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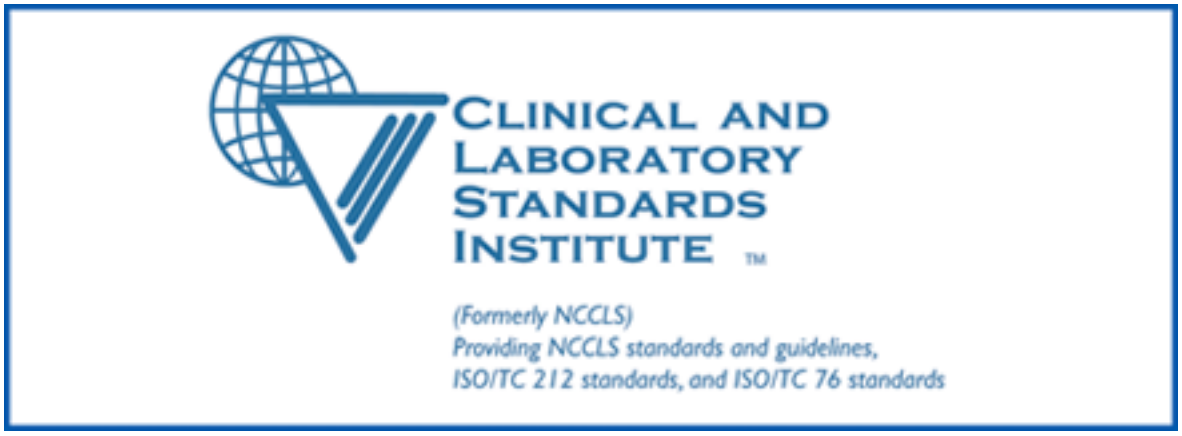
Western Blot Assay for Antibodies to *Borrelia burgdorferi;* Approved Guideline



This document addresses technical and interpretive considerations for use of Western blot assays that detect antibodies to *Borrelia burgdorferi* and other *Borrelia* species that cause Lyme disease.

A guideline for global application developed through the NCCLS consensus process.

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Western Blot Assay for Antibodies to *Borrelia burgdorferi;* Approved Guideline

Abstract

Western Blot Assay for Antibodies to Borrelia burgdorferi; *Approved Guideline* (NCCLS document M34-A) is intended for use as a critical tool in the diagnosis of Lyme disease for laboratorians who perform Western blot assays within clinical and reference laboratories. The document addresses the advantages and disadvantages of Western blot assays; antigen preparation; electrophoresis of antigens; transfer of antigens to the matrix; calibration of blots; quality control and proficiency testing; scoring the blot; reporting the results; and interpretation of the report. While the document specifically deals with Western blot assays for antibodies to *Borrelia burgdorferi* in the diagnosis of Lyme disease, the document's generic recommendations are applicable to other situations in which Western blot assays are applied in the clinical or reference laboratory.

NCCLS. *Western Blot Assay for Antibodies to* Borrelia burgdorferi; *Approved Guideline*. NCCLS document M34-A (ISBN 1-56238-415-5). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2000

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Western Blot Assay for Antibodies to *Borrelia burgdorferi;* Approved Guideline

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Foreword

The intended audience for M34-A—*Western Blot Assay for Antibodies to* Borrelia burgdorferi*; Approved Guideline*, is clinical and reference laboratories that perform Western blot assays for antibodies. The document details technical and interpretive considerations for use of Western blot assays for antibodies to *Borrelia burgdorferi* and other *Borrelia* species that cause Lyme disease. The outline of M34-A and its generic methodological considerations may also be applicable to the use of Western blot assays for detecting antibodies to other microorganisms or antigens.

The Western blot assay for antibodies was first widely used as a second-tier test method for detecting specific antibodies or confirmatory assay for the detection of antibodies to HIV. In this role, the Western blot assay was used as a highly specific assay with which to further evaluate a positive or borderline reaction in an enzyme immunoassay (EIA). With this rationale, the Western blot assay has been applied as a second-tier test for antibodies to *Borrelia burgdorferi* to provide serologic evidence to aid in the diagnosis of Lyme disease. In a situation in which a very complex set of antigens from whole bacteria was used instead of a handful of antigens from a retrovirus, the difficulties and challenges of the Western blot assay became more apparent. The increased use of the assay and increased dependence on results for providing diagnostic evidence of infection has caused considerable confusion with performance of the assay and interpretation of its results.

This document provides a set of recommendations to minimize, if not eliminate, these difficulties. It also provides a framework for setting national or international guidelines for performing and interpreting Western blot assays detecting antibodies to *Borrelia burgdorferi.* While it has been noted that differences in interpretation of the Western blot assay used for diagnosis of Lyme disease exist between North America and Europe, it is the subcommittee's intention to make every effort to foster harmonization in interpretation in future versions of M34. To begin to fill this identified void in standardization, the Area Committees on Microbiology and Molecular Methods will seek opportunities to develop guidelines and standards for the performance and interpretation of Western blot assays that have applicability to a broad variety of antibodies to other organisms and antigens for application worldwide.

Key Words

Antibody, antigen, Western blot assay

Western Blot Assay for Antibodies to *Borrelia burgdorferi;* Approved Guideline

1 Introduction

1.1 Principle

The Western blot or immunoblot assay is a qualitative or semiquantitative immunologic test for antibody to a nonself- or self-antigen. It has six steps:

(1) one-dimensional electrophoretic separation of antigens (proteins, carbohydrates, and/or lipids) primarily on the basis of molecular size;

(2) capillary or electrophoretic transfer of the separated components to a solid matrix, usually a microporous membrane;

(3) blocking of uncomplexed protein-binding sites on the membrane;

(4) incubation of the blocked membrane with antibodies in a body fluid, usually serum or cerebrospinal fluid, that is suspected of containing antibodies to antigens on the membrane;

(5) colorimetric, fluorescent, chemiluminescent, phosphorescent, radiometric, or electronic detection of the bound antibodies on the membrane by using labeled second antibody or other ligand with specificity for the immunoglobulins (usually IgM and IgG) of interest; and

(6) visual assessment or image analysis of the developed blot, identification of the detected antibody-antigen complexes on the blot using antigen standards, and comparison of the reactions of clinical specimens to reactions of positive and negative control specimens.

Material of various degrees of complexity is subjected to electrophoretic separation in the first step. Lysed material may be whole cells, viruses, organelles, subunit fractions, or combinations of recombinant-derived antigens of infectious agents, allergens, or animal tissues.

The test result is the binding or lack of binding to a combination of selected antigens in the electrophoretically separated sample. Identification of bands that are observed in the developed blot depends either on monospecific antibodies to the selected antigens or purified preparations of native or recombinant forms of the selected antigens run in parallel on the gel. A Western blot test is usually interpreted as “positive” when a certain number of the selected antigens are represented as bands of a minimum intensity on the developed membrane, photographic film, or digital image. The Western blot assay is usually but not always performed for immunological testing for infectious diseases and autoimmune diseases as a highly specific assay for antibodies after a screening or otherwise highly sensitive antibody assay, such as EIA.

The Western blot assay, like other antibody assays used as a second step or supplemental procedure, has its highest positive predictive value when the *a priori* likelihood of the disease is high on the basis of clinical and epidemiologic criteria. The negative predictive value of the Western blot will also vary according to the population tested; because of the variability of the antibody response among infected individuals, it cannot be as well-defined clinically as the positive predictive value of the assay.

1.2 Scope

M34-A is intended to serve as an adjunct in the serologic diagnosis of Lyme disease. To achieve this goal, this guideline presents a comprehensive test methodology for the performance of the Western blot assay for antibodies against the organism *Borrelia burgdorferi* and other *Borrelia* spp. which have been implicated as causative agents of Lyme disease. The Western blot methods outlined within M34-A also have broad applicability over a range of other antigens and antibodies. While the interpretive guidelines contained in M34-A are specific to *Borrelia* species that cause disease in endemic areas in North America, it is anticipated that future expansion of these guidelines to address *Borrelia* species implicated as Lyme etiologic agents in other areas of the world outside of the United States will foster further global harmonization of these methods.

1.3 Definitions[a]

Accuracy// Measurement accuracy// Accuracy of measurement, *n* - Closeness of the agreement between the result of a measurement and a true value of the measurand {/analyte}.

Affinity, *n* - A measure of the attraction, or force of association, between a single antigenic site and a single antibody to that site; **NOTE:** a) The affinity constant is usually expressed as the equilibrium constant for the receptor + ligand reaction. Because of their heterogeneity, average or mean affinity constants are usually described for polyclonal antisera.

Antibody, *n* **-** The functional component of antiserum, composed of a population of Y-shaped protein molecules, each member of which is capable of reacting with (binding to) a specific antigenic determinant.

Antigen, *n* **-** Any substance that can stimulate the production of antibodies by an organism and combine specifically with them.

Epitope//antigenic determinant//(determinant), *n* **-** 1**)** The minimum molecular structure of the antigenic site that will react with a monoclonal antibody; **2)** Any site on an antigen molecule at which an antibody can bind; the chemical structure of the site determining the specific combining antibody.

Antiserum, *n* - A serum produced in animals or human beings that contains antibodies to one or more antigens of interest.

Band, *n* - A discrete region of the developed Western blot that corresponds to an antigen of a particular molecular size and the binding of antibody to that antigen.

Blocking, *n* - The reaction of uncomplexed binding sites or of coupling agents to prevent nonspecific binding of test reactants.

Blot development, *n* - The detection of the binding of antibody to an antigen on the blot by colorimetric, fluorescent, phosphorescent, chemiluminescent, radiometric, or electronic signal.

Conjugate, *n* **-** A material produced by attaching two or more substances together.

Control//Control material, *n* - A device, solution, or lyophilized preparation intended for use in the quality control process.

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

Cross reactivity, *n* - The reaction of an antibody with an antigen other than that which elicited its function, as a result of shared, similar, or identical antigenic determinants.

Cut-off control, *n* - A serum or other antibody source that predictably yields one or more detectable bands with a signal at or near the lower limit of visualization.

Denaturation, *n* - Loss of native structure or configuration of a macromolecule, usually with resulting loss of biological or immunological reactivity or solubility.

Densitometry, *n* - A photographic or digital recording of the band and subsequent image analysis for quantitation.

Efficiency, *n* - The percentage (number fraction multiplied by 100) of results that are true results, whether positive or negative.

Enzyme conjugate, *n* - One of the reagents of an immunoassay that has either an antigen or antibody complexed to an enzyme by a covalent linkage.

False negative result//False negative, FN, *n* **-** A negative test result for a patient or specimen that is positive for the condition or constituent in question.

False positive result//False positive, FP, *n* **-** A positive test result for a patient or specimen that is negative for the condition or constituent in question.

Hybridoma, *n* - A cell made in the laboratory by fusing a normal cell with a cancer cell to combine certain features of each; **NOTE:** Most specifically**,** a cell line derived by the fusion of a B cell and a plasmacytoma cell, usually for the production of monoclonal antibodies.

Immunoassay, *n* - **1)** Any laboratory method for detecting a substance by using an antibody reactive with it; **2)** A ligand-binding assay that uses a specific antigen or antibody capable of binding to the analyte.

Immunogen, *n* - Any substance that elicits a cellular and/or humoral immune response and the production of antibody in a biological system).

Label, *n* - **a** substance that is linked to a reagent (e.g., antigen or antibody) to facilitate detection in an immunoassay system using either a measurable property of the label or an entity produced by the label; **NOTE:** In EIA the label is the enzyme; in FIA the label is a fluorescer; in RIA the label is the radionuclide.

Ligand, *n* - An entity that binds to a receptor; **NOTES:** a) For example, an atom, ion, molecule, antibody, hormone, or drug; b) In immunological testing, the terms "ligand" and "analyte" are frequently used synonymously.

Matrix, *n* - The milieu of the sample, such as serum, plasma, urine, etc., that can influence the behavior of an assay by specific or nonspecific interferences; **NOTE:** Matrix can also refer to the solid support on which the test is performed, e.g., the plastic-coated card of an agglutination test.

Membrane, *n* - A microporous, usually flat solid matrix that binds the transferred antigen for subsequent incubations with first a source of antibodies and second antibody-detection reagents.

Monoclonal, *adj* - Arising from a single clone of cells.

Nonspecificity, *n* - The reactivity of an agent in a test system to substances other than the analyte of interest.

Polyclonal, *adj* - Pertaining to cells or cell products derived from different clones and having affinity for different epitopes on the same antigen and/or different antigens.

Precision, *n* **-** The closeness of agreement between independent test results obtained under prescribed{/stipulated} conditions.

Predictive value of a negative test result//Predictive value negative, *n* - The probability that a subject with a negative test result actually does not have the disease.

Predictive value of a positive test result//Predictive value positive, *n* - The probability that a subject with a positive test result actually has the disease.

Qualitative assay, *n* **-** Reports only the presence or absence of the analyte, without quantification. A positive test result implies only that the assay signal exceeds the analytical threshold (detection limit), or a cut-off point set to give an arbitrary combination of sensitivity and specificity. In simplistic and idealistic terms, detection of the analyte should correlate with the presence (and nondetection with the absence) of the infectious agent or of related antibodies, resulting from either natural exposure or immunization.

Quantitative assay, *n* - Generates a spectrum of signal responses that correlate with the concentration of the analyte of interest. If analyte preparations with known concentrations are available for calibration, the actual concentration of the analyte can be determined.

Reactivity, *n* - The qualitative assessment of binding of an antigen or antibody with another substance **NOTE:** Sometimes used as a synonym for "positive" when reporting test results.

Recombinant-derived antigen, *n* **-** Peptides or proteins produced by the introduction of genetic material into cells of another genus, species, or class of organism.

Run, *n* - **1)** An interval within which the accuracy and precision of a testing system is expected to be stable, but cannot be greater than 24 hours or less than the frequency recommended by the manufacturer.

Scoring the blot, *n* - The examination of the developed blot membrane to (1) assess the quality of the assay and (2) identify and count bands that meet or exceed a minimum intensity; the results of the Western blot assay.

Semiquantitative assay, *n* – A qualitative assay with an additional option for response range (degree of positivity, dilution to which positive results are obtained, or comparison to a color chart).

Seroconversion, *n* - The conversion of a patient's serum from negative to positive in a given test for antibodies to the infectious agent in question.

Sensitivity, *n* **- 1)** ***In Quantitative testing,*** The change in response of a measuring {system or} instrument divided by the corresponding change in the stimulus; **NOTES:** a) A significant scientific dispute exists regarding this term, its underlying concept and its definition, with the opposing view defining **Sensitivity** in a manner similar to **VIM93**'s definition for **Limit of Detection**. While ordinarily, a **VIM93** citation as given would be sufficient to settle the dispute, a significant case has been made on both sides. Consequently, until the dispute is scientifically settled, the definition above is a standard only if it is clearly stated in the context of its use in a document. b) The sensitivity may depend on the value of the stimulus; c) The sensitivity depends on the imprecision of the measurements of the sample. **2)** ***In Qualitative testing,*** The test method's ability to obtain positive results in concordance with positive results

obtained by the reference method; **NOTE:** d) If the true sensitivity of a device is better than the reference method, its apparent specificity will be less and the level of apparent false-positive results will be greater; **3) Clinical sensitivity,** *n* - The proportion of patients with a well-defined clinical disorder whose test values are positive or exceed a defined decision limit (i.e., a positive result and identification of the patients who have a disease). **NOTE:** e) The clinical disorder must be defined by criteria independent of the test under consideration**.**

Signal//Measurement signal, *n* - A quantity that represents the measurand and which is functionally related to it.

Specificity, *n* **- 1)** The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering phenomena/influence quantities; **2) Analytical specificity,** *n* - For quantitative tests, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample; **3)** For qualitative or semiquantitative tests, the method's ability to obtain negative results in concordance with negative results obtained by the reference method; **NOTE:** a) ***In Immunology,*** specificity is an antiserum quality defining its reactivity with defined antigens and lack of specificity is the inaccuracy introduced by cross-reacting and/or interfering substances, because cross-reacting substances compete with the analyte for antibody-binding sites; **4) Clinical specificity,** *n* - The proportion of subjects who do not have a specified clinical disorder whose test results are negative or within the defined decision limit.

Standard, *n* **- 1)** An authoritative "document" setting forth criteria for performance and characteristics; **NOTES:** a) This definition is compatible with the use of "standard" in the U.S. Code of Federal Regulations (CFR) as well as in the context of NCCLS use; b) As a consequence of the conflicting use of the term as defined in definition 1, versus the following definitions, any use of the term **standard** shall be appropriately specified in context. **2) Primary standard,** *n* - A standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity; **3) Secondary standard,** *n* **-** A standard whose value is assigned by comparison with a primary standard of the same quantity; **4) Reference standard,** *n* - A standard, generally having the highest metrological quality available at a given location or in a given organization from which measurements made there are derived**; 5) Working standard,** *n* - A standard that is used routinely to calibrate or check material measures, measuring instruments, or reference materials; **NOTES:** a) A working standard is usually compared against a reference standard; b) A working standard that is used routinely to ensure that measurements are being carried out correctly is called a "check standard."

Strip, *n* - A section of membrane with antigens that is incubated with an individual antibody specimen and developed.

Titer, *n* **-** The reciprocal of the dilution factor required to produce a defined outcome in a defined system.

1.4 Advantages

The following are advantages of the Western blot assay:

(a) There is increased specificity compared to a mixture of antigens (e.g. EIA or dot-blot), because antigens of higher individual specificity for the diagnosis of the disease in question are physically separated from antigens that are more broadly cross-reactive.

(b) Expansion or diminution of the complexity of the serum immune response can be monitored over time.

(c) Patterns of protein binding may correlate with specific disease manifestations and/or prognosis.

(d) The Western blot assay can be used in patients who have received a recombinant or other subunit vaccine against the infectious disease under consideration. Whole-cell assays like the EIA or immunoflourescent assay (IFA) may contain the vaccine immunogen as a component.

1.5 Limitations

The following are limitations of the Western blot assay:

(a) The Western blot, as usually performed for diagnostic or epidemiological purposes, is a qualitative immunological test. It is more difficult to obtain an end-point titer than with an EIA or IFA because of the test format.

(b) The protein, lipoprotein, or glycoprotein antigens are usually denatured, and, consequently, the detection of binding of antibodies to conformational epitopes may be under-represented.

(c) Antigens that are carbohydrates, glycolipids, very large proteins, or very small proteins may not be represented on the blot membrane after transfer.

(d) Bacteria, viruses with large genomes, parasites, fungi, and mammalian cells produce many antigens, and some of these may migrate to the same positions in one-dimensional gels. An antigen providing high predictive value may produce a band identical to an antigen providing low predictive value because of broad cross-reactivity.

(e) In comparison to an EIA, there may be greater subjectivity and interobserver variation in decisions of whether a band is intense enough to be scored in the Western blot.

(f) More time is required for training in the performance and laboratory interpretation of the test results of the Western blot than for an EIA.

(g) A solid-phase matrix immunoassay like a Western blot does not measure functional activity as do some other antibody assays such as complement fixation, agglutination, or bactericidal tests. The Western blot assay may not indicate immunity to a pathogen as well as an antibody assay that measures bactericidal activity, for instance.

(h) It is more difficult to standardize a Western blot assay than an EIA because of the greater number of steps and variables. For this reason, there are also greater opportunities for errors in performance and variation both within and between laboratories.

(i) Proteolysis of proteins can lead to additional bands and to lower amounts of the selected antigens of diagnostic importance. These proteolytic products might be less of a problem where there is an unresolved mixture of the antigens, as in an EIA.

(j) Preparation of the antigen substrate for Western blot assay is generally more costly in materials and is more labor intensive than for EIA or IFA.

2 Preparation of Antigens

2.1 General Considerations

Growth *in vitro* of a pathogenic bacterium is not equivalent to growth of the organism in its animal host. There is evidence from several bacterial systems that there are sets of proteins and other components that are expressed only in a host cell or animal. Recovery of organisms grown under these conditions, while desirable, is nonetheless difficult to achieve. For many bacteria, including the *Borrelia* spp. that cause

Lyme disease, the usual practice for antigen preparation is to grow the organism in axenic cultures. If *in vivo*-specific antigens are identified and the genes for them are cloned, the recombinant versions may supplement or replace culture-derived antigens.

Antigens should be of a standardized composition, purity, and concentration. For infectious agents, the strain and passage, and growth conditions (including medium composition, and duration and temperature of growth) will affect the character of the resulting blot. All antigens of diagnostic importance should be produced in sufficient quantity to permit sensitive detection of antibodies to them when a blot is developed. The electrophoretic migration of each of the diagnostic antigens to be scored should be precisely documented with respect to molecular mass markers and reference reagents such as monoclonal antibodies or purified native or recombinant antigens. The relative migration of each diagnostic antigen should be verified if an organism is serially cultivated or received from another laboratory. In order that antigen preparations may be standardized and for performances of laboratories to be compared, it is desirable to use only one or a few well-characterized strains. Preferably, the chosen strain(s) will be one(s) for which monoclonal antibodies, recombinant proteins, and DNA sequences are available to confirm the identity of the strain and facilitate immunoblot calibration. These strains should be deposited with the American Type Culture Collection (ATCC) or equivalent reference service. The strain(s) should be representative of the agents that cause disease in populations served by the testing laboratory.

2.2 Choice of Strain for Antigen Preparations

At least three genospecies of *B. burgdorferi sensu lato* are responsible for Lyme disease worldwide: *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*. Only *B. burgdorferi sensu stricto* has been isolated to date in North America from humans. The etiologic role of other genospecies of *B. burgdorferi sensu lato* in human disease in North America remains to be determined. If one or more of these genospecies are shown to be etiologic agents of human disease, then these genospecies may need to be included as additional antigen preparations for Western blot analyses. Although there is substantial antigenic cross-reactivity between genospecies when serum specimens of individuals infected with one of the genospecies are examined, the sensitivity of the assay may be lower when a different genospecies is used as the sole source of the antigen.

The strain of *B. burgdorferi* selected for blot preparation should express well all the antigens to be scored. Strains B31 (ATCC® 35210), 297 (ATCC® 53899), and 2591 are examples of suitable choices for *B. burgdorferi sensu stricto*. They are well characterized by different investigators and laboratories, there is substantial published experience with them, and they are available from recognized depositories. For future development of assays the strain should be cloned by limiting dilution or colony plating; isolates from nature sometimes consist of mixtures of strains. Because infectivity for mammals (generally laboratory mice) and expression of some proteins is often lost from cultured *B. burgdorferi* after as few as ten passages *in vitro*, use of cells passed fewer than ten times (or fewer than about 60 generations) after animal passage is desirable.[1] More important than passage level is demonstration that the isolate expresses all molecules of diagnostic interest (see below). Once a strain and passage level have been selected, a master seed stock should be established and sufficient vials of working stocks prepared so that cells of identical history can be consistently used.

NOTE: Hereafter in this document, "*B. burgdorferi"* will mean *B. burgdorferi sensu stricto* unless otherwise stated. It is anticipated that the guidelines will also be appropriate for laboratories testing for antibodies to the other genospecies of Lyme disease agents.

2.3 Growth Conditions for *B. burgdorferi*

Expression of some antigens is dependent on temperature and cell density *in vitro*. Since the level of expression of diagnostic antigens will affect test sensitivity, these variables should be investigated and standardized. Suitable conditions are growth at 33 to 35 °C in BSK II medium or equivalent,

supplemented with 6 to 7% partially hemolyzed rabbit serum, and harvested at late log phase of growth, about 1 x 10^8 spirochetes/mL.[2,3] Other important variables in the cultivation of *Borrelia* spp. are the concentrations of oxygen and carbon dioxide. The appropriate conditions are usually achieved by growth of cells in liquid medium in tightly capped tubes or bottles with only a small space for ambient air. For the latter method, the size of the vessel and air space should be standardized. An alternative is to use the defined gas mixture of 4% O_2, 5% CO_2, 91% N_2.[4] Good quality culture medium supports the growth of *B. burgdorferi* from a single cell. The quality of each lot of culture medium, whether it is made in the laboratory or purchased commercially, should be assessed by growth of serial dilutions of a low-passage spirochete stock. The lot numbers of complex and perishable ingredients such as bovine serum albumin and rabbit serum should be recorded for reference in case of suboptimal performance of the medium.[5] If the shelf-life dates of the ingredients have been exceeded, they should not be used. Once optimum culture inocula and growth conditions have been identified, they should be used consistently.

Whatever exact conditions are used, spirochete numbers in the culture should be monitored by dark-field or phase-contrast microscopy. Infectious *B. burgdorferi* has a tendency to clump together in culture, making accurate enumeration difficult. An alternative to cell counts is the determination of total cellular protein in the cell harvest.

2.4 Demonstration that a Culture of *B. burgdorferi* is Pure at Harvest

The accuracy of any Western blot assay depends on the consistency of the antigen. Because the antigens for the assays are almost always derived from living cells, be they the pathogen itself or a recombinant DNA host, there is always the chance of contamination with other microorganisms. This contamination can both reduce the yield of the desired antigen and introduce antigens that are not specific for the infection in question, thereby leading to either lower sensitivity or specificity of the assay. Only pure cultures should be used for antigen production.

In the case of antigen preparation for the Western blot assay for antibodies to *B. burgdorferi*, the spirochetes cultures should be inspected by dark-field or phase-contrast microscopy at harvest for evidence of contamination with other microorganisms. The thinness of the organisms prevents them from being well visualized by Gram stain. Culture purity should be assessed further by incubating aliquots of *B. burgdorferi* in standard microbiologic media, preferably brain-heart infusion broth, trypticase soy agar, mycoplasma media, thioglycollate broth, and chocolate agar, each held at 37 ºC for one week. Additionally, spirochete cultures should be streaked on Sabouraud dextrose agar and incubated at 25 °C for two weeks for evidence of fungal contamination. Occasionally, there is contamination of cultures from mycoplasmas; this may be suspected (1) after a sooner-than-expected change in the phenol red indicator from red to yellow and the observation of increased quantities of small amorphous particles by microscopy; or (2) the detection of small, slow-growing colonies on chocolate agar.

2.5 Preparation of *B. burgdorferi* Cell Lysates

During preparation of antigens from cell harvest the following errors and consequences can occur: (a) centrifugation at insufficient speed or for insufficient time lowers antigen yield; (b) prolonged storage or repeated cycles of freeze-thawing can lead to autolysis of the cells with antigen degradation; (c) inadequate washing retains culture constituents, such as bovine albumin and rabbit serum, into the antigen preparation.

Cells of *B. burgdorferi* can be recovered by centrifugation for 30 minutes at 10,000 x g. Residual medium components, particularly bovine serum albumin and rabbit immunoglobulins, are removed by washing the cell pellet two to three times with buffer containing divalent cations to stabilize the bacterial membrane (e.g., PBS supplemented with 5mM $MgCl_2$). Suspensions of washed cells may be prepared in buffer compatible with sample electrophoresis buffer (e.g., 10 mM Tris HCl, pH 7.5-1 mM EDTA) and frozen until use, preferably at -70 ºC or colder. Samples should be stored in small aliquots to avoid repeated

cycles of freeze-thawing. The protein concentration of the cell suspension should be determined by a widely accepted method, such as the Bradford procedure, using a standard curve, and then adjusted to a convenient value (e.g., 2 mg/mL).

Samples are prepared for electrophoresis by adding a buffer in a volume equal to or two- to threefold lower than that of the cell suspension. The buffer contains sodium dodecyl sulfate (SDS) and a reducing agent, and the sample in the buffer is then usually boiled.[6] A minimum of 1.4 g SDS per g of protein is required to solubilize most polypeptides and enables them to migrate in a polyacrylamide gel of the correct porosity in proportion to their molecular mass; a substantial excess by weight of SDS over protein is desirable. To avoid electrophoresis artifacts, use SDS of high purity. Dithiothreitol or 2-mercaptoethanol is a suitable reducing agent for disruption of disulfide bonds in proteins. Dithiothreitol has the advantages of being nearly odorless, less prone to auto-oxidation, and effective at a lesser concentration than 2-mercaptoethanol. Boiling of samples for more than five minutes or repeatedly can lead to hydrolysis of proteins and should be avoided.

Samples should be treated as potentially infectious until they are boiled in an SDS-containing sample buffer.

3 Separation of Antigen Constituents by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.1 General Considerations

A variety of conditions for electrophoresis may give satisfactory results if optimized and standardized. Relevant variables include the concentration of polyacrylamide and cross-linker; lengths of resolving and stacking gels; ionic strength and pH of buffers; SDS concentrations in the gel and running buffers; voltage and current during electrophoresis; and amount of protein loaded per unit of cross-sectional area of the gel. A discontinuous gel system is usually used; the stacking gel allows colinear migration of antigens prior to separation. In general, desirable electrophoretic conditions should yield a straight-line, semilogarithmic relationship between the migratory distances of the proteins in the gel and their actual molecular weights. Electrophoretic conditions should be selected to permit adequate heat dissipation so that protein migration rates are uniform across the gel without peaks and valleys. Unequal migration can also occur when the gel is inadequately mixed before polymerization and when there is leakage of current between chambers in the gel apparatus. Overloading of the gel with proteins may marginally increase the sensitivity of the assay but at the cost of resolution and irregular migration patterns.

3.2 SDS-PAGE of *B. burgdorferi*

Electrophoresis of whole-cell antigens of *B. burgdorferi* should resolve antigens ranging in apparent or actual molecular masses from 10 kDa to 100 kDa. This can be satisfactorily achieved in gradient gels ranging in concentration from 7.5 to 15% wt/vol acrylamide.[7] Single concentration gels (e.g., 12.5%) are also suitable, providing that all antigens to be scored are well resolved and distinct. Molecular weight standards, preferably at least six proteins, should be used to calibrate the 10 to 100 kDa range. A lane containing these standards and a thin strip of separated *B. burgdorferi* antigens in the gel should be stained with Coomasie Blue or other stain of equal or better sensitivity, such as silver- or copper-based stains, to assure adequate separation and resolution of the proteins and possibly other antigenic constituents. If the antigen preparation is the same across the gel, the sample can be layered over the top of a long well stretching most of the width of the gel.

4 Transferring the Antigens, Blocking the Membrane, Incubating the Specimen, and Developing the Blot

4.1 Transfer of Antigens to the Membrane

Following electrophoresis of the polyacrylamide gel, the gel is carefully removed from between the glass gel supports. Care should be taken at this step to prevent tearing of the gel. The gel is then placed on top of or beneath the membrane. Air bubbles between the membrane and the gel, which would block transfer in this area of the gel, should be gently removed prior to the transfer process. The transfer of antigens from the gel to the membrane may occur through capillary action alone, but transfer is achieved more rapidly by application of a direct electric current perpendicular to the plane of the gel and adherent membrane.

Nitrocellulose has been the most common membrane material. Polyvinyl difluoride and nylon have also been used. The transfer can be done under "wet" conditions, meaning completely suspended in a buffer in a glass or plastic chamber. The buffer for this purpose should allow transfer of the antigens, usually proteins, on the basis of charge and molecular size. Achieving this aim may require inclusion of an ingredient, such as methanol, that will remove some of the SDS from the proteins. Although much of the SDS may be gone by the time the protein reaches and binds to the membrane, all or many of the proteins should still be considered in a denatured state. The binding of proteins to the membrane is through the hydrophobic and/or electrostatic forces. Blot transfers are also done under so-called "dry" or "semidry" conditions.

In general, transfer of large proteins (e.g., over 100 kDa) and small proteins (e.g., under 20 kDa) from the gel to the membrane with approximately equal efficiency should be obtained. This means continuing the electrophoretic transfer long enough for the largest proteins to migrate out of the gel but not so long that proteins already bound to the membrane are removed. Small proteins rapidly move out of the gel, but their small size may result in the passage through membrane pores instead of binding to the membrane. For that reason, membrane pores are usually 0.45 micron or less. The adequacy of transfer can be assessed by staining of the post-transfer membrane with Amido Black or Ponceau Red stains and the post-transfer gel with Coomasie Blue.

4.2 Blot Preparation

Following transfer of the antigens to the membrane, the membrane is removed from atop the gel and placed in a solution to block all remaining protein-binding sites. Blocking solutions generally are Tris- or phosphate-buffered saline solutions containing BSA, gelatin, Tween 20, heterologous serum, casein, and/or nonfat milk. Membranes are kept in the blocking solution for sufficient time to ensure clear backgrounds in the developed blot. This usually means an incubation overnight at 2 to 8 ºC. The blocking step is longer than subsequent primary and secondary antibody incubations, because the time to achieve 99% saturation is several-fold greater than the time to achieve 50% saturation. The blocking step requires nearly complete saturation of the unbound sites, while antibody binding at 50% of capacity usually yields acceptable sensitivity.

Prepared blots may be stored dry or wet, but a dry blot retains greater activity than a wet blot over the same amount of time of prolonged storage. Wet strips can be stored in blocking buffer, plus 0.04% sodium azide at 4 °C for up to one week. The blots should be washed in blocking buffer without azide prior to use in the assay. Dry strips can be stored at 4 °C or colder for months. Blots can be divided into strips ranging from 2 to 4 mm in width, using a straight edge and scalpel or paper cutter. Prior to cutting the membrane, a suitable mark should be placed on the front side to ensure proper orientation throughout the assay. The side of the membrane that was next to the gel should be kept face-up in the various incubations and washes.

4.3 Suitable Samples

Serum rather than plasma should be used. Lipemic sera may be centrifuged at 60,000 x g to clarify the serum prior to use or storage. Serum may be stored for up to 14 days at 2 to 10 ºC before testing. IgM testing is best performed on unfrozen specimens that have been stored at 2 to 10 ºC for no more than five to seven days. Serum and cerebrospinal fluid for IgM testing should be kept at –70 ºC. Samples should be kept in tightly capped or sealed containers to avoid desiccation. Frost-free freezers that periodically rise above the freezing point should be avoided for storage. Specimens that have been refrozen should not be used. Control serum samples and antigen standards should be stored frozen in small aliquots rather than in large volumes, which would subject these reagents to repeated freeze-thaw cycles. After storage serum and cerebrospinal fluid specimens should be mixed prior to use to homogenize the solution. For shipments, specimens should be sent with a cold pack or frozen.

Inhibition of enzymatic reactions during the development phase of the assay may occur with some preservatives. Organic mercurials like merthiolate inhibit alkaline phosphatase activity. Sodium azide and sodium hypochlorite (household bleach) will inhibit horseradish peroxidase activity. If contamination of the serum or cerebrospinal fluid is a concern, the sample may be filter-sterilized using a low protein-binding membrane filter before storage.

4.4 Dilution of Samples

In a Western blot, serial dilutions to achieve an end-point titer are usually not done. Testing several different dilutions would raise the cost of the assay prohibitively for other than research purposes. Instead, a certain dilution of serum is used for all specimens. The appropriate dilution is established during test development. In general, the goal is to achieve high sensitivity and high specificity. It is unusual to find a negative control serum that does not have antibodies that bind to one or more antigens in the blot when used at a low dilution. Nonspecific or cross-reactive binding is more likely to occur when the antigen mixture is complex, as it is for *B. burgdorferi*, a bacterium that expresses 500 or more proteins and nonproteinaceous antigens. In contrast, the Western blot assay for antibodies to the HIV 1 virus comprises only four diagnostic antigens out of nine total viral proteins. The serum specimens should be diluted sufficiently that negative control serum specimens bind to few if any bands and that there be minimal background binding to the membrane itself. Some serum samples contain antibodies, particularly rheumatoid factors and those occurring during acute Epstein Barr virus infection, that are more inherently "sticky" than others and produce a dark background to the entire blot. In most assays, a 1:50 or 1:100 dilution of serum is used. More concentrated serum samples, e.g., 1:10 or 1:25, often produce excessive background, even with prior blocking. Cerebrospinal fluid can be more concentrated than serum, because there are fewer and less diverse kinds of antibodies present. The diluent should always be added to incubation chambers or channels before the sample when preparing dilutions directly. This avoids nonhomogeneous direct contact of the serum with the blot with consequent uneven backgrounds.

Diluents for Western blots are generally buffers such as phosphate, Tris or glycine with a pH range of 7.0 to 8.2. These buffers generally contain isotonic saline and a blocking agent such as gelatin, nonfat milk, casein, serum albumin, or heterologous serum. Surfactants, such as Tween 20 at a concentration of 0.5% vol/vol, may also be used. These sample diluents should be made fresh; preservatives described above should be avoided.

4.5 Assay Conditions

Western blot assays, like other indirect immunoassays, consist of a primary antibody incubation, washes, secondary antibody-conjugate incubation for binding to the first antibody, washes, and development to reveal the first antibody bound to the antigen on the blot. One of the goals of a clinical laboratory is to use and/or develop tests that can be completed or repeated within a single eight-hour shift or adjacent shifts. If incubations are longer than one hour, a test with multiple incubations becomes more difficult to schedule.

Primary antibody incubations should range from 15 to 60 minutes and be carried out at room temperature or 37 °C. Incubations that require one hour or longer for adequate signal detection indicate suboptimal conditions. Washes following these incubations routinely consist of at least three thorough washes with the sample diluent buffer or equivalent buffer at room temperature. The incubation of the second antibody or second ligand conjugate, also diluted with sample diluent, should range between 15 to 30 minutes to minimize nonspecific binding.

Orbital rotators and vibrational mixers should not be used for Western blotting procedures due to blotchy and nonhomogeneous results. Channeled reaction trays in conjunction with monodirectional platform rockers are recommended.

4.6 Blot Development

For testing human serum specimens, enzyme conjugates of choice have been antihuman IgG and/or IgM Fc-specific antibody conjugated with either horseradish peroxidase (HRP) or alkaline phosphatase. Antibodies conjugated with biotin or digoxigenin, conjugates of protein A and/or G, and radiolabeled ligands are less widely used. Avidin-biotin conjugates are not generally required, because they complicate the design of the assay and do not offer an advantage over a more direct enzyme label. Protein A and Protein G conjugates can be used for Western blot development but are less specific than polyclonal antibodies to immunoglobulins. Antibody conjugates with specificity for IgG or IgM Fc fragments will assure the monospecificity of the assay. Antibody conjugates that are heavy and/or light chain specific have potentially a higher degree of cross-reactivity. Both HRP and alkaline phosphatase have advantages and disadvantages but generally can be used interchangeably at the discretion of the user.

As with all enzyme-based immunoassays, time and temperature parameters are crucial in the Western blot. Substrate reaction is more susceptible to changes in temperature and time than is the binding of antibodies with the antigen on the membrane. Substrate reaction rates for both alkaline phosphatase and HRP reactions are extremely temperature-dependent. Consequently, environmental conditions should be as consistent as possible from one assay run to another. The timing of the substrate reaction should depend on the development of the cutoff control bands. A substrate reaction or development time of 5 to 15 minutes is usually sufficient, but the reaction should be stopped when the cutoff control band or bands are first visible, even if this occurs sooner than 5 minutes after the start of the reaction. Although an invariable time for incubation of this step is not recommended, reactions that require more than 30 minutes for detection of the cut-off control indicate suboptimal conditions or reagents. The reaction is stopped by washing with reaction diluent, followed by distilled water.

4.7 Blot Considerations Specific for *B. burgdorferi* and Lyme Disease

The diagnostically important antigens identified to date are proteins or lipoproteins. Some infected individuals have antibodies to small glycolipids of *B. burgdorferi,* but these antigens are usually not considered in the scoring of Western blots. Monomeric proteins larger than 120 kDa have not been described in *B. burgdorferi.* Therefore, there is not concern about inadequate representation of large antigens. Few studies have been done on nondenatured antigens, such as might be detected by immunoprecipitation in nonionic detergents. Consequently, it is not known how frequently antibodies directed against conformational epitopes that are not well retained by the Western blot procedure are missed.

5 Calibration of Blots

In the production of a Western blot assay, antigens in the gels and on the blots are identified and confirmed by both molecular weight standards and specific monoclonal antibodies to selected antigens. (The process for selecting the antigens is described in Section 7, Scoring the Blot.) The molecular weight standards, especially those that are prestained, also provide a rapid measure of the consistency between gels and for the preliminary characterization of antigens against which a specific monoclonal antibody is not available. Most antigens now selected for Western blot reading were originally characterized by their electrophoretic migration. For example, the OspA protein of *B. burgdorferi* was first called the "31K protein" on the basis of its apparent molecular mass in gels. Some proteins used for blot interpretation are still identifiable by their apparent size in the SDS-PAGE gels. Molecular mass markers covering the range of 10 kDa to 100 kDa should be used; this may require up to three different ranges of standards that are commercially available. Size standards covering different ranges can be combined into a single tube to achieve the desired range for the Western blot assay.

Preferably, the actual molecular weights of the standards are known from the DNA sequence of the gene. This will minimize differences between manufacturers. However, even when actual molecular weights are known, there may be differences between standards from different sources and between lots from the same source because of such variables as glycosylation, multimerization, or the effects of proteolysis during manufacture. Thus, whenever standards from a different lot or manufacturer are used, they should be checked for differences from the standards currently in use or originally used for optimizing the blot assay. The apparent molecular masses of the standards may also vary with differences in acrylamide concentration, pH, SDS concentration, or ionic strength of the buffer and gel used for electrophoresis.

Monoclonal antibodies to specific antigens, particularly those used in blot interpretation (see Section 8), are critical for interlaboratory and intralaboratory blot standardization. Examples include antibodies to *B. burgdorferi* proteins OspA, OspB, OspC, OspD, flagellin, GroEL, P39, P66, and P83.[8] Some of these proteins, such as OspB and OspC, have considerable variability in sequence between strains within a genospecies, and, consequently, the monoclonal antibodies should be demonstrated to bind to the relevant proteins of the particular strain in use. Preferably, there is a different monoclonal antibody that is specific for each protein in the sets of proteins used for blot interpretation. As of late 1998, there is not a nonproteinaceous antigen included in the sets of antigens used in blot interpretation for *B. burgdorferi*, but this does not exclude use of such an antigen, such as a glycolipid, in the future.

An alternative method for band identification in Western blots is to use recombinant proteins of *B. burgdorferi* as markers for the native protein's migration. This may be difficult to achieve, though; the recombinant protein may be processed differently. For instance, cleavage of the signal peptide and subsequent acylation of the Osp proteins may not be as efficient in *E. coli* as it is in the *B. burgdorferi* and, thus, the recombinant Osp protein would migrate at a different rate from the native protein. Other recombinant proteins are only available as fusion proteins. Possibly, recombinant proteins will find use in Western blot assays as a defined combination of recombinant proteins that completely replaces a cell lysate of *B. burgdorferi*.

Once the Western blot assay has been calibrated using the approaches described above, the location of the selected antigens on the blot should be indicated for the end user, such as a clinical laboratory in a hospital. This can be achieved by marks on each strip of the membrane or a separate calibrated strip in which each of the antigens is visible after blot development.

6 Quality Control and Proficiency Programs

Positive and negative control specimens are critical both in the production of a Western blot assay and in its subsequent application. Since the Western blot assay usually is used as a highly specific test, information about the control specimens should be accurate. Misdiagnosis of another disorder as Lyme

disease may reduce specificity. Inclusion of specimens from individuals with subclinical *B. burgdorferi* infection among negative controls may reduce the sensitivity of the assay.

Positive or "strongly reactive" control serum specimens, used for band identification, should be obtained from patients whose cases are definite Lyme disease by criteria of an expert panel. The positive control sample should contain antibodies that reproducibly react with at least the minimal number of diagnostic antigens in the Western blot assay as described in Section 7.

A second positive specimen, a band intensity or cutoff control (also called "minimally reactive" or "1+"), also should be provided with each kit supplied by a manufacturer or included by each laboratory that develops its own Western blot assay. The cutoff control serum specimens should be obtained from patients with documented *B. burgdorferi* infection and EIA or IFA titers in the low or borderline ranges and barely detectable bands with one or more antigens of diagnostic importance in the Western blot assay. These cutoff control specimens should be unadulterated serum samples or dilutions of strongly reactive samples in normal human serum. (Dilutions of strongly reactive serum specimens in anything other than normal human serum may cause significant changes in the background because of unnatural protein and immunoglobulin levels.) Each run needs its own cutoff control. A visual reading or image analysis that is obtained in one run should not be applied to the results of another run. Other positive control serum samples of varying reactivity should be run during test development or evaluation, but need not be included in each run.

The third type of control specimen, a negative or background control, should be obtained from healthy blood donors and collected, handled, and stored in the same manner and dilution as the patient serum specimens for testing. These serum specimens should be from persons who have a negligible risk of *B. burgdorferi* infection by clinical and epidemiological history. Because asymptomatic infection or remote, inactive infection with *B. burgdorferi* occurs, the serum specimens should be confirmed to be negative by EIA or IFA and then Western blot. Negative control serum specimens should be used in each run and at the same dilutions as patient specimens. They should not be diluted to produce a cleaner background. High dilutions of control serum specimens change protein and immunoglobulin levels and thus are not representative of uninfected patients.

The three types of control specimens outlined above also are used for analysis of cerebrospinal fluid (CSF) by Western blot. However, obtaining CSF in sufficient quantities from patients with *B. burgdorferi* infection of the central nervous system is difficult. In the absence of a positive control CSF specimen, a positive control serum should substitute. However, CSF from patients without *B. burgdorferi* infection should be more readily available and should be used as the negative control.

Because of the antigenic complexity of *B. burgdorferi*, it is not uncommon to find that antibodies in serum specimens from negative controls bind to some proteins in the blot, probably due to cross-reactions with antigens of other infectious agents. These reactions usually produce bands of low intensity or bands that are not identifiable with antigens of diagnostic importance. One example of a *B. burgdorferi* antigen that is commonly bound by antibodies in serum specimens from negative control persons is the flagellin or "41 kDa" protein. It is because of these frequent cross-reactions that several, well-defined antigens are used for scoring the developed blot. For example, reaction with flagellin has predictive value only in the context of reactivity with other, more specific *B. burgdorferi* antigens.

In addition to the three kit or procedural controls described above, each laboratory should have laboratory-specific positive and negative controls to evaluate test consistency and reproducibility over time. Preferably, these control serum specimens should be obtained in a volume sufficient to last at least a year. Laboratory-specific positive and negative controls should be run regularly, preferably once with each new set or lot of prepared strips and reagents. They can also be used as needed to assess intraoperator and interoperator performance within the laboratory.

Quality assurance requires that blinded samples be tested in a defined periodic schedule. Laboratories performing and reporting the Western blot for antibodies to *B. burgdorferi* should participate in an approved proficiency program.

7 Scoring the Blot and Reporting the Results

The results of a Western blot assay are presence or absence of bands on the developed blot. When the expected bands are observed with the positive control but no bands are observed on the strip with a patient's serum, scoring (or reading) the blot as negative is simple. More difficult is the scoring of a strip on which one or more bands are present. The calibration process allows estimation of the apparent molecular masses of the bands and, in cases for which there is a monoclonal antibody and/or recombinant antigen available, actual identification of the band as a certain antigen. But even if every band could be identified with a particular protein, only a minority of these proteins is used for blot scoring. This is because most of the proteins or other antigenic components of the bacterium seldom or never elicit an antibody response by infected persons, are so broadly cross-reactive with the antigens of other microorganisms that they are insufficiently specific, or are not well represented on the blot for technical reasons, such as comigration with another protein or migration off the gel.

In the development of a Western blot assay, selection of a set of antigens of high predictive value for Western blot scoring requires the examination of serum specimens from a large number patients who have been infected with *B. burgdorferi*. For this battery of sera, the diagnosis of infection should be documented either by cultivation of the microorganism or by detection of the organism with a highly specific, stringently controlled, and reproducible polymerase chain reaction assay. If, as an alternative to direct detection by these means, clinical and epidemiological criteria alone are used, the case definition should be that of an expert panel. The specimens should be from patients who have different stages of the infection; in the case of Lyme disease, this would be early localized, early disseminated, and late disseminated infections with *B. burgdorferi.*

The specificity of the antigens, individually and in combinations, should be investigated through inclusion in the same development studies of serum specimens from healthy individuals or patients with other diseases, especially those that may be confused with *B. burgdorferi* infection on clinical, epidemiological, and/or serologic grounds:

(a) patients with other spirochetal illnesses, such as syphilis, relapsing fever, and leptospirosis;

(b) patients with other tick-borne infections, such as rickettsial diseases and babesiosis;

(c) patients with disease whose clinical symptoms mimic early or late Lyme diseases, such as neurological illnesses, fibromyalgia, and rheumatoid arthritis; and

(d) patients whose diseases may result in the presence of antibodies that bind to borrelial antigens and/or the membrane itself as the result of polyclonal B cell activation, such as occurs in Systemic Lupus Erythematosus, Acquired Immune Deficiency Syndrome, and infectious mononucleosis.

Since not all persons with present or past B*. burgdorferi* infection will have detectable antibodies to each of the selected antigens in the set, the binding to a subset of the proteins determines whether a Western blot is positive or not. For example, one currently used criterion for a positive Western blot for IgG antibodies specifies that there be bands for at least five of ten selected antigens in a set. These are the following proteins that are identified by name or estimated molecular mass: 18 kDa, OspC, 28 kDa, 30 kDa, 37 kDa, P39, 45 kDa, 58 kDa, P66, and 93 kDa proteins.[9,10] Some investigators recommend

inclusion or substitution of OspA and OspB proteins in the set.[11] A criterion for IgM antibody Western blot testing specifies that two of three of the following bands be present: flagellin, OspC, and P39.

Although a semiquantitative or quantitative estimation by eye or image analysis of the intensity of the bands can be made, this is only marginally more useful than whether or not there is a band. But for even this "yes-no" determination there should be standards for accepting or rejecting faint bands and for assessing whether there are artifacts. The erroneous scoring of a faint band is a common reason for false-positive readings of Western blots. The scoring of the Western blot is most accurate when there is a low background on the membrane itself. When serum produces a high background on the membrane itself, many, if not all of the bands may be artifactual. The scoring of the Western blot is performed in the laboratory independently of other considerations, such as the patient's history or other laboratory results.

The reporting of the Western blot as either "positive" or "negative" is based upon the number and identities of bands that are scored. If a report of "borderline" or "indeterminate" is used, it should be of proven predictive value for diagnostic, therapeutic, prognostic, and/or epidemiological purposes. The term "indeterminate" is best reserved for blots with high membrane background or with suspected artifactual bands. Certain bands in the Western blot assay may in the future be shown to have significance for diagnosis and/or prognosis and are optionally recorded.

Included with the laboratory's report of the blot as "positive" or "negative" should be a listing of the antigens in the scoring set and which bands were bound by the patient's antibodies. This information allows comparison of the results of one Western blot assay of one manufacturer or laboratory with that of another and also whether the antibody response is broadening to include more antigens or narrowing over time. A particular pattern of binding may be of prognostic or therapeutic relevance as further data on the natural history of the infection is gathered.

8 Interpretation of the Report

The laboratory's report of the Western blot results is interpreted in a clinical and epidemiological context by the healthcare provider, public health professional, or clinical researcher. As with other tests that measure antibody responses, the Western blot is an indirect method for supporting a disease diagnosis and serves as an adjunct to other methods, such as the history and physical examination. With the criteria for test positivity in common use at this time, the Western blot assay for anti-*B. burgdorferi* antibodies has higher specificity but lower sensitivity than other methods of detecting antibody responses, such as EIA or IFA. If the number of bands required for test positivity were reduced below five (to take the example above), or the number of selected antigens in the set was increased above ten, it is likely that the sensitivity of the Western blot would increase but at the expense of lower specificity. In any case, the "analytical" sensitivity of the Western blot for detecting antibodies to individual proteins will not correlate with clinical sensitivity for identifying infected individuals.[12] To properly interpret Western blot results in a clinical context it is important to have an understanding of the evolution of the antibody response in that illness, the factors which influence that antibody response, other illnesses which can cause cross-reactive antibodies and lead to false-positive results, and the way that the Western blot is employed in clinical practice.

A Western blot that is reported as positive on the basis of the criteria established for the test may be clinically irrelevant. The presence of IgG antibodies to the microorganism may be the result of a past infection, which is inactive because of an adequate immune response or treatment with antibiotics. A Western blot may be positive for months to years after successful therapy of the infection. The presence of either IgG or IgM antibodies may also be the result of a recent but asymptomatic infection. Another situation in which an individual has antibodies to *B. burgdorferi* in the absence of Lyme disease is after immunization with a vaccine to *B. burgdorferi*. In these cases, the antibodies themselves do not necessarily indicate an active infection, disease attributable to the infection, or immunity to the infection.

Indeed, a more appropriate name is "Western blot assay for antibodies to *B. burgdorferi*" and not "Western blot assay for Lyme disease."

The positive test may also rarely be on the basis of cross-reactive antibodies and not on past or present infection with *B. burgdorferi.* This is a true false-positive and may occur even with the most careful adherence to test protocols and with impeccable quality control. To completely eliminate such false positives, the test specificity would have to be set so high that the assay would have too many false-negative results. On the other hand, the positive result may also be on the basis of either past *B. burgdorferi* infection or an infection that is latent or inactive from a clinical point of view. In this latter case, the Western blot assay achieved its goal of detecting antibodies to the agent and is not false positive for this purpose or perhaps the purpose of an epidemiological serosurvey of exposure to *B. burgdorferi*. The prevalence of a true positive test for anti-*B. burgdorferi* antibodies in patients who do not have active clinical Lyme disease depends on the frequency of an asymptomatic seroconversion and the duration of a true positive test in a previously symptomatic infected individual. This is a diagnostic issue encountered in regions of the country highly endemic for Lyme disease. The prevalence of seropositivity in communities with high focal endemicity may be 5% or higher.[13,14] Thus, in patients with atypical symptoms, positive test results must be interpreted with caution.

8.1 Predictive Value of a Positive and Negative Western Blot

The predictive value of a positive laboratory test is related not only to test specificity, i.e., the false-positive rate, but also the pretest probability or likelihood that the patient has the illness in question on clinical and epidemiological grounds alone.[15] The pretest probability that a given individual has Lyme disease depends upon both the nature of the presenting symptoms and the likelihood of exposure to *B. burgdorferi*, that is, residence in or travel to an endemic area and the prevalence of *B. burgdorferi* infection in the area. In an endemic area like coastal regions of the northeastern United States, patients with a flu-like syndrome in the winter are unlikely to have early Lyme disease. Even with a higher seasonal risk of acute *B. burgdorferi* infection in an endemic area, the likelihood that Lyme disease is the cause of flu-like symptoms is low.[16] The IgM Western blot has in comparison to the IgG assay a slightly lower positive predictive value more than a month after start of the infection. The IgG may be true positive on the basis of remote, inactive infection and not an active infection. Western blot crude results and laboratory readings must be interpreted in the light of the clinical and epidemiological setting.

8.2 Use of the Western Blot Test to Detect Antibodies in Clinical Practice, Epidemiological Surveys, and Research

Because the Western blot is more expensive, more labor-intensive, and less suited to automation than EIA or equivalent technology, it is not generally used as the primary or first test to detect antibodies. Another problem is the usually qualitative and at best semiquantitative nature of Western blot analysis and the subjectiveness of reading band intensity. The reading of faint bands as positive or negative varies between individuals and laboratories. Band intensity may vary for technical reasons even in the same laboratory on different days. For this reason, the Western blot may be better suited to further characterize the antibody response to *B. burgdorferi* antigens in individuals who have tested "positive," "borderline," "equivocal," or "indeterminate" on tests performed by other methods, usually EIA or IFA.[8,17]

If a two-tiered strategy is implemented, the first, or screening test should be very sensitive, since the Western blot will be used as the more specific second test. However, the test systems are not independent of one another: both EIA and Western blot measure antibodies, and neither are direct detectors of infection, as is isolation of *B. burgdorferi* in culture or its detection by polymerase chain reaction. Theoretically, this two-step strategy provides for high positive predictive value for infection (at some time) but may not be definitive for current infection, nor is a negative result definitive for ruling out current infection.

There is less experience with use of the Western blot assay as a primary or sole test for antibodies to *B. burgdorferi* or to other pathogenic microorganisms. There is no reason why the Western blot in its present or modified form could not function well in this way, especially if the cost per test was comparable to that for EIA. Moreover, a primary Western blot assay may also have a role in clinical research, such as the evaluation of subunit vaccines against *B. burgdorferi.* In this case antibodies to the subunit may need to be identified in assessments of seroconversion. However, the sensitivity and specificity of a Western blot assay used in this way in clinical practice and epidemiological surveys would have to be equal or superior to the two-tier assay for this testing strategy to be acceptable for clinical practice. A stand-alone Western blot assay would likely require different criteria and better understanding of the immune response than at present.

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NCCLS consensus procedures include an appeals process that is described in detail in Section 9.0 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

M34-P: *Western Blot Assay for Antibodies to* Borrelia burgdorferi; *Proposed Guideline*

General

1. This document contains very valuable instructions for the performance of Western blot assays and of Western blots for antibodies to *Borrelia burgdorferi* in particular. We appreciate this effort very much since we ourselves are preparing a document on the microbiological diagnosis of Lyme borreliosis. The final draft of our document is currently being reviewed and will contain guidelines for interpretation of Western blots applicable for the European situation. We expect its publication during this year.

   The NCCLS document is focusing on "technical and interpretive considerations for use of Western blot assays for antibodies to *Borrelia burgdorferi* and other *Borrelia* species that cause Lyme disease." Additionally authors state "It also provides a framework for setting national or international guidelines for performing and interpreting Western blot assays detecting antibodies to *Borrelia burgdorferi*" and in chapter 2.2: "It is anticipated that the guidelines will also be appropriate for laboratories testing for antibodies to the other genospecies of Lyme disease agents." Thus, it is intended that the approved document would hold not only technical but also interpretive recommendations for all *Borrelia* species.

   We all know that the epidemiological situation is different in Europe. In contrast to the US, European human isolates belong to at least three species, *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*. It has been shown by various authors that immunoblot patterns are quite different depending on the strains used as antigen and that the immune response appears to be restricted to fewer antigens. In addition it has been recently shown that appropriate interpretation criteria must be established for the strain used as antigen. Thus the suggested criteria established for the *B. burgdorferi* s.s. strains and for American patients recommended in the NCCLS document cannot be simply transferred to the European situation.

   Therefore, we kindly ask you to inform the readers of the guidelines that "The suggested criteria in these guidelines are designed for use in North America and are not applicable in its present form for interpretation of Western blot results in Europe."

- **The subcommittee agrees in principle with this comment. There are potential differences in the interpretation of the Western blot for *Borrelia burgdorferi* in Europe and United States. Some of the interpretational differences may be developed out of scientific opinion rather than scientific fact. This is easily demonstrated in the US where scientific opinion still raises a controversy over the "correct" interpretation for Western blot. This guideline, however, is meant to outline a generic Western blot test method that has potential applicability across a number of pathogens, not just strains of *B. burgdorferi* that are endemic to North America. The committee recognizes that there are differences in interpretation in Europe and the US. As the differences in Western blot interpretation are harmonized, it is anticipated that this will be expanded upon in future revisions in M34.**

Summary of Delegate Comments and Responses

M34-A: *Western Blot Assay for Antibodies to* Borrelia burgdorferi*; Approved Guideline*

General

1. A diagram of the "blot" would be helpful.

- **Unfortunately, the subcommittee did not have access to a suitable diagram of a Western blot at the time M34-A was drafted. The subcommittee believes that adequate resources (textbooks, the Internet, etc.) are available to users of M34-A who are in need of this information.**

Section 4.3

2. Page 8: Storage temperature for IgM samples should be –70 °C rather than –20 °C is not adequate for IgM.

- **The subcommittee agrees with this comment. The text has been modified to read: "Serum and cerebrospinal fluid for IgM testing should be kept at –70 °C."**

Section 7

3. The definition of indeterminate Western blot needs to be expanded to include weak bands that subjectively cannot be clearly determined as positive compared to the positive control bank.

- **The subcommittee disagrees. The definition of an "indeterminate" Western blot band is very clearly differentiated from "borderline" in Section 7. "Indeterminate" reports are meant to apply to situations where high membrane background and/or suspected artifactual bands exist. Weakly positive bands, as outlined in Section 7, fall into the "borderline" category, not "indeterminate." The subcommittee, when addressing this issue, wanted to avoid any confusion between the two categories, and the terms are unambiguously defined in this manner.**

Related NCCLS Publications*

I/LA18-A **Specifications for Immunological Testing for Infectious Diseases; Approved Guideline (1994).** This guideline outlines specimen requirements; performance criteria; algorithms for the potential use of sequential or duplicate testing; recommendations for intermethod comparisons of immunological test kits for detecting infectious diseases; and specifications for development of reference materials.

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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