobilization of Antibody

Matrix	Function group on the beads	Group to be coupled	Products[a]
Cross-linked agarose	-CHO	$-NH_2$	Aminolink coupling gel (P)
	diaminopropylamine	-COOH	Immobilized diaminodipropylamine (P)
	cyanate	$-NH_2$	CNBr activated matrices (S)
			CNBr-activated Sepharose 4B (A)
	Imidazolyl carbamate	$-NH_2$	carbonyldiimidazole affinity resins (S)
			Reacti-gel (P)
	N-hydroxysuccinimide	$-NH_2$	HNS activated matrices (S)
			Affi-gel 10 (B), Affi-gel 15 (B)
	iodoacetyl	-SH	Ultralink iodoacetyl (P)
	hydrazide	-CHO	Carbolink (P)
			Affi-Gel Hz (B)
			Agarose Adipic Acid Hydrazide (A)
Bis-acrylamide/azlactone		-NH2, -SH, -OH,	Ultralink biosupport medium (P)
Acrylic	*N*-hydroxysuccinimide	$-NH_2$	Affi- Prep 10 (B)
Pressure stable polymer	hydrazide	-CHO	Affi-Prep Hz (B)

[a]The letter in parenthesis indicates the abbreviation of the commercial sources. A: Amersham Pharmacia Biotechnology; B: Bio-Rad; P: Pierce; S: Sigma. This is not an exhaustive list, but serves to provide examples of the most commonly used activated supports.

on specific applications; we routinely use plastic columns having a 5-mL capacity and pack them with 1 mL of support. Normally we pack the columns using gravity flow; but depending upon the support either pressure or vacuum assisted packing techniques might be appropriate. An optimum flow rate (approx 0.5 mL/min) permits equilibration between the immobilized antibody and the analyte.

2.4. Antibody Preparation

Either monoclonal or polyclonal antibodies may be used to prepare immunoaffinity columns. A variety of techniques may be used in the isolation of antibody from ascites, serum, cell medium, or other sources. Purification of antibody will reduce complications later in the analysis. Dependent on the properties of the antibody and the matrix from which it is isolated, ammonium sulfate precipitation ***(5)***, ion-exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, or affinity purifications using protein A or protein G are commonly used to purify the immunoglobulin ***(6)***. Protein concentration can be determined by UV-absorbance at 280 nm or colorimetrically using Bradford ***(7)*** or modified Lowry methods ***(8)***. The purity of the isolated immunoglobulin can be assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis based on the molecular weight of IgG (150,000 D).

2.5. Gel Bead Conjugation

1. CNBr-activated Sepharose 4B beads.
2. Antibody-coupling buffer (0.1 *M* sodium bicarbonate buffer, pH 8.3).
3. Blocking solution (0.1 *M* Tris-HCl buffer, pH 8.0, or 0.1 *M* ethanolamine).
4. Washing solutions (0.1 *M* Tris-HCl buffer, pH 8.0, with 0.5 *M* NaCl and 0.1 *M* acetate buffer pH 4.0 with 0.5 *M* NaCl).
5. Storage solution (phosphate-buffered saline [PBS], pH 7.2, with 0.02% sodium azide, each liter contains 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4). (*Caution*: sodium azide is very toxic; read the label prior to use and handle with care.)

3. Methods

3.1. Immunoaffinity Gel Generation

1. Weigh 3 g of freeze-dried CNBr-activated Sepharose 4B powder into a 50-mL centrifuge tube; add 20 mL of 1 m*M* HCl (pH 2.0–3.0) to suspend the gel. Three grams of freeze-dried powder will swell to approx 10 mL of gel and the scale of the preparation may be altered to suit the application. Pour the gel onto a sintered glass filter (porosity C) and wash carefully with 600 mL (200 mL per gram of gel) of 1 m*M* HCl over a 15-min period. The washing solution should be added in several aliquots and great care should be taken to prevent the gel from drying. If necessary, flow through the funnel may be increased by using a slight vacuum, but it is important to wash slowly to properly prepare the gel.
2. Mix 50 mg of IgG (*see* **Note 2**), dissolved in 20 mL of 0.1 *M* $NaHCO_3$, pH 8.3/0.5 *M* NaCl, with 10 mL of gel (**Subheading 3.1.**, **step 1**) and divide into two 50-mL centrifuge tubes (*see* **Note 3**). Rotate the slurry with end-to-end tube rotator (for example, Roto-Torque Heavy Duty Rotator Model 7637-01, Cole-Parmer Instrument Company, Vernon Hills, IL) at 4°C for approx 14–16 h or 2 h at room temperature.
3. Centrifuge the gel at 200*g* for 5 min. Combine the supernatant from each tube and retain for measurement of unreacted antibody.
4. Wash away unbound IgG with coupling buffer (0.1 *M* $NaHCO_3$, pH 8.3/0.5 *M* NaCl). Save the wash supernatants for the determination of unreacted antibody. The coupling buffer wash solution should be at least five times the gel volume.
5. Mix the gel with 10 mL of 0.1 *M* Tris-HCl, pH 8.0, per tube to block remaining unreacted groups on the cyanogen bromide activated gel. Using an end-to-end rotator, allow the gel to react for 2 h at room temperature. The Tris-HCl buffer contains primary amino groups that will react with any remaining active groups on the gel.
6. Wash the gel with 0.1 *M* acetate buffer pH 4.0/ 0.5 *M* NaCl followed by 0.1 *M* Tris-HCl, pH 8.0/0.5 *M* NaCl. Repeat twice more for a total of three cycles of the two-buffer wash. The washing solution should be at least five times the gel volume for each cycle.

7. Store the finished product at 4°C in PBS, pH 7.2, 0.02% sodium azide until used.
8. Determine the efficiency of IgG immobilization to the Sepharose beads by measuring protein concentration in the supernatants obtained from **steps 3**. The protein remaining in these supernatant fractions represents antibody that was not immobilized. The efficiency of conjugation may be calculated by:

Percentage Bound = 100 [(total mg IgG added) – (mg IgG in supernatants)]/(total mg IgG added)

Efficiencies less than 70% indicate that inefficient IgG immobilization has occurred. The cause of inefficient coupling is often the presence of a buffer component (free amines in Tris-HCl buffers, for example) that competes with primary amines of the antibody for binding at the active sites of the cyanogen bromide activated Sepharose beads.

3.2. Immunoaffinity Column Application

1. Pack the gel into a plastic or other suitable column after allowing the gel to warm to room temperature and being careful to keep the column free of air bubbles or channels. Normally, a visual assessment for air bubbles or channels is adequate; if air bubbles are present then the column may be gently tapped against a hard surface or the column may be repacked. We routinely pack 1 mL of immunoaffinity gel into 5-mL columns equipped with a bottom frit (200–300 µm) to retain the gel. We do not normally use a top frit although other researchers find this protects the column.
2. Carefully apply the sample (*see* **Notes 4** and **5**) to the column. The sample may be applied using gravity flow or with a peristaltic pump, but use care not to disrupt the gel in the column. Theoretically, there is no limit to the volume of sample added, but the sample amount should not exceed the binding capacity of the column.
3. Wash the column with a solution that is strong enough to wash off the interferences but not the analyte(s). We commonly use 10-column volumes of 10% MeOH in water. Column washing is a crucial step in the process because it is the step in which most interference are removed while the analyte remains bound by the immobilized

antibody. Determination of an appropriate wash solution is an empirical process.

4. Elute the analyte (*see* **Note 6**) by applying 10-column volumes of 50 m*M* glycine HCl, pH 2.8, or another eluent sufficiently strong to remove the analyte, but mild enough to avoid damage to the column. Again this is a critical step, because you wish to elute the analyte and nothing else. In addition, the integrity of the immobilized antibody on the column should be preserved to allow reuse of the column. We have found the glycine buffer works well to remove the analyte without damaging the immobilized antibody. Again, the user may need to experiment with other solutions dependent on the particular antigen and antibody they are using. The analyte–antibody interaction is controlled by nonbonded interactions, that is, hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic bonding. The solution that elutes the analyte must disrupt these forces, so changes in pH, ionic strength, or increased organic solvent content have been used.
5. Regenerate the column by applying five-column volumes of 0.1 *M* Tris-HCl pH 8.0/0.5 *M* NaCl followed by five-column volumes of 0.1 *M* acetate buffer pH 4.0/0.5 *M* NaCl. This cycle of a two buffer wash is repeated an additional two times. It is important to remove all analyte from the column to prevent carryover during column reuse.
6. Store the column at 4°C in PBS, pH 7.2, with 0.02% sodium azide.

3.3. Immunoaffinity Column Validation Steps

1. Calculation of binding capacity: if IgG is the antibody used for immobilization the molar ratio between the IgG and the antigen (analyte) is 1:2 because IgG is bivalent. Thus, the theoretical binding capacity of the column is as follows:

$$\text{Binding capacity (mass)} = (\text{mass IgG} \times 2 \times \text{mol. wt. analyte})/\text{mol wt. IgG}.$$

It should be noted that the mass of IgG used in the numerator refers to the mass of IgG immobilized per column (50 mg in our example), the multiplier of "2" denotes the bivalent nature of IgG, and the molecular wt. of the analyte should be provided on a millimole basis (i.e. mg/mmol). The nominal molecular weight of 150,000 mg/mmol for IgG is used in the denominator.

For small molecule analytes (*see* **Note 6**) for which a radiotracer form is available, sequentially load a known quantity of tracer dissolved in buffer and determine the amount of analyte in the eluant. When the radioactivity not retained by the immunoaffinity column plateaus, the column binding sites are saturated. Wash the column, and elute the retained radioactivity. The mass of analyte in the eluted volume is the apparent column capacity. In many instances a radiolabeled analyte may not be available. In such cases, high-performance liquid chromatography, UV spectroscopy, or any other analytical tool capable of selectively quantifying the analyte may be used to determine column capacity.

2. Determination of column stability: test the column stability by applying a known amount of analyte; perform sample loading, column wash, analyte elution, column regeneration, and storage cycle steps for multiple analyses. Initially we test the columns' reusability daily for a week, then weekly for a month, then monthly for up to 3 mo.

4. Notes

1. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.
2. The most common method of preparing the antibody is to dialyze the isolated antibody into 0.1 *M* $NaHCO_3$, pH 8.3/0.5 *M* NaCl buffer and adjust the concentration to give 50 mg/20 mL of buffer for cyanogen bromide Sepharose conjugation. It is important to measure the concentration of the solution so the total amount of antibody exposed to the gel is known in order to calculate the efficiency of coupling. The antibody must not be dialyzed or reconstituted into buffers that contain primary or even secondary amines such as Tris-HCl, which will react with the activated group on the gel, thereby reducing the efficiency of IgG immobilization. Inactivation of activated gels occurs for other buffer and gel combinations as well, most manufacturers will discuss specific incompatible combinations and the scientist must be aware of the chemistry involved in the activation of the gels.
3. The amount of antibody reacted with the gel is critical for good binding of the analyte. If too little antibody is used only nonspecific bind-

ing will be observed (use more than 1 mg antibody per mL of gel). If too much antibody is used the gel will show poor binding efficiency presumably because of steric hindrance. An appropriate amount of the antibody for the immobilization step is 5–10 mg of IgG/mL of gel.

4. Sample preparation is important to the longevity of column. Centrifuge the sample to eliminate any precipitate, after which the samples should be passed through 0.45-micron filters (and/or 0.22 micron) to prevent column fouling.
5. The analyte binding efficiency is matrix dependent. Some matrices, such as urine and tissue extracts, can be directly loaded onto the column, other matrices such as milk may need sample processing prior to loading onto an immunoaffinity column. The simplest sample preparation method is dilution; this method has been applied to serum, liver, and kidney extracts after removal of particulates. Sometimes dilution alone is not sufficient to eliminate the matrix effect and classical sample preparation techniques (solvent/solvent extraction, solid phase extraction, etc.) will be necessary prior to immunoaffinity chromatography. We found milk often needs this type of treatment.
6. The conditions required to elute the analyte from the IAC are empirically determined. Several common methods can be examined:
   a. Change the pH of the elution buffer; we have often used 50 m*M* glycine-HCl, pH 2.8. Low pH solutions have been utilized as an analyte elution solution more often than high pH solutions.
   b. Increase ionic strength, most often by increasing the salt concentration, 1M NaCl has commonly been used.
   c. Use of a competitive eluent that contains structural elements of the analyte can often selectively elute the analyte.
   d. Use of an organic modifier of up to 10 % dioxane (*caution*: carcinogen), 50% ethylene glycol, or in some cases high concentrations of ethanol or methanol may be used.
7. When a radiolabeled form of the analyte is available, the amount in the wash and sample elution steps is easily measured, thereby facilitating optimum development. Other analytical techniques can be used, although care must be taken to avoid interference from the sample matrix. The ability of IAC to separate analyte from interferences can be assessed through either LC or GC determination for small molecule determination or SDS-PAGE for large molecule determination where co-elution of other substances can be easily observed.

References

1. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.
2. Hage, D. S. (1998) A Survey of recent advances in analytical applications of immunoaffinity chromatography. *J. Chromatogr.* **715,** 3–28.
3. Pound, J. (ed.) (1998) *Immunochemical Protocols*, Humana Press, Totowa, NJ.
4. Real, F. X., de Bolos, C., and Oosterwijk, K. (2000) Polyclonal and monoclonal techniques. *Methods Mol. Biol.* **125,** 353–368.
5. Heide, K. and Schwick, H. G. (1978) Salt fractionation of immunoglobulins, in *Handbook of Experimental Immunology* (Weir, D. M., ed.), Blackwell Scientific Publications, Oxford.
6. Boschetti, E. and Jungbauer, A. (2000) Separation of antibodies by liquid chromatography, in *Separation Science and Technology* (Ahuja, S., ed.), Academic Press, San Diego, CA.
7. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
8. Markwell, M. K., Haas, S. M., and Tolbert, N. E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87,** 206–210.

20

Immunocapture-Polymerase Chain Reaction

Vincent Mulholland

Summary

Antibody capture of viruses can be used as a preparatory step in nucleic acid amplification techniques. Immunocapture of virus particles can be used to streamline and/or optimize the concentration, purification and specificity requirements of polymerase chain reaction assays.

Key Words: Immunocapture; PCR; reverse transcription; potato; *Solanum tuberosum*; virus.

1. Introduction

Immunocapture-polymerase chain reaction (IC-PCR) is a synthesis of two commonly used diagnostic tools. This method exploits the high-affinity binding of antibodies to provide a facile method of purification, usually from a complex matrix, supplying the substrate for PCR detection. PCR exponentially amplifies a deoxyribonucleic acid (DNA) template in a temperature-dependent fashion by the annealing of oligonucleotide primers, enzymatic extension of bound primers by a heat-stable polymerase, followed by denaturation of

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

the product. This three-stage process, performed in a thermocycler, is repeated sufficient times to allow amplification to proceed to levels, which will allow detection of the PCR product.

One advantage of this hybrid method is that the antibodies used in the immunocapture step do not need to provide the ultimate level of specificity required for the assay. For example, a genus-specific capture antibody may be used to provide the template for a species-specific PCR assay. In fact, each step of the technique can be tailored to provide the required level of discrimination.

IC-PCR has been used extensively in plant pathogen diagnostics, particularly in relation to viruses ***(1–11)***. The methodology outlined below was developed for use in the diagnostics of viral infection of potatoes (*Solanum tuberosum*). As the potato virus genomes involved were single-stranded ribonucleic acid (RNA), an additional step is needed between the immunocapture and PCR phases (RNA is not a suitable substrate for direct use in PCR). The action of reverse transcriptase (RT) on oligonucleotide-primed RNA allows the synthesis of complementary DNA (cDNA), required to permit detection using PCR. This technique is referred to as IC-RT-PCR.

2. Materials

1. Universal extraction bags 12 × 14 cm with synthetic intermediate layer (Bioreba AG).
2. Dulbecco's phosphate-buffered saline without magnesium or calcium (PBS; pH 7.4): 8 g of NaCl, 0.15 g of KH_2PO_4, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, 0.2 g of KCl per liter.
3. Sample extraction buffer (SEB): PBS (pH 7.4) containing 0.05% Tween-20, 2% (w/v) polyvinylpyrrolidone 25000, 0.2% (w/v) ovalbumin, and 0.5% (w/v) bovine serum albumin.
4. HOMEX 6 Plant Tissue Homogenizer (Bioreba AG).
5. PCR plates (96 well).
6. Coating buffer (pH 9.6): 0.16% (w/v) Na_2CO_3, 0.29% (w/v) $NaHCO_3$.

7. Antibodies at 1 mg/mL, diluted to a working concentration of 1-in 100 in coating buffer (pH 9.6).
8. Plate seals.
9. Washing buffer: PBS + 0.1% (v/v) Tween-20.
10. Enzyme solution: Cellulase "onozuka" RS/Macerozyme R-10 (Yakult Pharmaceuticals) 0.1 g of each enzyme in 1 mL of RNase-free water.
11. 1X RT buffer: 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$.
12. 5X RT buffer: 250 m*M* Tris-HCl (pH 8.3), 375 m*M* KCl, 15 m*M* $MgCl_2$.
13. Moloney murine leukemia virus reverse transcriptase.
14. *Not*I poly dT primer; 5'-CAATTCGCGGCCGCT$_{18}$-3'.
15. dNTPs, 10 m*M* each with respect to dATP, dCTP, dGTP, and dTTP.
16. RNase-free H_2O.
17. 100 m*M* dithiothreitol (DTT).
18. Recombinant RNasin ribonuclease inhibitor (Promega).
19. 10X PCR buffer.
20. 25 m*M* $MgCl_2$.
21. PCR primers at 5.0 pmoles/µL.
22. Taq DNA polymerase 5U/µL.
23. Electrophoresis equipment.

3. Methods

The methods outlined below consist of 1) tube coating with the capture antibody, 2) sample extraction from potato, 3) virus capture in the coated tubes, 4) cDNA synthesis from the captured viruses and 5) PCR amplification of the cDNA. Tube coating requires an overnight incubation, so should be set up on the day prior to extractions.

3.1. Tube Coating

Coat 96-tube PCR plates, which are certified DNase- and RNase-free) with 50 µL of virus-capture antibody (1 mg/mL) diluted 1:100

in coating buffer pH 9.6, cover with a plate sealer (Dynatech Laboratories), and incubate overnight at 4°C.

3.2. Sample Extraction

1. Using a scalpel (*see* **Note 1**), remove approx 0.8 g of tissue from the heel-end of each potato tuber, in thin slices (*see* **Note 2**).
2. Place the slices into Universal extraction bags, and add 1 mL of sample extraction buffer (SEB) to each.
3. Slot the polythene bags containing the tuber sample and SEB into the frame of a HOMEX 6 Plant Tissue Homogenizer, and grind.

3.3. Virus Capture

1. After antibody coating, wash the PCR plate wells three times with 200 µL of washing buffer, then block with 100 µL of SEB for 1.5 h at 37°C.
2. Knock the SEB out onto a paper tissue pad and wash the plates twice with 200 µL of wash buffer.
3. Add 50 µL of tuber extract to individual tubes on the plate.
4. To this extract, add 50 µL of enzyme solution diluted in SEB (*see* **Note 3**). Incubate the plates overnight at 4°C.
5. Remove the extracts from the tubes by knocking the plates on an absorbent pad, then wash three times with 200 µL of wash buffer. Should the sample adhere to the wells, soak the plates for 3 min during the wash stage.
6. Rinse with 200 µL of 1X RT buffer and flick out the remaining liquid before proceeding with the cDNA synthesis step.

3.4. cDNA Synthesis

3.4.1. NotI Poly dT Primer Binding

For each reaction (*see* **Note 4**) required, add the appropriate multiples of the following into a master mix (*see* **Note 5**):

Ingredient	Volume per sample
5X First-strand buffer	6 µL
DTT solution (100 m*M*)	3 µL
RNasin (40U/µL)	0.15 µL
*Not*I poly dT primer (1 µg/mL)	3 µL
H_2O	17.85 µL

Dispense 30 µL of this master mix into each tube. Incubate plates (in a PCR machine) at 65°C for 5 min, then 24°C for 30 min to allow primer annealing.

3.4.2. First-Strand Synthesis

For each reaction required, add the appropriate multiples of the following into a master mix:

Ingredient	Volume per sample
5X First-strand buffer	2 µL
dNTPs (10 m*M*)	2 µL
DTT (100 m*M*)	1 µL
RNasin (40 U/µL)	0.05 µL
M-MLV RT (200 U/µL)	0.5 µL
H_2O	4.45 µL

Dispense 10 µL of this master mix into each tube. Incubate plates in a PCR machine at 37°C (*see* **Note 6**) for 2 h for RT, 95°C for 5 min to stop the reaction and a final 4°C hold.

3.5. PCR Amplification

The example of a Carlavirus-specific PCR assay ***(12)*** is set out below. However, there are numerous PCR assays available for

potato viruses that may also be carried out from the cDNA produced using the method previously described.

The two primers used for the PCR were the *Not*I Poly dT primer (as used for production of cDNA) and a Carlavirus-specific primer ***(12)***, Carla-Uni, 5'-GGAGTAACYGAGGTGATACC-3' (where Y stands for a C or G nucleotide).

Make up the PCR master mix as follows:

Ingredient	Volume per PCR
H_2O	14.8 µL
10X PCR buffer	2.5 µL
$MgCl_2$ (25m*M*)	2.5 µL
dNTPs (10m*M*)	0.5 µL
*Not*I poly dT primer (5 pmol/µL)	1.0 µL
Carlavirus primer (5 pmol/µL)	1.0 µL
Taq DNA polymerase (5 units/µL)	0.2 µL

Transfer 22.5 µL of this master mix to each of the PCR tubes, and add 2.5 µL of cDNA to the reaction. Seal the tubes and perform amplification under the following conditions; 95°C for 5 min; 94°C for 30 s, 50°C for 1 min, 72°C for 1 min (35 cycles for each); and 72°C for 5 min.

After PCR amplification, perform electrophoresis of the products through a 1.5% (w/v) agarose gel in 1X TBE buffer and then stain with ethidium bromide at 0.5 µg/mL ***(13)***.

Examine the gel for a band of approx 120 bp (dependent on the virus used), which indicates the presence of a Carlavirus. Include positive and negative controls to ensure that the assay is working and that contamination has not occurred (**Fig. 1**).

The range of potato viruses that can be detected include potato virus S, potato virus M ***(12)***, and potato latent virus ***(10)***. Further members of the Carlavirus genus can be detected in other plant species ***(12)***.

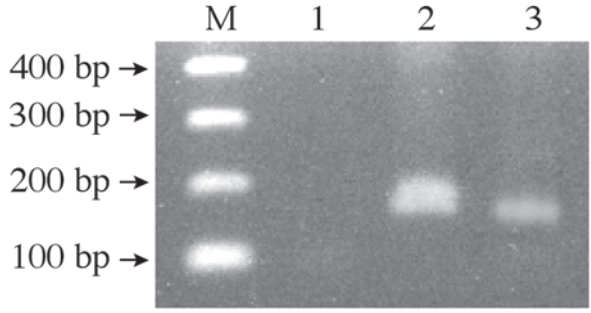


Fig. 1. IC-RT-PCR of potato latent virus. Agarose gel analysis of IC-RT-PCR assays from infected potato plants, immunocaptured using a PVS° polyclonal antibody, which also had affinity to potato latent virus ***(10)***. Lane M 100-bp molecular weight standard (SuperLadder-Low; ABgene); lane 1, negative control (water); lane 2, positive control (potato virus S); and lane 3, potato latent virus.

4. Notes

1. To increase the speed of the process, several scalpels were used in rotation. The scalpel blades were decontaminated by successive washes of 1 min in duration in the following solutions; water, 0.2 *M* NaOH, water, 96% ethanol, and then briefly flamed to remove the ethanol.
2. Potatoes are sampled on a disposable surface (such as a polythene bag) on top of a ceramic tile, giving a clean cutting surface for each tuber.
3. For 150 samples, 2X 1 mL of enzyme solutions were made in 1.5-mL microfuge tubes and mixed on a culture wheel at room temperature until dissolved (the enzyme solution should be made in advance as it takes some time to dissolve). Before the addition to the tuber extract, 2 mL of the enzyme solution was mixed with 8 mL of SEB in a pipet trough, to give the correct dilution.
4. To allow for inaccuracies in pipetting, the master mixes for the RT step, and subsequently for PCR, should be prepared for more than

the actual number required. As an example, a master mix sufficient for one hundred samples should be prepared for use in for 96 reactions.

5. Because of the sensitivity of PCR assays, it is most important to minimise the potential danger of cross-contamination. Ideally, PCR laboratories should consist of two separate rooms, each containing their own equipment (e.g., pipetors). One room should be dedicated to the setting up of RT reactions and PCR assays only. Both laboratories should use aerosol-resistant tips to prevent carry-over of sample within the barrel of the pipet. Post-PCR tubes should never be opened in the room used to set up PCRs as this is probably the most potent source for potential contamination. A laminar-flow cabinet, particularly one designed for PCR, should be considered a requirement for a PCR room in situations where a large number of samples will be processed.
6. Some viruses have secondary structure, which can prevent the production of cDNA detectable in a PCR assay by early termination of the synthesis reaction. To overcome this problem one can raise the temperature of incubation used in first-strand synthesis to 42°C or higher. This will reduce some secondary structures, but will also reduce the half-life of the reverse transcriptase. AMV reverse transcriptase may be used instead, because it has an optimal temperature of 42°C. Unfortunately, AMV RT has more endogenous RNaseH activity than M-MLV RT, thus on average AMV RT produces shorter cDNA fragments. RNaseH deficient RT enzymes are also available (e.g., the SuperScript enzymes from Invitrogen), and there is some evidence that these may be the most sensitive type of RT enzymes for PCR assays. The RT conditions required for the efficient detection of individual viruses can only be determined empirically.

Acknowledgments

I would like to thank Dr. Ed Canning and Mrs. Marian McNeil (Scottish Agricultural Science Agency) for the development and testing of methods presented in this work.

References

1. Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., and Dunez, J. (1992) A highly sensitive immunocapture polymerase chain

reaction method for plum pox potyvirus detection. *J. Virol. Methods* **39,** 27–37.

2. Nolasco, G., de Blas, C., Torres, V., and Ponz, F. (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J. Virol. Methods* **45,** 201–218.
3. Kokko, H. I., Kivineva, M., and Karenlampi, S. O. (1996) Single-step immunocapture RT-PCR in the detection of raspberry bushy dwarf virus. *Biotechniques* **20,** 842–846.
4. Mumford, R. A. and Seal S. E. (1997) Rapid single-tube immunocapture RT-PCR for the detection of two yam potyviruses. *J. Virol. Methods* **69,** 73–79.
5. Jacobi, V., Bachand, G. D., Hamelin, R. C., and Castello J. D. (1998) Development of a multiplex immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic tobamoviruses. *J. Virol. Methods* **74,** 167–178.
6. Pasquini, G., Simeone, A. M., Conte, L., and Barba, M. (1998) Detection of plum pox virus in apricot seeds. *Acta Virol.* **42,** 260–263.
7. Sefc, K. M., Leonhardt, W., and Steinkellner, H. (2000) Partial sequence identification of grapevine-leafroll-associated virus-1 and development of a highly sensitive IC-RT-PCR detection method. *J. Virol. Methods* **86,** 101–106.
8. Sharman, M., Thomas, J. E., and Dietzgen, R. G. (2000) Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J. Virol. Methods* **89,** 75–88.
9. Helguera, P. R., Taborda, R., Docampo, D. M., and Ducasse, D. A. (2001) Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of Prunus necrotic ring spot virus in the peach. *J. Virol. Methods* **95,** 93–100.
10. Brattey, C., Badge, J. L., Burns, R., Foster, G. D., George, E., Goodfellow, H. A., et al. (2002) Potato latent virus: a proposed new species in the genus Carlavirus. *Plant Pathol.* **51,** 495–505
11. Ptacek, J., Skopek, J., Dedic, P., and Matousek, J. (2002) Immunocapture RT-PCR probing of potato virus Y isolates. *Acta Virol.* **46,** 63–68.
12. Badge, J., Brunt, A., Carson, R., Dagless, E., Karamagioli, M., Philips, S., et al. (1996) A carlavirus-specific PCR primer and partial nucleotide sequence provides further evidence for the recognition of

cowpea mild mottle mosaic virus as a whitefly-transmitted carlavirus. *Eur. J. Plant Pathol.* **102,** 305–310.

13. Sambrook, J. and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

21

Magnetic Cell Sorting

Maciej Zborowski and Jeffrey J. Chalmers

Summary

Antibodies reacting to specific cell surface markers can be bound to magnetic beads and used to specifically capture cells exhibiting the marker. This approach allows the selective enrichment of specific cell subpopulations and as the methods are amenable to use in blood, tissue fluids and culture medium living cells can be recovered that can subsequently be subcultured. Negative cell separation can also be used where undesirable cell types are tagged using magnetic beads and removed from the desirable cell population.

Key Words: FACS; lymphocyte; beads; magnetic; separation; enrichment; fluorescent; cell marker.

1. Introduction

Early experimentation on magnetic cell separation was conducted on phagocytic cells ***(1)*** and red blood cells ***(2)***. Currently, the literature devoted to magnetic cell separation has reached a few thousand publications and shows the diverse benefits that the technology has made to life sciences and clinical research during the past 15 yr

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

(3–5). Information on magnetic cell separation protocols is also maintained on commercial websites (Miltenyi Biotec, Nexell, Immunicon, Bangs Polysciences, Dynal, Cortex Biochem, Pharmingen, StemCell Technology, Molecular Probe, to name a few; *see also* **Table 1**). Some important sources of information on the development of clinical applications of magnetic separation are hematology meetings (such as those organized by American Society of Hematology and the International Society for Experimental Hematology) and cellular therapy meetings (International Society for Cellular Therapy).

Methodologies are typically based on high-gradient magnetic separators *(6)* or open gradient separators *(7)*. The desired cell population is tagged with magnetic particles in the micrometer to submicrometer size range, such as Dynabeads *(8)* or MACS beads *(9)*, respectively, with a considerable selection of other types of magnetic particles also available *(10,11)*. The ever-expanding field of cell surface marker characterization by immunophenotyping, with the resulting discoveries of new clusters of differentiation (CD) molecules, provides a rich source of candidate cell markers for magnetic particle tagging *(12)* using monoclonal antibodies as linkers.

Negative cell separation, by depletion of the unwanted cell population tagged with magnetic beads, provides a relatively simple and effective method for purging bone marrow of residual cancer cells (both the tagged cells and the free beads in solution are removed by the application of the external magnetic field *[13,14]*). A drop in fractional cell concentration of unwanted cells by more than five orders of magnitude (more than "five-log" depletion) has been reported *(4)*. Perhaps more demanding and technically difficult is positive cell separation, in which the desired cell population is tagged with the magnetic beads and separated. A complication of this method is that the beads remain with the enriched cell population and removal of them may be needed prior to the cells being used for further work. Additionally, the antibody binding to the cell surface receptor may lead to a cell signaling cascade and undesirable cell activation. The main advantage is the high selectivity of the separation defined by the specificity of the targeting antibody,

Table 1
Examples of Commercially Available Magnetic Particles for Cell Separation[a]

Particle trademark	Manufacturer	Diameter (μm)	Representative ligands	Website
Dynabead	Dynal Biotech, Oslo, Norway	4.5	Monoclonal antibodies	www.dynalbiotech.com
BioMag	Bangs Laboratories, IN (part of Polysciences Inc., Warrington, PA)	1	Monoclonal antibodies	www.bangslabs.com
MACS	Miltenyi GmbH, Bergisch Gladbach, Germany	0.05–0.1	Monoclonal antibodies	www.milenyibiotec.com
BD IMag	BD Biosciences, San Jose, CA	0.2	Monoclonal antibodies	www.bdbiosciences.com
Captivate	Molecular Probes, Eugene, OR (in association with Immunicon Corporation, Huntingdon Valley, PA)	0.2	Monoclonal antibodies	www.probes.com
EasySep	StemCell Technologies, Vancouver, BC, Canada	0.2	Tetrameric–antibody complex	www.stemcell.com

[a]This table is intended for illustration purposes. Additional information is available on the Internet, in particular, at the website, www.magneticmicrosphere.com.

with the additional benefits of high depletion rates of unwanted cells. The enrichment rates (the ratio of the final to the initial fractional cell concentration) in excess of 100 (or "two-log"), with concomitant depletion rates of four order of magnitudes ("four-log") have been reported ***(9)***. Further increases in the desired cell fraction purity can be obtained by a combination of negative and positive separation steps.

An important part of magnetic cell separation is the determination of product purity and recovery (or yield) by cell cytometry, typically using the automated, differential cell counts by fluorescence-activated cell sorting (FACS; **ref. *15***). This requires additional cell labeling with fluorescent antibodies against cell surface marker epitopes not occupied by the targeting antibodies used for magnetic separation. Alternatively, a sandwich labeling method has been adapted in which the primary antibody used for cell targeting is conjugated to a fluorescent label, and the secondary antibody specific to epitopes on the primary antibody carries the magnetic particle ***(16)***. "Immuno–fluoro-magnetic" sandwich labeling is particularly well suited to the use of small, colloidal magnetic particles because they do not interfere with the optical properties of the labeled cells and therefore do not require removal prior to FACS analysis.

The advantages of using submicrometer magnetic particles for tagging include formation of stable suspensions and a relatively short reaction time of the antibody–particle complex with the target cell marker ***(9)***. They have been used for blood cell progenitor enrichment prior to transplantation in cancer patients, detection of rare cancer cells in circulating blood, detection of fetal cells in maternal blood, and for monitoring changes in cellular content of grafts used for cellular therapies ("graft engineering"; **refs. *4*,*9***). The advantages of the larger, micrometer-sized magnetic beads includes the use of small, inexpensive magnets that may be used in combination with standard laboratory tubes and simplicity of the separation process. Automated magnetic separators have made possible the

expansion of cellular therapies to smaller clinical centers, and the exploration of new cellular therapy modalities.

A typical magnetic cell separation protocol includes a number of steps that can be grouped as follows: cell sample preparation, cell labeling and tagging, magnetic separation, cell product post-processing, and cell product characterization. The actual implementation depends on the type of cell sample and the type of magnetic tagging bead. It may require running a number of initial tests in order to achieve the optimal experimental conditions. Typical optimization parameters include target cell purity, recovery (or yield), percent viability and the speed of the overall process. As an example, a protocol for a positive selection of natural killer cells from human peripheral blood is described ***(17)***.

2. Materials

1. MiniMACS magnetic isolation system using MS+ column (Miltenyi Biotec, Auburn, CA)
2. NK cell-targeting, primary antibody: mouse anti human CD56-phycoerythrin (PE) antibody conjugate, isotype IgG1 (Immunotech, Marseille, France).
3. Secondary, magnetic tagging antibody: mouse anti PE-MACS microbeads antibody conjugate, isotype IgG1 (Miltenyi Biotec, Auburn, CA).
4. Ficoll-Hypaque preparation (Accurate Chemical and Scientific Corporation, Westbury, NY).
5. Degassed solution of phosphate-buffered saline (PBS) without calcium and magnesium, supplemented with 0.1% (w/v) bovine serum albumin and 2 m*M* ethylene diamine tetraacetic acid.
6. Lysis buffer: 154 m*M* NH_4Cl, 10 m*M* $KHCO_3$, 0.1 m*M* ethylene diamine tetraacetic acid.
7. Other laboratory equipment and reagents: visible light microscope, hemacytometer, Trypan blue solution, laboratory balance, refrigerated centrifuge, household bleach 10% solution, automated cell counter (Coulter Z1 Counter, Beckman-Coulter, Miami, FL), flow cytometer (EPICS Elite II, Beckman-Coulter).

3. Methods

3.1. Peripheral Blood Lymphocyte Preparation

1. Transfer 30 mL of the peripheral blood to two 50-mL conical tubes and add 20 mL of PBS solution to each tube. Cap the tubes and mix the contents gently by inverting tubes several times.
2. Place the tubes in a centrifuge and spin at 300*g* for 15 min at room temperature.
3. Carefully aspirate and discard two thirds of the top plasma layer (supernatant) without disturbing the underlying cell mass.
4. Replace the discarded plasma with an equal volume of PBS buffer solution, cap the tubes and mix well by gently inverting the tubes.
5. Mix well the contents of the Ficoll bottle, wipe the rubber cap with 70% ethanol then inject approx 15 mL air with a 20-mL syringe and needle and withdraw 20 mL of the Ficoll solution. Equally divide the Ficoll solution between two new 50-mL Falcon tubes.
6. Layer the blood mixture on top of the Ficoll solution using a 10-mL pipet and pipettor. Proceed carefully so as to not disturb the Ficoll-blood interface by keeping the pipet tip against the tube wall close to the blood surface. The Ficoll to blood volume should be approx 2:3.
7. Place the tubes with the layered blood on Ficoll in the centrifuge. Centrifuge for 30 min at 350*g*, room temperature (with the brake off).
8. Using a 10-mL pipet and pipetor, carefully aspirate the plasma layer from the Ficoll tubes and discard. Leave approx 5 mL of plasma on top of the mononuclear cell layer to avoid cell loss. Carefully aspirate the mononuclear cell layer and transfer to two new 50-mL Falcon tubes. A total of approx 12 mL of the mononuclear cell volume should be obtained. Move the pipet tip in a circular, continuous manner over the mononuclear cell layer when aspirating avoiding red blood cell contamination. Discard the red blood cell contents in to the waste container containing 10% bleach solution.
9. Add 15 mL of PBS buffer to the mononuclear cell suspension, mix well by aspirating gently with the 10-mL pipet.
10. Place tubes with the mononuclear cell suspension in the centrifuge, spin for 18 min 350*g* at room temperature. Decant the supernatant, blot on a paper towel, resuspend in 6 mL of PBS buffer.
11. Deplete the cell suspension of monocytes and macrophages by incubation in 150-cm^2 tissue culture flask for 2 h at 37°C (monocytes and macrophages will adhere to the surface of the flask). Wash off the nonadherent cells with PBS buffer and collect.

12. Optionally, remove the residual red blood cells by the addition of 25 mL of lysis buffer. Incubate at room temperature for 5 min and wash with PBS buffer by centrifugation.
13. Count the cells using a hemacytometer (or an automated cell counter, such as a Coulter counter, if available). Adjust the cell concentration to 10×10^6 per mL. Set aside two 1×10^6 cell aliquots for FACS controls: a cell auto-fluorescence control, and an irrelevant primary antibody-PE conjugate binding control. Optionally, add a T-cell control using anti CD3 antibody, a monocyte control using anti CD14 antibody, and a leukocyte control using anti CD45 antibody.

3.2. Cell Staining With Primary Antibody-Fluorescent Conjugate

1. To stain with primary antibody-fluorescence conjugate add 5 µL of anti-CD56-PE antibody conjugate per 10^6 cells, incubate at 4°C for 30 min, wash twice with the PBS buffer.

3.3. Cell Tagging With Secondary Antibody-Magnetic Particle Conjugate

1. To tag with secondary antibody-magnetic particle conjugate add 10 µL of anti PE-MACS microbeads antibody conjugate per 10^6 cells, incubate at 4°C for 15 min and wash twice with the PBS buffer. Set aside 1×10^6 cell aliquot for FACS analysis. Determine the total cell concentration, and population viability using a hemacytometer and Trypan blue stain.
2. Determine the volume of cell suspension using a laboratory balance.

3.4. Magnetic Separation

1. Place the MS+ column in to the magnet according to the manufacturer's instructions.
2. Flush the magnetic column with at least 0.5 mL of PBS buffer by applying it to the top of the column and allowing to run through. Discard the tube with the eluted PBS buffer and replace it with a new tube.
3. Load the column with 0.5 mL of the labeled cell suspension using a pipet, allow the cell suspension to run through, follow by 1.5 mL of buffer solution. The eluted cell suspension is depleted of $CD56^+$ cells, and is designated as the negative cell fraction.

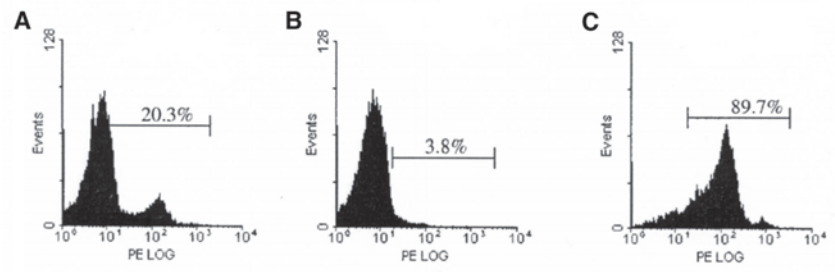


Fig. 1. Magnetic separation of NK cells characterized by CD56 cell surface marker. FACS histograms of the PBL sample labeled with anti CD56-PE primary antibody and anti PE-MACS bead secondary (**A**), and the negative (**B**), and positive (**C**) fractions from the magnetic separation experiment as described in the text. Note enrichment of the $CD56^+$ cells in the positive cell fraction.

4. Remove the column from the magnet, place over a new tube, and flush the column content with 1 mL of PBS buffer using plunger supplied by the manufacturer. The eluted cell suspension is enriched in the $CD56^+$ cells, and is designated as the positive cell fraction.
5. Optionally, repeat **step 4** to increase the $CD56^+$ cell recovery in the positive cell fraction.
6. Optionally, apply the positive cell fraction to a new MS+ column by repeating **steps 1–4** to increase the purity of $CD56^+$ cells in the positive cell fraction.
7. Set aside 1×10^6 cell aliquots from the positive and negative cell fractions for FACS analysis. Determine total cell concentration, cell suspension volume, and population viability in the separated cell fractions.

3.5. Purity and Recovery Analysis of the Separated Cell Product

1. Using appropriate excitation and emission light settings on the FACS flow cytometer set up for PE fluorescence detection, and adjust gating settings using forward and side light scatter to distinguish PBL from red blood cells and platelets. Determine PE-positive cell percent fraction in the original sample and in the separated cell fractions. Report $CD56^+$ cell purity as the percent $CD56^+$ cell fraction in the sample, as illustrated in **Fig. 1**.

2. Calculate $CD56^+$ cell number in the original and in the separated fractions by multiplying their cell concentrations by the corresponding cell volumes. Report fractional $CD56^+$ cell recovery as the ratio of the $CD56^+$ cell number in the separated fraction to that in the original sample.
3. Report cell population viabilities as measured by the Trypan blue exclusion test in the original and sorted cell samples.

References

1. Levine, S. (1856) Magnetic techniques for in vitro isolation of leucocytes, *Science* **123,** 185.
2. Melville, D., Paul, F., and Roath, S. (1975) High gradient magnetic separation of red cells from whole blood. *IEEE Trans. Magn.* 1701.
3. Uhlen, M., Hornes, E., and Olsvik, O. (1994) *Advances in Biomagnetic Separation*, Eaton Publishing Co., Natick, MA
4. Gee, A. P. (1998) Immunomagnetic cell separation using antibodies and superparamagnetic microspheres, in *Cell Separation Methods and Applications* (Recktenwald, D., and Radbruch, A., eds.), Marcel Dekker, Inc., New York, pp. 175.
5. Zborowski, M. (1997) Physics of magnetic cell sorting, in *Scientific and Clinical Applications of Magnetic Carriers* (Haefeli, U., Schuett, W., Teller, J., and Zborowski, M., eds.), Plenum Press, New York, pp. 205.
6. Hatch, G. P. and Stelter, R. E. (2001) Magnetic design considerations for devices and particles used for biological high-gradient magnetic separation (HGMS) systems. *J. Magnetism Magn. Mater.* **225,** 262.
7. Coey, M. and Weaire, D. (1998) Magnets, markets, and magic cylinders. *The Industrial Physicist* September, 34.
8. Ugelstad, J., Stenstad, P., Kilaas, L., Prestvik, W. S., Herje, R., Berge, A., et al. (1993) Monodisperse magnetic polymer particles. New biochemical and biomedical applications. *Blood Purif.* **11,** 34.
9. Kantor, A. B., Gibbons, I., Miltenyi, S., and Schmitz, J. (1998) Magnetic cell sorting with colloidal superparamagnetic particles, in *Cell Separation Methods and Applications* (Recktenwald, D., and Radbruch, A., eds.), Marcel Dekker, Inc., New York, pp. 153.
10. Liberti, P. A., Rao, C. G., and Terstappen, L. W. M. M. (2001) Optimization of ferrofluids and protocols for the enrichment of breast tumor cells in blood. *J. Magnetism Magnetic Mater.* **301,** 301.

11. Thomas, T. E., Richards, A. J., Roath, O. S., Watson, J. H. P., Smith, R. J. S., and Lansdorp, P. M. (1993) Positive selection of human blood cells using improved high gradient magnetic separation filters. *J. Hematother.* **2,** 297.
12. Dyer, P. A., Brown, P., and Edward, R. (1998) Immunomethods: magnetic, column and panning techniques, in *Cell Separation: A Practical Approach* (Fisher, D., Francis, G. E., and Rickwood, D., eds.), Oxford University Press, Oxford, p. 191.
13. Treleaven, J. G., Gibson, F. M., Ugelstad, J., Rembaum, A., Philip, T., Caine, G. D., and Kemshead, J. T. (1984) Removal of neuroblastoma cells from bone marrow with monoclonal antibodies conjugated to magnetic microspheres, *Lancet* **1,** 70.
14. Pole, J. G., Gee, A., Janssen, W., Lee, C., and Gross, S. (1990) Immunomagnetic purging of bone marrow: a model for negative cell selection. *Am. J. Pediatr. Hematology/Oncol.* **12,** 257.
15. Shapiro, H. M. (1995) *Practical Flow Cytometry,* Wiley-Liss, Inc., New York.
16. Miltenyi, S., Müller, W., Weichel, W., and Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry* **11,** 231.
17. Comella, K., Nakamura, M., Melnik, K., Chosy, J., Zborowski, M., Cooper, M. A., et al. (2001) Effects of antibody concentration on the separation of human natural killer cells in a commercial immunomagnetic separation system. *Cytometry* **45,** 285.

22

Cell Enrichment and Immunochemical Staining

Oscar R. Lara and Jeffrey J. Chalmers

Summary

Cell populations can be enriched using a number of physicochemical methods based on characteristics, such as density, size, and volume. Centrifugation using density gradients is typically applied to isolate populations with similar characteristics. Cells obtained using these methods can be further authenticated using specific antibodies that react to markers on the cell surface, allowing the identification of cell subtypes.

Key Words: Buffy coat; lymphocyte; carcinoma; enrichment; cell culture; polycarbonate membrane.

1. Introduction

Cell-enrichment processes not using fluorescent or magnetic technology are based on cell physicochemical properties, such as density, size, electrophoretic mobility, or surface composition (phenotype) ***(1)***. One of the earliest and still most commonly used methods is based on the use of centrifugal force to exploit density differences and deplete erythrocytes from whole blood. Further

From: *Methods in Molecular Biology, vol. 295: Immunochemical Protocols, Third Edition.*
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

refinements of this approach include the introduction of density gradients for increased resolution in cell density-based separations ***(2)***. This important advancement allowed routine isolation of blood mononuclear cell fraction for use in research ***(3)***. The use of modified silica coated with polyvinylpyrolidone (PVP) allows the use of continuous or discontinuous gradients, and these have been successfully applied in the density determination of several cell types, including leukocyte subsets ***(4)***. Cells isolated by these techniques can be authenticated by the use of antibodies that react specifically to surface markers, allowing the identification and quantification of cell types and subtypes.

It is necessary to take into account several factors when using density gradient centrifugation. Among these are the osmolality of the gradient, potential toxicity of the gradient, temperature, the effect of the gradient cushion on the cell population to be recovered, and cell concentration load ***(5)***. The cell density depends on the properties of the suspension medium, as well as the cell function. High medium osmolality (relative to the cell) can result in cell dehydration and subsequent changes in cell density. Additionally, when there is a mixture of cells in the suspension, osmolality effects may be different for each cell type and therefore bias the distribution of the cells separated by this process. Temperature affects the density as well; the lower the temperature, the higher the density of a liquid.

Cell suspension concentration effects have been studied empirically and theoretically optimized for several applications which, demonstrates it to be one of the most important parameters during density gradient centrifugation. Early studies on the hydrodynamics of particle sedimentation have shown the influence of particle size and density in centrifugation ***(6)***. For instance, high-density particles tend to produce a drag effect over low-density particles, pulling them to the high-density zone. This in turn decreases the purity of the high-density population and reduces the yield of the low-density population. Another important effect is the concentration of the cell suspension as the apparent viscosity of the solution increases with the increasing cell concentration ***(7)***.

Enrichment of rare cancer cells from peripheral blood samples is an application that typically requires density gradient centrifugation as a first step. Application of this technique addresses two objectives: depletion of erythrocytes and depletion of polymorphonuclear cells. It is expected that cancer cells undergo sedimentation with the mononuclear cell fraction because of their similar density. However, some studies have found that cancer cells are also lost in the polymorphonuclear fraction or the erythrocyte fraction ***(8,9)***. Optimization of the density gradient sedimentation step is an important issue in such an application, because it will determine the recovery of rare cells from blood and affect the chances of their detection by immunochemical means.

The following section describes the steps necessary to isolate carcinoma cells (cell line, MCF-7) from a buffy coat. A filtration step is added at the end of the density gradient centrifugation procedure to concentrate cells onto a polycarbonate membrane, which is a convenient vehicle for analysis by immunocytochemistry.

2. Materials

1. Human carcinoma cells (MCF-7, from American Type Culture Collection, Manassas, VA).
2. Buffy coat (leukocyte fraction collected from peripheral blood available from a Red Cross or a Blood Bank).
3. Trypan blue (Invitrogen).
4. Eagle medium (Invitrogen).
5. Accutase (Innovative Cell Technologies).
6. Hank's balanced salt solution (HBSS; JRH Biosciences).
7. Phosphate-buffered saline (PBS; JRH Biosciences).
8. Ethylene diamine tetraacetic acid (EDTA; Invitrogen Corporation).
9. Bovine serum albumin (BSA; Invitrogen Corporation).
10. Accuprep (Accurate Chemicals and Scientific Corporation).
11. Anti-HEA fluorescein isothiocyanate (Miltenyi Biotec, cat. no. 130-080-301).
12. Rabbit-anti FITC phosphatase alkaline (cat. no. A4843; Sigma, St. Louis, MO).

13. Fast Red™ TR-Naphtol AS-MX (cat. no. F-4846; Sigma, St. Louis, MO).
14. Distilled water.
15. Polycarbonate membranes (Whatman; cat. no. 7060-1313).
16. Membrane holders (Swinnex; Millipore, cat. no. SX0001300).
17. Labeling buffer: PBS supplemented with 0.5% bovine serum albumin and 2 m*M* ethylene diamine tetraacetic acid.
18. Syringe pumps.
19. Particle counter (Beckmann Coulter).
20. Capillary centrifuge.
21. Capillary tubes.
22. Syringes (5–30 mL).

3. Methods

The following method will allow researchers to develop the use of density gradient cell separations using "spiked" buffy coat preparations. Final recovery of carcinoma cells from the buffy coats can be compared with initial known percentages added to samples, allowing refinements of the technique before use with actual samples.

The described methods are divided into following subheadings: 3.1. Cell Harvesting and Cell Suspension Production, 3.2. Density Gradient Separation, and 3.3. Cell Detection Through Immunocytochemistry.

3.1. Cell Suspension Production

1. Two days before the actual separation procedure, transfer the carcinoma cells to new flasks to keep them in log phase growth (*see* **Note 1**). Use Accutase as described in **step 2**.
2. On the day of the separation, check that the carcinoma cells are subconfluent in the flasks and then detach cells from the flask using Accutase diluted in cold PBS. Follow the manufacturer's instructions for optimum concentration and incubation time (*see* **Note 2**).
3. Wash cells twice with labeling buffer by centrifugating at 300*g* and proceed to determine cell concentration using a particle counter. Also, perform a viability test using a Trypan blue exclusion method. Keep cells in cold storage (4°C) until used.

4. Buffy coat (white blood cell fraction) obtained from Red Cross must be kept in cold storage until use. Take an aliquot of the buffy coat and dilute with HBSS to determine cell concentration. Either a hemacytometer or an automated particle counter can be used for cell count (*see* **Note 3**).
5. Based on the total number of cells present in the buffy coat, seed viable carcinoma MCF-7 cells to a final percentage of 0.1% (*see* **Notes 4** and **5**).

3.2. Density Gradient Separation

1. Dilute the cell suspension (buffy coat spiked with the cancer cells) with HBSS at different ratios prior to centrifugation. Suggested ratios range from 1:1 to 1:5, cell suspension:HBSS.
2. Mix the suspension thoroughly and proceed to determine the hematocrit of the suspension by using the capillary tube method.
3. The density gradient separation protocol was first developed for 50-mL conical tubes but any tube size can be used as long as the amounts used are scaled accordingly. For 50-mL conical tubes, place 18 mL of the density gradient cushion (Accuprep) into the tube, and then lay 22 mL of the cell suspension on top of the gradient cushion. Care should be taken to not mix the cell suspension with the gradient cushion. Centrifuge the tubes for 30 min at 350*g* at room temperature with the centrifuge brake off (*see* **Note 6**).
4. Discard the plasma layer carefully by aspiration using a pipet leaving about 1 cm of plasma on top of the mononuclear cell layer.
5. Using a sterile pipet, collect the cell layer, trying to remove all of the layer as a single aspirate while avoiding contamination from the polymorphonuclear cell layer. It is recommended to wash recovered cells at least once with labeling buffer by centrifugation at 300*g*.
6. Resuspend the cell pellet in labeling buffer (about 5 mL final total volume) and proceed to count recovered cells in triplicate (*see* **Note 7**). An automated particle counter is highly recommended because it is possible to set size gates for cancer cells, erythrocytes, and leukocytes.
7. Plot a graph of percentage recovery vs hematocrit; an example of the graph obtained is shown in **Fig. 1**. The data show a peak in recovery at approx 22% hematocrit; this value should be used for further experiments.

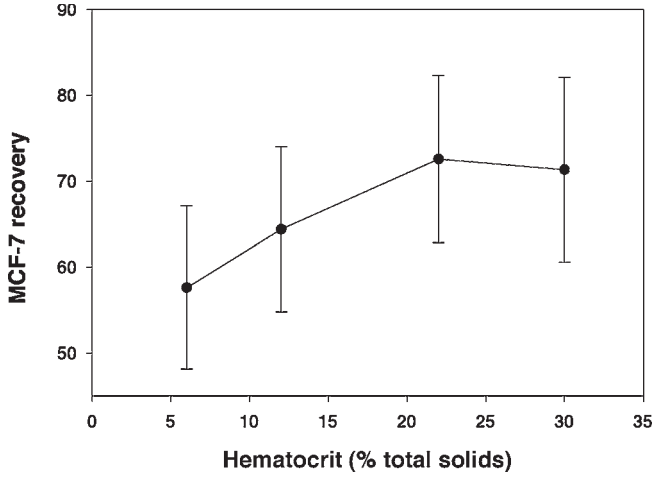


Fig. 1. Recovery of MCF-7 cells seeded at a concentration of 0.13% in buffy coat samples as a function of hematocrit. Samples were loaded on a Ficoll-Hypaque gradient at the concentration specified by the experimental point.

3.3. Cell Detection Through Immunocytochemistry

Immunomagnetic cell separation can be used to further debulk the mixture of white blood cells obtained from the density gradient separation and thus further enrich the suspension with rare cancer cells (*see* Chapter 21). Before immunochemical staining, it is usual to concentrate the cells onto polycarbonate membranes so that they are immobilized (*see* **Note 8**).

1. Place cell suspension into a syringe (syringe size will depend on the volume recovered).
2. Assemble the membrane holders with the polycarbonate membrane previously moistened with labeling buffer, attach syringes, and mount in a syringe pump. Set flow rate to a maximum of 1 mL/min (*see* **Note 9**).
3. While the syringe pump is running, prepare antibody–enzyme and substrate solutions. Usually a dilution of 1:1000 of the antibody–enzyme is enough to successfully stain cells (*see* **Note 10**); to prepare substrate, follow manufacturer's recommendations.

4. Once all cell suspension has passed through the filter, detach the membrane holders from the syringe and using a 1-mL syringe, add enough antibody–enzyme solution to cover the membrane. Incubate in the refrigerator (dark, cool place) for about 30 min.
5. Reconnect to the syringe pump and wash the membrane with labeling buffer (again, flow rate not higher than 1 mL/min) and then add enough substrate solution to cover the membrane using a syringe. Incubate for approx 15–20 min. Push liquid out of the membrane holders and recover the membrane. Analyze under light microscope. A picture showing recovered cells is shown in **Fig. 2**.

4. Notes

1. It is important to avoid confluent cultures in this protocol. Confluent cultures contain few cells growing, and there might be differences in density within the same population. Cultures in growing phase will have a better chance to yield a cell population with a consistent density.
2. The use of trypsin is common to detach cells from adherent cultures; however, in this case it is not recommended. MCF-7 cells treated with trypsin tend to form clumps, which are difficult to breakup. The use of Accutase yields a mostly single-cell suspension that will not form clumps even after it has been washed with labeling buffer. Total cell count and viability assays will also be easier for the researcher to carry out.
3. Determining cell concentration in a buffy coat sample using a particle counter can be difficult. If the laboratory has access to hematological material, it is recommended to use the Unoppette system (Becton Dickinson), after diluting the sample. Results from particle counter and manual counting should not differ by more than 5%.
4. The seeding of carcinoma cells must be performed based on a live, healthy cell count. Dead cells (those that stained with Trypan blue) are not to be considered because they are expected to sediment in the high-density region.
5. This protocol mentions a 0.1% of rare cells seeded into the buffy coat. It is possible to increase or reduce the percentage of cells. However, less than 0.1% of cells might make it difficult to obtain reliable recovery data, and a larger percentage would not be a good representation of "rare" cells circulating in blood.

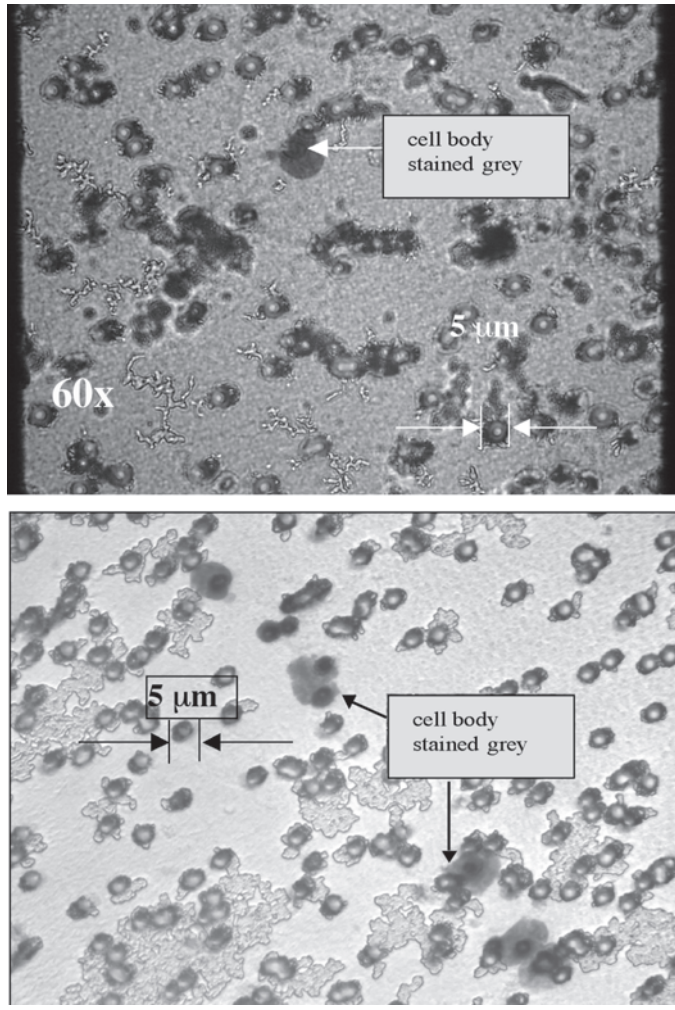


Fig. 2. Photographs of MCF-7 cells recovered from the enriched fraction after immunomagnetic sorting and stained with anti-HEA FITC as primary antibody anti-FITC phosphatase alkaline as secondary antibody and enzyme substrate (FastRed™). In this image, cells appear as medium-grey bodies. In specimens viewed in the laboratory, the cell bodies would appear stained red as opposed to grey. The dark, defined circles are pores in the membrane.

6. It is very important to maintain density gradient and cell suspension at room temperature before and during centrifugation. Temperature affects density and therefore the separation and yield.
7. Depending on the number of cells used in the centrifugation step, there will be a variable cell concentration in the tubes to test. Dilutions may be necessary.
8. To carry out immunomagnetic cell separation, cells will be stained with microbeads that target the CD45 receptor. At the same time, cancer cells must have been labeled with anti-HEA FITC.
9. In any case do not exceed 1 mL/min flow rate for the syringe pump. Higher flow rates could damage recovered cells because they could have been squeezed into the 5-µm pore size of the membrane. However, filtration through this pore size will eliminate some remaining erythrocytes and debris present in the sample.
10. Test every lot of antibody received; the best results are obtained with a titrated antibody response.

References

1. Fisher, D., Francis, D. E., and Rickwood, D. (1998) *Cell Separation. A Practical Approach*, Oxford University Press, Oxford.
2. Bøyum A. (1968) A one-stage procedure for isolation of granulocytes and lymphocytes from human blood. General sedimentation properties of white blood cells in a 1g gravity field. *Scand. J. Clin. Lab. Invest.* **97,** 51–76.
3. Todd, P. and Pretlow, T. G. (1991) Separation of living cells. *ACS Symposium Series* **464,** 1–24.
4. Ulmer, A. J. and Flad, H. D. (1979) Discontinuous gradient separation of human mononuclear leucocytes using Percoll™ as gradient medium. *J. Immunol. Methods* **30,** 1–10.
5. Pertoft, H. (2000) Fractionation of cells and subcellular particles with Percoll™. *J. Biochem. Biophys. Methods* **44,** 1–30.
6. Batchelor, G. K. and Wen, C. S. (1982) Sedimentation in a dilute polydisperse system of interacting spheres. Part 2. Numerical results. *J. Fluid. Mech.* **124,** 495–528.

7. Frömmer, D. and Lerche, D. (2002) An experimental approach to the study of the sedimentation of dispersed particles in a centrifugal field. *Arch. Appl. Mech.* **72,** 85–95.
8. Partridge, M., Phillips, E., Francis, R., and Li, S. (1999) Immunomagnetic separation for enrichment and sensitive detection of disseminated tumor cells in patients with head and neck SCC. *J. Pathol.* **189,** 368–377.
9. Krüger, W., Datta, C., Badbaran, A., Togel, F., Gutensohn, K., Carrero, I., et al. (2000) Immunomagnetic tumor cell selection-implications for the detection of disseminated cancer cells. *Transfusion* **40,** 1489–1493.

Index

C