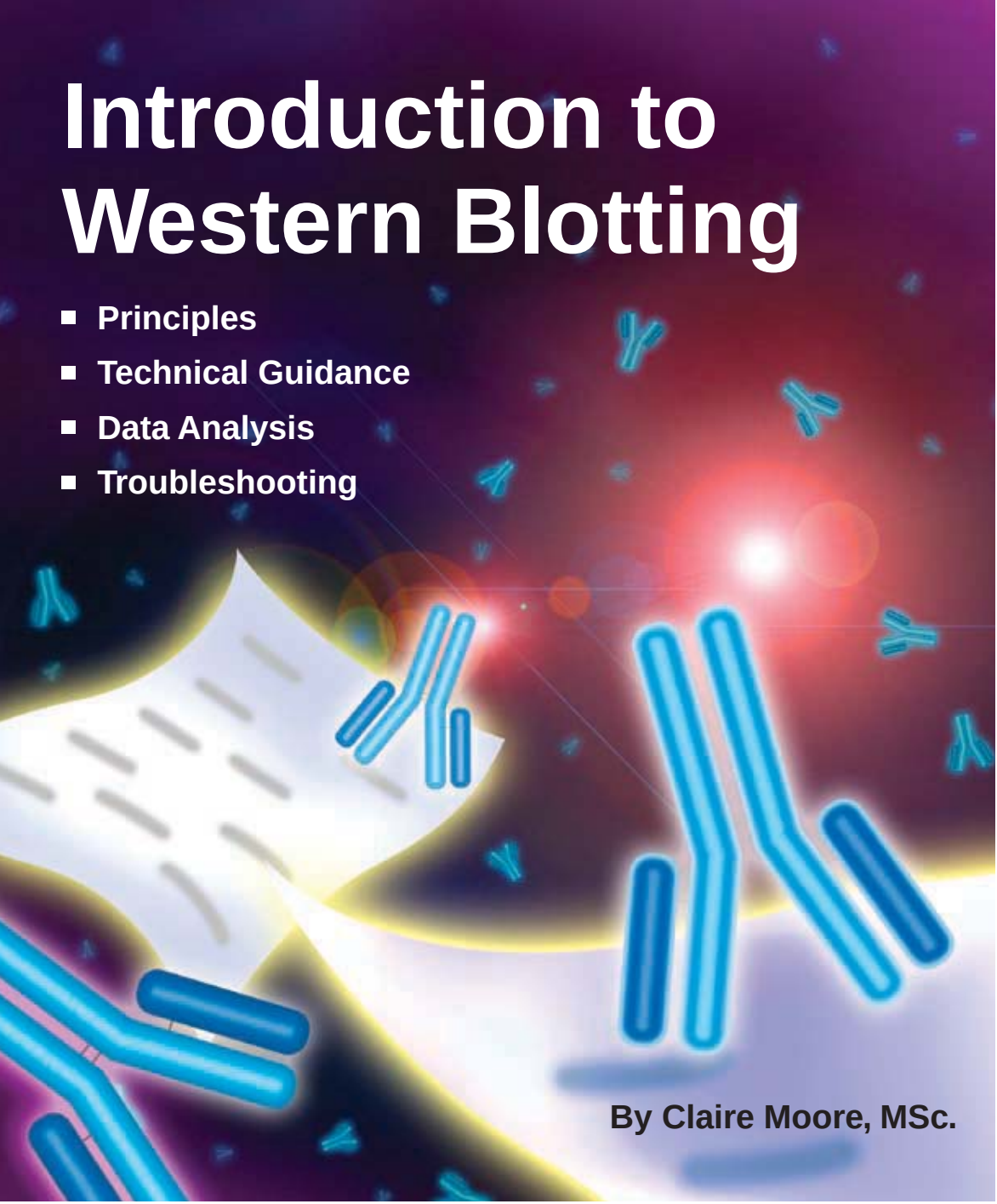
Introduction to Western Blotting

- Principles
- Technical Guidance
- Data Analysis
- Troubleshooting



By Claire Moore, MSc.

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Contents

Preface

Since Western blotting is one of the most prevalent laboratory procedures in use today, we have produced this handy reference guide to provide a basic overview of the key technical considerations that go into performing successful Western blots. Beyond basic theory, we have included some typical experiments, as well as troubleshooting advice and time-saving pointers to make things go more smoothly. We hope you will find this manual useful, and would greatly appreciate your feedback to **marketing@abdserotec.com.**

For those who are not already familiar with our products, we specialize in antibodies, and have been manufacturing high-quality immunological reagents for over 25 years. As a result we think a lot about antibodies, not just how they are created, produced and used, but also what is so special about them. If you can imagine the difficulty of finding a single needle in haystack, you can begin to appreciate the task accomplished by the antibody as it successfully finds its corresponding antigen amongst the collection of other potential targets present in cellular materials.

If you are choosy about your antibodies and want to find a perfect match for your experiment, then please give us a try. We focus on antibody production in small and large scale across a broad range of research areas, and for a variety of applications. We are so confident in our antibodies and in our ISO-certified production facilities, that we offer a performance guarantee for all the applications listed on our detailed datasheets. We are also available to help, offering technical support from trained scientists experienced in the use of antibodies. We can address queries that arise while carrying out your experiments, and can also assist in selecting the proper antibodies and controls. Please contact our technical team at **tech.uk@abdserotec.com**.

Finally, if you do find yourself without access to the antibody you are seeking, remember that we are able to identify new specificities using the latest technology with our parent company MorphoSys. Our HuCAL® recombinant antibodies are identified by screening over 45 billion potential specificities using highly sophisticated techniques to produce new monoclonals faster than with traditional methods.

Please visit us at **www.abdserotec.com/westernblot** to learn more about our Western blotting products, and to search our catalog of over 14,000 antibodies.

Introduction

Chapter 1

Overview

Western blotting, also known as immunoblotting or protein blotting, is a core technique in cell and molecular biology. In most basic terms, it is used to detect the presence of a specific protein in a complex mixture extracted from cells. The Western blotting procedure relies upon three key elements to accomplish this task: the separation of protein mixtures by size using gel electrophoresis; the efficient transfer of separated proteins to a solid support; and the specific detection of a target protein by appropriately matched antibodies. Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film, or an imaging system.

Since Western blotting is accomplished rapidly, using simple equipment and inexpensive reagents, it is one of the most common laboratory techniques. The results achieved are also easy to interpret, unique, and unambiguous. Therefore, it is routinely used on its own, or along with other immunoassays, in research and clinical settings. An overview of the technique is shown in the diagram below:

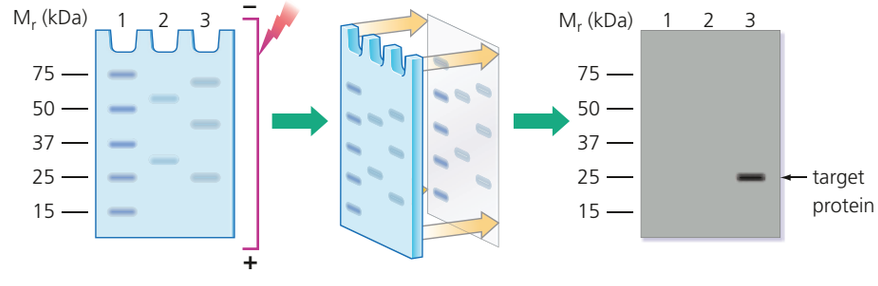


FIGURE 1 **Overview of Western Blotting.** Separation of protein mixtures by electrophoresis; transfer to a blotting membrane; and detection of target protein, which only becomes visible in the final stage as a band similar to that shown in lane 3. **Lane 1:** Prestained molecular weight standards. **Lanes 2&3**: Protein mixtures.

Why Use a Western Blot?

Western blotting is an extremely powerful technique, despite its overall simplicity, because it provides additional information not readily gathered from other key immunological laboratory techniques. Since proteins are separated by size during the gel electrophoresis stage, and then detected by a specifically directed antibody, the procedure essentially confirms the identity of a target protein. Furthermore, when data does not match expectations, there may be clues as to what should be investigated to determine the reason. Is the band of lower or higher molecular weight than expected? Is there a single band, or are there several bands? A smaller than expected band could indicate that the protein has been cleaved or is degraded. Conversely, when bands are seen at higher levels than expected, this may indicate an actual increase in mass due to glycosylation or multimer formation. Alternate splicing may also cause unexpected size variations, as may the particular combination of charged amino acids found in the protein.

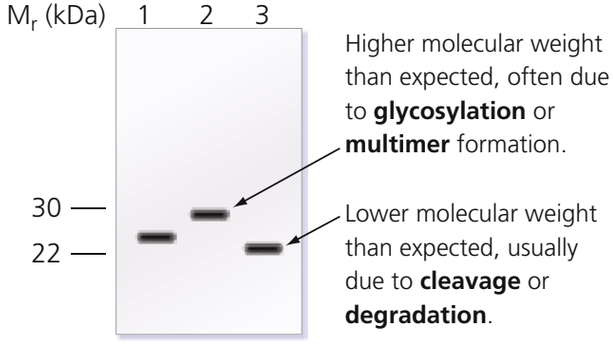


FIGURE 2 **Expected vs. Actual Molecular Weight.** **Lane 1:** Target protein band at the expected molecular weight. **Lanes 2&3:** Common alterations that modify the molecular weight.

Western blots are in wide use across a broad range of scientific and clinical disciplines. Their ability to clearly show the presence of a specific protein both by size and through the binding of an antibody makes them well-suited for evaluating levels of protein expression in cells, and for monitoring fractions during protein purification. Likewise, they are helpful for comparing expression of a target protein from various tissues, or seeing how a particular protein responds to disease or drug treatment.

In many cases, Western blots are used in combination with other key antibody based detection techniques, such as ELISAs or immunohistochemistry. In these instances, Western blots provide confirmation of results both in research and diagnostic testing. For example, with HIV and prion disease, Westerns are used as a key supplemental screen since their results are less ambiguous, and quicker, than other methods. Moving forward, Western blots continue to be of value in confirming results from antibody arrays, making them suitable for use in proteomics research. Specific results and data analysis will be addressed in further detail in Chapter 4.

Antibody Considerations

One of the critical features of any successful Western blot is the highly specific interaction between an antibody and an antigen. The **antigen**, usually a protein or peptide, is the target of the antibody. The precise point of interaction is between a small region of the antigen, an **epitope**, and the recognition sites found on the arms of the antibody molecule.

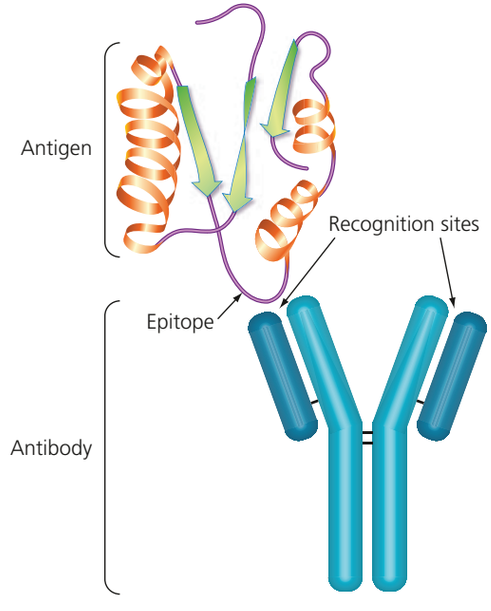


FIGURE 3 **Antibody-Antigen Interaction**

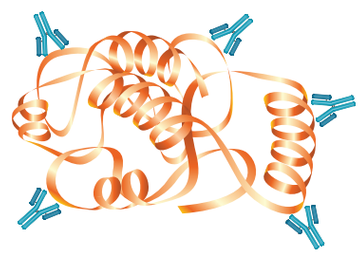
Antibodies selected for immunodetection should be Western blot tested if possible, with attention paid to the experimental conditions recommended by the antibody supplier. Usually, Western blot positive antibodies recognize a short linear sequence of amino acids found within the target protein that remains intact, or becomes visible, when the target protein is fully unraveled. This is because most Western blots are carried out under denaturing and reducing conditions which remove all higher order protein structure. In contrast, some epitopes can be conformational, forming a three-dimensional structural configuration of amino acids that will be lost upon denaturation of the protein. Thus, not all antibodies work in a typical Western blot. Since Western blot procedures allow for flexibility in choosing gel electrophoresis and blotting conditions, it is possible to modify buffers to retain enough higher order protein structure for detection by some antibodies. The antibody datasheet should indicate which buffer conditions are best-suited to the particular antibody-antigen interaction.

Monoclonals vs. Polyclonals

The antibodies used to detect the target protein in a Western blot will be either monoclonal or polyclonal. Both types of antibody are typically created when an antigen, usually a protein or peptide, is injected into an animal and its immune system responds by producing antibodies specifically targeted against that antigen (or more precisely to various epitopes found on that antigen). Polyclonal antibodies consist of a mixed pool of immunoglobulin molecules that bind to several different epitopes found on a single antigen. Polyclonals are usually produced in rabbits, donkeys, sheep, and goats, and are purified from serum.

In contrast, monoclonal antibodies bind to a single epitope within a target antigen. They are composed of homogeneous cloned immunoglobulin molecules, rather than the heterogeneous antibody mixture typical of polyclonals. Monoclonals are made by fusing antibody producing cells from the spleen of the immunized animal (usually a rat or mouse) with an immortalized cell line to produce single specificity antibodies that can be purified from tissue culture supernatant. Both monoclonals and polyclonals are used in Western blotting, and offer various advantages and disadvantages that are summarized in the accompanying table.

Comparison of Monoclonal and Polyclonal Antibodies

Monoclonal	Polyclonal
Specificity for a **single** epitope. 	Varying specificities to **multiple** epitopes. 
Identifies whether a particular region of a protein is present.	Identifies the entire target protein via binding at multiple sites. Since multiple epitopes are targeted, there is a higher likelihood of detection of the target.
May cross-react with other proteins that share this epitope, such as isomers or common motifs.	Higher background and cross-reactivity possible due to detection of multiple epitopes, any of which may be shared by related proteins.
Usually less sensitive since only a single antibody molecule binds to each target.	More sensitive because signal is amplified through the binding of several antibodies per target.
More expensive to produce initially, but available in an unlimited supply.	Less expensive to produce initially, but supply is limited to immunized animal(s). There will be greater variability between preparations.

Genetically Engineered Antibodies

In addition to traditional monoclonal and polyclonal antibodies targeted against specific proteins, there are other means of antibody generation and protein detection available as the result of numerous advances in genetic engineering technology.

It is now possible to create and produce antibodies using fully *in vitro* techniques such as phage display in conjunction with highly complex libraries which represent the vast array of potential antibody binding regions. At AbD Serotec, we routinely produce human recombinant monoclonal antibodies for research, clinical, and diagnostic applications with our **HuCAL® Technology**.

Epitope Tags

If there are no antibodies available to the protein of interest, it is still possible to carry out a range of immunodetection techniques, including Western blotting, by using epitope tags and matched epitope tag antibodies.

This elegant strategy works by adding a small sequence of DNA that codes for a known antigenic epitope during cloning of the protein of interest. Since matched antibodies already exist that will specifically bind to this epitope, the target protein can be detected because it also expresses the appropriate epitope. Therefore, immunodetection can be carried out quickly and without the need to wait for the generation of unique antibodies to a newly identified target protein. This technology is also of significant benefit when working in organisms where few specific antibodies are readily available. One downside to this technology is that the target protein is altered by the addition of tag, and thus it is not identical to native forms of the protein.

There are a wide variety of epitope tag antibodies available, including: His-6 (MCA1396), V5 (MCA1360), c-myc (MCA2200), and others which are supplied by AbD Serotec. Epitope tag antibodies are available with a range of common antibody labels allowing one to switch experimental techniques or detection systems without having to modify the target protein. In the Western blots shown below, myc-tagged KSR (Kinase Suppressor of Ras) is detected through the use of either an anti-myc antibody or an anti-KSR antibody.

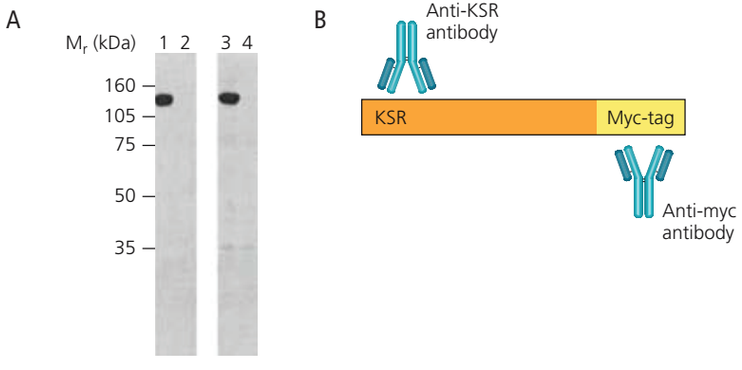


FIGURE 4 **Western Blot and Model of Myc-tagged KSR.** **PANEL A:** Myc-tagged murine KSR expressed in COS cells is detected using either anti-KSR (MCA2106) in lanes 1&2, or anti-myc epitope (MCA2200) in lanes 3&4. **Lanes 1&3**: myc-KSR vector. **Lanes 2&4**: vector alone control. Note that an identical band is produced with both antibodies. **PANEL B:** KSR target protein with c-myc epitope tag. KSR-myc fusion is detectible by both anti-KSR antibodies and anti-myc antibodies.

Samples, Gels, and Blotting

Chapter 2

Overview

A typical Western blot, or immunoblot, relies upon a purified, semi-purified, or crude extract of cellular proteins containing a target protein that can be detected by antibodies. Several key steps are required to take the sample from the cellular starting point to a detectible band on a Western blot. This chapter focuses on the preparative stages that are accomplished prior to immunodetection by antibodies. Throughout these processes it is essential that the cellular protein is prepared and stored carefully, since this will significantly impact the experimental results.

The three key preparative stages are:

- **Sample production** by lysis or homogenization to solubilize and release cellular proteins.
- **Separation** of protein mixtures using gel electrophoresis.
- **Transfer** of separated proteins to a blotting membrane which can be manipulated more easily than a gel.

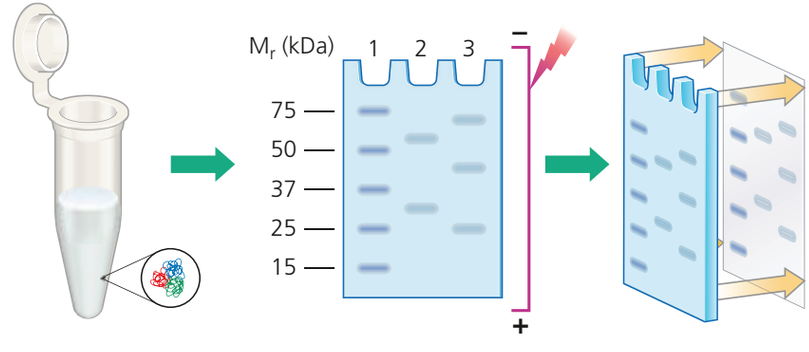


FIGURE 5 **Sample Preparation, Electrophoresis, and Transfer**

Western blots are effective in detecting low nanogram to low picogram amounts of target protein, depending on the antibodies used and the detection substrate chosen. If the target is suspected to be of very low abundance, or if there is no detectible signal on the blot, then it may be necessary to concentrate, immunoprecipitate, or fractionate the starting material.

Sample Preparation

Cell Lysates

Crude cellular lysates are the most common direct source of starting material used in Western blotting. They can be prepared from immortalized cell lines known to express the target protein, or from transfected cells carrying a protein expression vector. Many different cell types (mammalian, insect, yeast, or bacteria) can be used to supply the protein needed with slight variations in the preparation procedure. In most cases, the cells are harvested, washed, and lysed to release the target protein. For best results, all these steps should be carried out in a cold room, or on ice. This will minimize proteolysis, dephosphorylation, and denaturation, since all begin to occur once the cells are disrupted. It is possible to simply lyse cells directly in gel loading buffer if a quick check is all that is required. However, sonication may be neccessary to disrupt the highly viscous cellular DNA. Usually, 20-50 µg of cellular lysate is loaded per lane for gel electrophoresis.

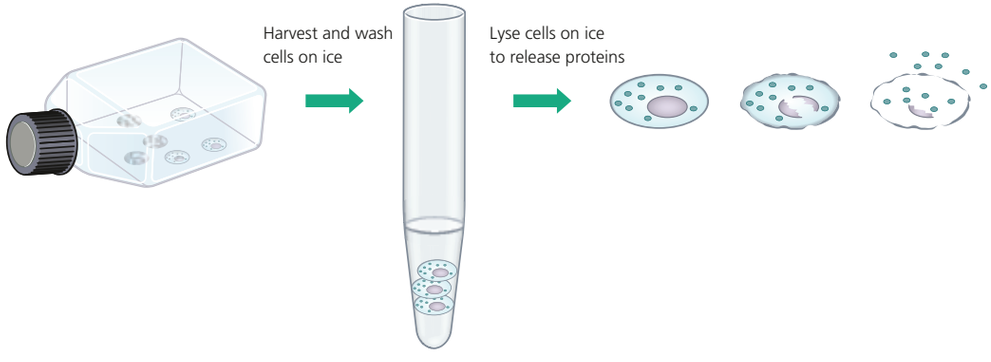


FIGURE 6 **Cells are Harvested, Washed, and Lysed to Release Proteins**

Choosing the proper lysis buffer and determining an appropriate volume is often a trial and error process that is affected by the type of protein being isolated as well as the particular cells used as a source. Lysis buffers vary from very gentle ones with no detergent to harsher solutions such as RIPA (Radio Immuno Precipitation Assay) buffer, which is denaturing and contains multiple detergents. Typically, NP-40 (Nonidet P-40) lysis buffer, with a milder non-ionic detergent, is used for the isolation of soluble cytoplasmic proteins. At other times, RIPA buffer is chosen because it reduces background, and because sometimes multiple detergents are required to fully release membrane bound or nuclear proteins. Consideration should also be given to the antibody-antigen interaction which may be affected by the changes to the target protein during lysis.

The amount of lysis buffer is determined based upon a cell count, or else it is estimated based upon the size of the tissue culture vessel. The accompanying table provides some suggested starting points. Sometimes mechanical disruption, such as with sonication or dounce homogenization, is required to fully release proteins from certain cell and tissue samples. Sonication is also used to break down cellular DNA which can interfere either due to its high viscosity or via non-specific binding. After lysis and centrifugation, the amount of protein in each lysate is measured.

Cell Lysis Volume Recommendations

Type of cells	Amount of material	Volume of lysis buffer
Tissue Culture	10^7 cells or 100 mm dish	1 ml
Whole Tissue	100 mg	Add 2 ml and sonicate or dounce homogenize
Bacteria	Spin sample, estimate volume	Add 10 volumes and vortex
Yeast	Spin sample, estimate volume	Add 10 volumes, then sonicate or vortex with glass beads

Tissue Samples

Tissue samples display a higher degree of structure than cultured cells and thus may require higher levels of mechanical intervention in order to release the protein of interest. They may also contain multiple cell types which are differentially responsive to the lysis buffer chosen. Smaller solid tissue samples (up to 100 mg) are placed in ice cold extraction buffer and homogenized on ice, usually with sonication or a douncing rod to facilitate cellular disruption. Alternatively, and more often with larger tissue samples, a blender is used to homogenize the tissue in PBS, and then cell lysis buffer is added. Once the tissue has been homogenized and lysed, the solubilized cellular components are clarified by centrifugation and tested for protein concentration prior to loading on a gel.

Purified or Semi-purified Extracts

The simplest source of starting material for Western blotting is purified or semi-purified protein samples that are produced in the course of protein purification. These samples rarely require any further manipulation and are simply mixed with gel electrophoresis loading buffer (Laemmli sample buffer). When using a purified or semi-purified protein preparation, it is possible to load a much smaller amount of total protein onto the gel. Usually 0.5-1 μg of purified or semi-purified protein is sufficient to observe a strong signal. If unsure, results can be improved by loading several dilutions of the sample.

Determining Protein Concentration

To ensure that samples are in the proper range of detection for the assay, and so they can be compared on an equivalent basis, it is important to know the concentration of total protein in each sample. There are various methods available for determining protein concentration using in-house or commercially supplied kits and reagents. The simplest method entails measuring the absorbance of the lysate solution at 280 nm or 205 nm. Alternatively, several protein assays are available which rely upon the reduction of metal ions by the peptide bond, e.g. the Lowry and BCA assays; or by dye binding, as with the Bradford assay. In all instances, a color change results that is proportional to the amount of protein in the sample. Protein concentration is determined by comparison of the target samples to a known standard, such as BSA (Bovine Serum Albumin) diluted in lysis buffer. To get the most accurate measure of protein concentration, it is advisable to test a few dilutions of the sample ensuring that the results lie in the linear range of the protein assay.

Loading Buffer

Once the protein concentration has been determined, samples are diluted in gel loading buffer, also called 2x Laemmli sample buffer. This buffer contains glycerol so that the samples sink easily into the wells of the gel, and a tracking dye (bromophenol blue) which migrates through the gel first to indicate how far the separation has progressed. For most routine Western blots, SDS (sodium dodecyl sulfate) and a reducing agent are also present in the gel loading/sample buffer to fully denature the protein and remove all higher order structure. See the accompanying table for the range of loading buffer options and the supplements they contain. Samples are heated in gel loading/sample buffer for either 5 minutes at 100°C, or 10 minutes at 70°C to aid in the denaturation. At this point, samples can remain at room temperature if they are to be used immediately, or placed at 4°C or -20°C for later analysis.

Sample Buffer Conditions

Gel Conditions	SDS	DTT or βME	Comments
Denaturing & Reducing	+	+	Removes all higher order structure, including disulfide bonds
Denaturing	+	-	Higher order structure is disrupted, but disulfide bonds are retained
Reducing	-	+	Disulfides are removed, but most higher order structure remains
Native	-	-	The protein retains higher order structure. Multimers and protein-protein interactions can be detected

Controls and Standards

It can be very useful to include a positive and a negative control on the gel along with the samples that are being evaluated. For a positive control it is typical to use a known source of target protein, such as purified protein or a control lysate, under conditions where it will be detectible by the antibody used in the experiment. The positive control is important for confirming the identity of the target since it will produce a reference band on the blot showing the expected migration of the target protein and confirming the activity of the antibodies. Positive controls are also helpful for troubleshooting, and for comparing the data between separate blots. Premade cellular and tissue lysates are now commercially available from a number of suppliers, including AbD Serotec, and can be used as a convenient positive control. In addition, commercially supplied tissue and cell lysates are suitable for troubleshooting protocols. If possible, it is also helpful to include a negative sample control, such as known null cell line, as a means of confirming that the signal is specific to the desired protein.

The final component required for the gel is a molecular weight standard since a key feature of Western blotting is to provide information on the size of the protein. Also known as molecular weight markers, these are premade mixtures of proteins with known molecular weights, usually 5-6 proteins spanning the range from 10 kDa to 200 kDa. Molecular weight standards come in a variety of formats, including unstained, prestained, multi-colored, or directly labeled for Western detection. They are an excellent means of monitoring progress while the gel is running, of checking transfer efficiency, and for orienting the immunoblot. Care should be taken not to overload the standards since they may obscure signal from the target protein.

Gel Electrophoresis

After the samples have been prepared, they are separated by size using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Since the samples have been denatured in gel loading buffer containing SDS detergent, the protein is uniformly negatively charged and will now migrate in an electric field through the gel and towards the positive electrode. Since the charge to mass ratio is equalized by the binding of SDS consistently along the length of the proteins, and higher structure has been removed, the proteins will be separated primarily by size. The key is to effect a separation such that the target protein will be properly resolved from the other components of the mixture. This makes it possible to clearly identify the target protein later through immunodetection with a specific antibody.

Most often the gel is made and run under denaturing conditions (25 mM Tris base, 192 mM glycine, 0.1% SDS, at pH 8.3). However, alternative gel conditions can be used, depending upon the protein under investigation and the aims of the experiment. Options include: non-denaturing, non-reducing, native, two-dimensional separations (by size and by isoelectric point), and buffer variations that are more suited to the separation of smaller or larger proteins. It is important to make sure that the buffer used is compatible with later steps in the procedure and the blotting materials.

SDS-PAGE gels (commercially supplied or made in-house) usually consist of a main gel, which is poured between two glass or plastic plates, and which is sometimes topped by a short stacking gel. Gels can be made with a uniform acrylamide percentage, or with a continuously varying gradient that yields improved resolution over a broader range of molecular weights. See the table below for some common gel percentages and their separation ranges.

Polyacrylamide Gel Percentage Separation Ranges

Acrylamide Concentration (%)	Separation Range (kDa)
5	60-210
7.5	35-95
10	15-70
15	4-45
4-12 gradient	5-200
4-20 gradient	4-200
10-20 gradient	3.5-110

The percentage and the thickness of the gel will impact the transfer of proteins out of the gel in the blotting phase, so using a thinner gel, or a lower percentage of acrylamide, may improve transfer results.

Once the gel sets, it is placed into the running apparatus. Small volumes of protein (5-20 µl) dissolved in gel loading buffer are added to each individual well. The gel is then connected to a power supply and allowed to run for a few hours in a buffer tank to separate the proteins. If the gel is run at too high a voltage it will overheat and distort the bands.
The accompanying gel shows cellular lysates which have been well-separated on a gradient gel, and stained with Coomassie dye to visualize all the separated protein bands.

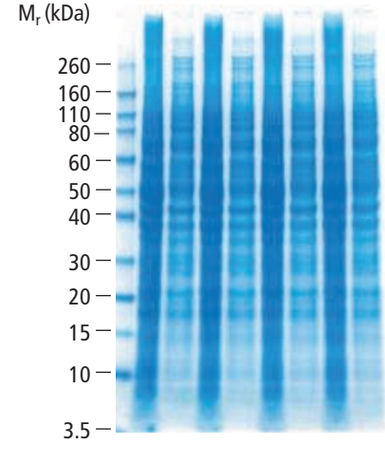


FIGURE 7 **HeLa Cell Lysates (LYS001) Separated on a 4-12% Gradient Gel**

Blotting

Following gel electrophoresis, the separated protein mixtures are transferred to a solid support for further analysis. While it is possible to use diffusion or vacuum assisted transfer, electroblotting *(Towbin et al., 1979)* is the method relied upon in most laboratories, due to the speed and efficiency of transfer. The electric field used for the transfer is oriented perpendicular to the surface of the gel causing proteins to move out of the gel and onto the blotting membrane, which sits between the gel surface and the positive electrode. The gel and blotting membrane are assembled into a sandwich along with several sheets of filter paper which protect the gel and blotting membrane and help to ensure close contact between their surfaces. It is imperative that the membrane is placed between the gel and the positive electrode so that the negatively charged proteins migrate from the gel onto the membrane. The transfer buffer used for electroblotting is similar to gel running buffer with the addition of methanol which helps proteins bind to the blot.

Electrophoretic transfer can be accomplished under wet or semi-dry conditions. In a wet transfer, the gel/blotting paper/filter paper sandwich is placed into a cassette along with protective fiber pads. The cassette is then immersed in a buffer tank and subjected to an electrical field. With semi-dry transfer, the gel/blotting paper/filter paper sandwich is assembled on large electrode plates which generate the electric field, and buffer is confined to the stack of wet filter papers.

Transfer times vary from 1 hour (semi-dry transfer) to several hours or overnight (wet transfer). Wet transfer is usually considered to be more reliable because it is less likely to dry out the gel, and is often preferred for larger proteins. Since there will be significant variation in the chosen transfer system, it is best to consult the manufacturer of the equipment used for specific instructions.

Blotting Membranes

The solid support onto which the separated proteins are transferred is usually of two types, nitrocellulose or polyvinylidene fluoride (PVDF) membrane, both of which bind proteins with high affinity. Nitrocellulose has been in use for a long time, and is sometimes preferred because of its excellent protein binding and retention capabilities. However, nitrocellulose is brittle and thus it is usually less effective when blots need to be reused. PVDF demonstrates superior mechanical strength making it suitable for stripping/reprobing and for further protein characterization techniques, such as sequencing and proteolysis Sometimes higher background staining is seen with PVDF membranes, and extra care must be taken to prevent this from occurring.

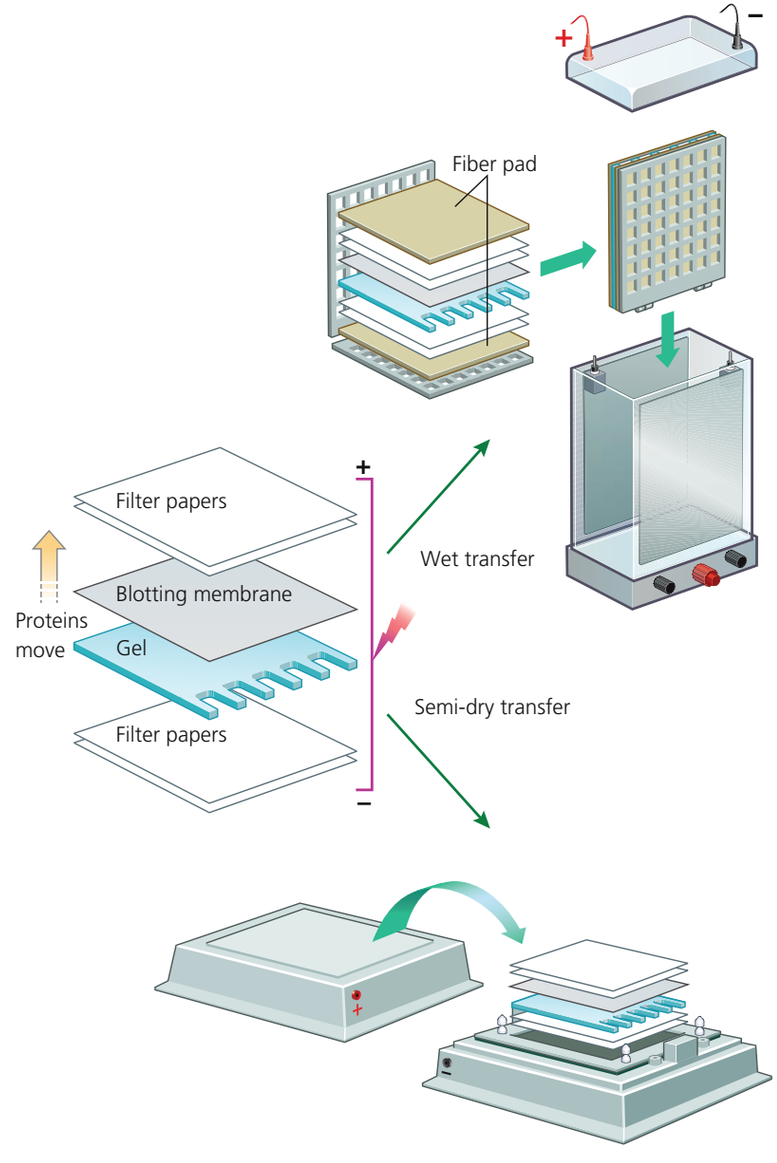


FIGURE 8 **Western Transfer Methods**

Setting Up the Transfer

While the gel equilibrates in transfer buffer, the blotting membrane is prepared. Often the blotting membrane is supplied precut to size, or else it is precisely cut to match the size of the gel. Since oils present on the hands will interfere with signal on the blotting membrane, it is advisable to wear gloves while handling the blotting membrane. Usually a corner is marked for later orientation, though this is not necessary when using pre-stained molecular weight markers in one of the side lanes. Prior to assembly in the transfer apparatus, the blotting membrane is prewetted. Nitrocellulose is prewetted by floating and then immersing in dH_20 or transfer buffer, and PVDF is prewetted in methanol. Both types of membrane are then soaked in transfer buffer and applied directly to the surface of the gel.

It is extremely important that no air bubbles are allowed to remain between the gel surface and the blotting membrane, since air bubbles will disrupt the transfer of any proteins in that area distorting the results. Air bubbles can be easily removed by pushing gently across the surface with a spatula or rolling with a pipet. It is wise to double check the orientation of the gel and blotting membrane with regard to the positive electrode so that the proteins migrate in the proper direction.

Size of the Target Protein

The size of the target protein should be considered when choosing transfer conditions. Generally, smaller proteins will transfer out of the gel faster, and may actually transfer through the blotting membrane into the filter papers beyond. Blotting membranes with a smaller pore size can be used for small proteins and peptides, and SDS can be reduced or eliminated from the transfer buffer to improve binding to the membrane. If there is a suspicion that the protein is transferring through the membrane, then a second membrane can be included behind the first to catch proteins that migrate through.

In contrast, large proteins can be slower to elute and may be retained within the gel, so overnight wet transfer is usually preferred. *Bolt et al.* have developed buffer conditions which improve transfer efficiency across a range of protein sizes, including very large proteins.

Confirming the Transfer

Once the blotting step has been completed, the apparatus is carefully disassembled and the success of the transfer is evaluated. The simplest method of confirming the transfer involves noting the appearance of prestained markers on the blot as compared to the gel. However, this is only a crude measure since it provides results for a single lane. A more reliable method of confirming transfer is through the use of a reversible stain which identifies the presence of protein bands directly on the membrane. Ponceau S, a red stain applied in an acidic solution, is typically relied upon for this purpose because it is compatible with all types of immunodetection labels and substrates. The procedure is very simple to use and yields results in about 10 minutes. When unlabeled molecular standards are used, it is necessary to mark the position of the molecular weight standards directly on the blot when they become visible with the Ponceau S dye since they will be needed for reference at a later point.

The only negative aspect of Ponceau S staining is that it doesn't always produce a good photographic record because the bands can be diffuse. In the accompanying stained blot, it is possible to see differing quantities of cell lysates that have been transferred along with multi-colored molecular weight markers at the far left.

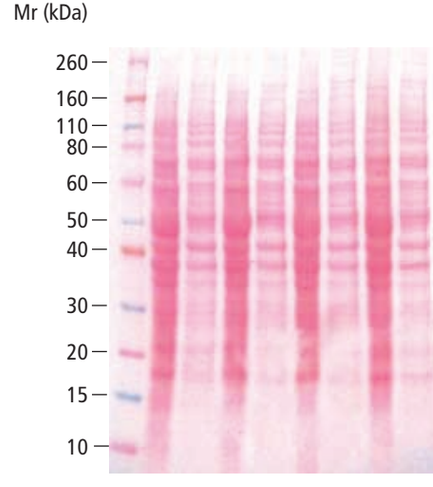


FIGURE 9 **Ponceau S Stained Western Blot of HeLa Cell Lysates (LYS001)**

There are several alternative methods that can be used in place of Ponceau S staining. One option is to use a metal ion stain, such as copper, or metal chelate stain. Another option is to stain the blot with India ink, which is very sensitive, inexpensive, and reliable. However, since India ink creates a permanent record it will obscure the signal from colorimetric detection reagents. Coomassie Blue stain is not typically used directly on a Western blot prior to immunodetection; instead, it is sometimes used to stain the gel after transfer in order to identify what remains behind.

Immunodetection

Chapter 3

Overview

After blotting, the target protein will be detected using appropriately matched and labeled antibodies. The typical immunodetection stage involves a few basic steps:

- **Blocking** - The blot containing the transferred protein bands is incubated with a protein or detergent solution which covers the entire surface so that antibodies do not bind non-specifically to the membrane.
- **Antibody incubation** - Labeled antibody binds to the target protein band present on the blot in a one-step or two-step procedure.
- **Detection with substrate** - The label attached to the antibody, usually an enzyme such as HRP (Horseradish Peroxidase), is detected using a substrate which produces a visible signal corresponding to the position of the target protein.

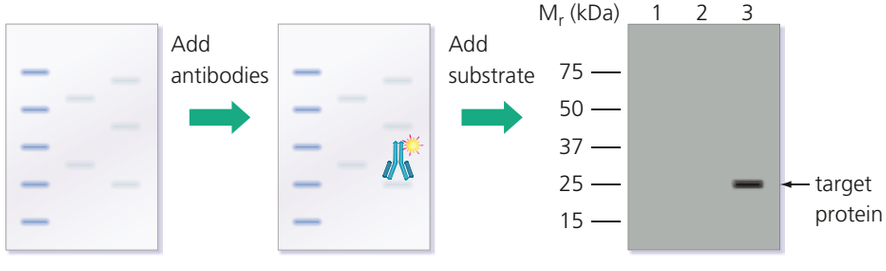


FIGURE 10 **Immunodetection Overview.** The Western blot is blocked, incubated with antibodies, and treated with substrate to make the target protein visible. Wash steps are carried out between incubations to remove excess unbound material and to minimize non-specific signal on the immunoblot.

Blocking

Blocking is a very important step in the immunodetection phase of Western blotting because it prevents non-specific binding of antibody to the blotting membrane. The most commonly used blocking solutions contain 3-5% BSA or non-fat dried milk (also known as Blotto or BLOTTO) in a solution of PBS (phosphate buffered saline) or TBS (tris buffered saline). Often, a small amount of Tween®20 detergent is added to blocking and washing solutions to reduce background staining, and the buffer is known as PBST or TBST. When choosing between these buffers, it is important to note that TBS/TBST is preferred with AP (Alkaline Phosphatase) labeled antibodies because PBS will interfere with the AP signal.

Non-fat dried milk is considered to be a good starting point when selecting a blocking solution because it is inexpensive and in very wide use. However, milk proteins are not compatible with all detection labels, so care must be taken to choose the appropriate blocking solution for the antibodies, buffers, and detection reagents. For example, BSA blocking solutions are preferred with biotin and AP antibody labels, and anti-phosphoprotein antibodies. This is because milk solutions contain casein, which is itself a phosphoprotein, and biotin, thus it will interfere with the assay results. Commercially supplied blocking solutions, such as Block Ace (BUF029) from AbD Serotec, are very convenient to use and can improve consistency of results, especially when non-specific background signal is an issue. After blocking, the blot is rinsed in wash buffer, usually TBST, with gentle agitation and in sufficient volume to keep the blot submerged. Please refer to Chapter 5 for detailed protocols.

Antibody Incubation

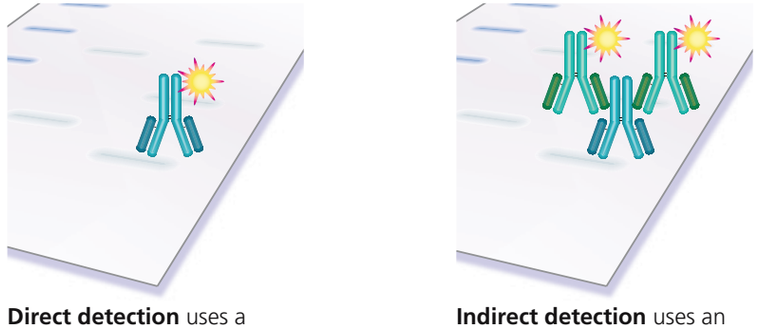
After blocking and washing, the blot will be incubated in a dilute solution of antibody, usually for a few hours at room temperature or overnight at 4°C. The antibody is diluted in wash buffer (PBST or TBST) or a diluted blocking solution, the choice depends upon the antibody. At AbD Serotec, we offer a HISPEC assay diluent (BUF049A) which can be used with primary and or secondary antibodies to reduce cross-reactivity and minimize non-specific binding. Since antibody preparations vary in their levels of purity and specific binding properties, there will be differences in the level of dilution required. For example, purified antibodies are usually diluted to a 1-10 μg/ml final concentration. The manufacturer's datasheet should provide dilution recommendations for a particular preparation. However, it is best to test a range of dilutions with each new antibody, optimizing conditions for the samples under consideration. Dot blots, slot blots, or test blots (see the end of this chapter) can be used for checking various

antibody concentrations. It is critical that all immunodetection steps (blocking, antibody incubation, substrate incubation, and all intervening washes) have a sufficient volume and gentle agitation to keep the blot evenly exposed to the reagents without drying throughout the process.

Indirect vs. Direct Detection

Antibody detection of the target protein is accomplished using a one-step or two-step protocol. The one-step procedure, **direct detection**, relies upon a single antibody which has been covalently joined to an easily detected label molecule (biotin, an enzyme, or a fluorescent dye). Labeled primary antibodies can be ordered directly from AbD Serotec or other commercial antibody suppliers. In addition, it is possible to directly label an antibody by using a commercially supplied labeling kit such as our **LYNX Rapid Conjugation Kits®**, or with in-house reagents.

With **indirect detection**, two different antibodies are used in sequence for the detection step. First, the Western blot is incubated with an unlabeled primary antibody directed against the target protein. After washing, a labeled secondary antibody is used to detect the presence of the primary antibody, and thus the target protein. The labeled secondary antibody is typically directed against the immunoglobulin class or subclass of the primary antibody's species. For example, one of our popular secondaries is STAR88P, a purified donkey antibody raised against goat/sheep immunoglobulin (IgG) which is coupled to an HRP label. Biotinylated primary antibodies also require a two-step detection procedure; however, the second step involves incubation with streptavidin, a bacterial protein, conjugated to HRP (or AP), rather than with a labeled antibody.



Direct detection uses a labeled primary antibody to identify the target protein.

Indirect detection uses an unlabeled primary followed by labeled secondary antibodies.

FIGURE 11 **Direct vs. Indirect Detection**

Comparison of Direct and Indirect Detection Methods

Direct Detection	Indirect Detection
Advantages	*Advantages*
▪ Faster overall, since there are fewer steps. ▪ Less chance of non-specific signal.	▪ Often gives a stronger signal because multiple secondary antibodies bind to each primary antibody. ▪ Easy to change label type or detection methods for a new experiment by swapping secondaries. ▪ Saves labeling time and expense, especially when all primary antibodies are made in the same species. ▪ Provides access to a wider range of labels.
Disadvantages	*Disadvantages*
▪ Coupling of label to the primary antibody may affect the antibody's ability to bind to the target protein. ▪ Labeling every primary antibody adds time and cost.	▪ More non-specific signal can arise from the binding of the secondary antibody to other proteins on the blot. ▪ Extra incubation and wash steps add time to the experiment.

Detection with Substrate

Now that the target protein has been specifically tagged with an appropriately labeled antibody, and excess antibody has been washed away, the label will be used to identify the location of the target protein on the blot. Some labels can be detected immediately, without any further processing. Fluorescent tags, for example, simply require the right equipment to observe and record the fluorescent signal. In Figure 12, a Western blot is probed with an anti-tubulin primary antibody and a DyLight® 800 (STAR117D800) secondary antibody. It is then detected using the LI-COR® Odyssey Infrared Imaging System. Note here that the molecular weight standards at the far left are labeled to produce a red signal allowing them to be easily distinguished from the samples. This highlights one of the advantages of fluorescent labels, which is that multiple labels can be seen on the same blot in the same experiment.

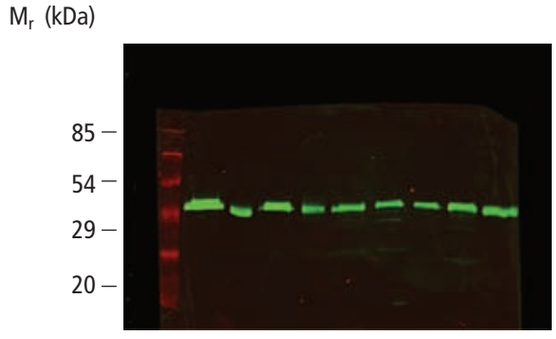


FIGURE 12 **Western Detection of Tubulin using STAR117D800**

The most common antibody label used in Western blots is HRP, a small, stable enzyme with high specificity and rapid turnover. HRP is deactivated by sodium azide, so it is imperative that no azide is present in the blocking, dilution, or washing solutions. At AbD Serotec we offer a specialized buffer for the long term storage and dilution of HRP labeled antibodies, BUF052A, which can be used with all antibodies except those generated against rabbit immunoglobulins. The HRP label is detected when it is exposed to a substrate solution in the final step of the immunodetection procedure. Substrate solutions for Western blotting are chemical reagents that are acted upon by the enzyme to yield a signal that can be easily measured. HRP label is typically detected with either colorimetric or chemiluminescent substrates.

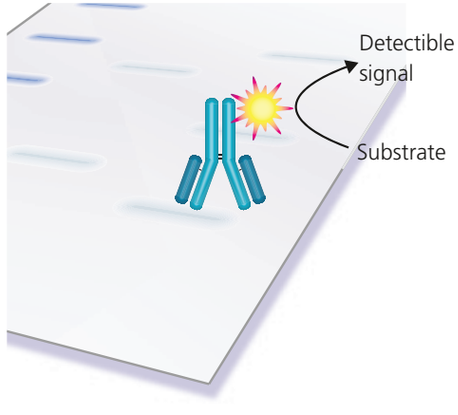


FIGURE 13 **Detection of the Antibody Label.** The antibody label is exposed to substrate in the final stages, creating a visible band either on the surface of the blot (colorimetric substrates), or as light emission (ECL substrate) captured on X-ray film or with a CCD camera.

Colorimetric substrates for HRP produce brown (DAB) or purple/black (4CN, TMB, NBT/BCIP) bands directly on the surface of the blot. These substrates are very easy to use and take from a few minutes to a few hours to produce visible bands. Detection limits for colorimetric substrates are in the low nanogram range. The colorimetric detection reaction proceeds until stopped, leading to the risk of overdevelopment and less flexibility of results produced. The signal also tends to fade over time, so the record is less permanent.

More routinely, HRP is used with ECL (enhanced chemiluminescence) detection. For ECL detection, the substrate is luminol which is oxidized by HRP in the presence of H_2O_2 and an enhancer to produce light. The emitted light is detected by exposing the Western blot to X-ray film, or by using a CCD camera for light capture. The emitted light forms a band on the film, or on the screen of the imaging system, indicating where the HRP-labeled antibody has bound to the target protein.

ECL detection of HRP is extraordinarily sensitive, allowing for the visualization of picogram to femtogram amounts of target protein. Furthermore, since multiple film or CCD exposures can be made with ease, little effort is required to produce a suitable permanent record for analysis or publication. Detection times for ECL substrates range from a few seconds to about 30 minutes and can usually be done or redone over a period of several hours. Some molecular weight markers are designed to produce a signal during ECL detection yielding a visible ladder that is convenient for identifying the bands produced during immunodetection. A discussion of Western blotting results is found in the next chapter.

Test Blots, Slot Blots, and Dot Blots

Test blots, as their name implies, are very simple Western blots that are created for the express purpose of optimizing or troubleshooting experimental conditions. They are usually produced by running multiple lanes of the same lysate or purified protein solution on a gel, and after transfer cutting the blot into strips to be tested individually. They provide a quick and efficient means of examining a range of antibody dilutions or detection substrates.

Dot blots and slot blots are also a very useful variation on the typical Western blot. They do not require gel electrophoresis, so there is no separation of proteins by size. Instead, the target protein or cell lysate mixture is added directly onto the surface of the nitrocellulose or PVDF membrane. Protein solutions can be applied directly in a small volume, or with a vacuum manifold to produce an orderly grid of samples similar to that seen in Figure 14. Each dot or slot blot would contain known amounts of target protein

or cell lysate. Once dry, dot blots and slot blots are subjected to the same immunodetection steps used for Western blotting, i.e. blocking, antibody incubation, and target detection with substrate. Grey and black spots on the figure below indicate which samples are positive for the target protein and correspond roughly to the bands produced on a Western blot.

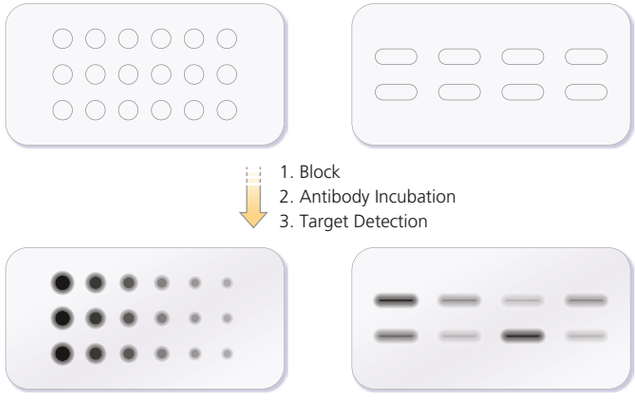


FIGURE 14 **Dot Blots and Slot Blots.** The dot blots at the left represent a dilution series of a sample, with smaller lighter dots corresponding to lower concentrations of target protein. The slot blots represent a group of random samples, the intensity of the signal corresponds to the concentration of the target protein in that sample.

By making a number of identical dots or slots of a single known protein sample, or a range of sample dilutions, one can quickly test several combinations and concentrations of primary and secondary antibodies. Dot blots and slot blots are also beneficial when screening a large number of samples, or if a simple answer will suffice. The main downside to slot blots/dot blots is that they provide no information about molecular weight. Thus, it is harder to detect false positive signals, or to tell whether modified forms of a protein are present.

Data Analysis

Chapter 4

Overview

The data produced with a Western blot is usually quite easy to interpret. In the majority of cases, bands corresponding to the target protein will become visible upon treatment of the blot with substrate. Their identity is confirmed by comparison to molecular weight markers (for size) and a positive control (size and signal). In some cases the data may be more complex, showing unexpected sizes, multiple bands, or alteration in bands following a particular treatment. The focus of this chapter is analysis and as such the data itself will be discussed along with examples of different types of Western blot data from research and clinical settings to demonstrate the flexibility of the technique. If there are no bands on the blot, or if there are unexplained blotches or uneven signal, troubleshooting advice is offered in Chapter 6.

Data

Quantitation

It is very important to be aware that the data produced with a Western blot is typically considered to be semi-quantitative. That is, it provides a relative comparison of protein levels, but not an absolute measure of quantity for a specific target protein in a particular experiment. The reason for this is two-fold: first, there will be variations in loading and transfer rates between the samples in separate lanes and on separate blots that will need to be normalized before a more precise comparison can be made; second, the signal generated will not be linear across the concentration range of samples due to substrate availability and linear responsiveness of the detection method. Since the signal produced is not linear, it should not be used to attach a precise concentration to a particular sample. ELISAs are more suitable for this purpose and generally more sensitive.

Normalization

In order to compare target protein expression levels between several different samples on the same blot or across blots, it is necessary to use a loading control to normalize the data. Loading controls are not actually anything that is loaded onto the gel; instead, they refer to a means of equalizing differences in gel loading and transfer rates between samples. They are not required for every gel that is run, but are necessary for publication quality work, and when the actual signal level between samples is compared. Typically, the blot is probed with an antibody to a well-characterized housekeeping gene which is used as a general measure of protein expression levels in the cells used as a source for a particular sample. Probing with the housekeeping gene antibody can be carried out along with the target antibody, separately by cutting a blot between the expected band locations, or later after the blot has been stripped of previously bound antibodies. Common loading controls measure the levels of GAPDH, beta actin, tubulin, and histones. These proteins vary in molecular weight and should be carefully chosen depending upon the target protein and experimental conditions since there can be some variation in their signal. In the Western blot below, Lnk (lymphocyte adaptor protein) expression is compared to non-infected cells and an unrelated protein as a negative control. The matched anti-tubulin probed section of the blot demonstrates that an equivalent amount of sample was loaded in each lane.

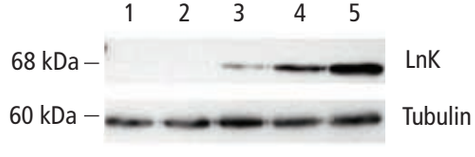


FIGURE 15 **Lnk Expression in Endothelial Cells Following Treatment with TNF α.** The Western blot was probed first with anti-Lnk antibody (AHP1003), showing an increase in Lnk expression over time in lanes 3-5. It was then stripped and reprobed with an anti-tubulin antibody to confirm loading equivalence. **Lane 1**: Non-infected cells. **Lane 2**: Negative transfection control-AdGFP. **Lanes 3-5**: 1, 2, and 3 hours post TNF treatment.

Densitometers and Analysis Software

In order to make a permanent record, or to get an objective measure of the signal generated on a Western blot, a densitometer is used to scan the blot or film. Imaging software is then used to compare the signal generated by the bands detected on the Western blot.

ECL signal can also be detected with CCD cameras, which usually have a better linear range than film and associated analysis software. Consult the manual for the densitometer or imaging system in use for specific instructions.

Western Blot Examples

Detecting or Characterizing Protein Expression

One simple and common use for Western blotting is to identify whether a particular protein is present or absent in a sample by looking for a band of the correct size on a Western blot. This routine type of Western blot is used to either test for endogenous cellular expression of a target protein, or to examine transfected cell lines to see if expression has been conferred by the introduction of a DNA construct. It is also used with techniques such as protein purification and cellular fractionation to identify which samples have the target protein, and thus is an aid to deciding which samples to combine or discard. After immunodetection, a band corresponding to the size of the target protein should become visible in positive test samples and the positive control, in comparison to a known null cellular sample. In the figure below, HEK293 cells transfected with three isoforms of the Homer 1 protein and are compared to rat cortical extract as a positive control. The blot shows that the antibody used, AbD Serotec's AHP737, recognizes Homer isoform 1c, but not 2b or 1a. The presence of untransfected cells (Lane 2) confirm that the signal is specific to transfection with the appropriate construct. The faint band seen in lane 4, is actually a small amount of spill over from lane 5.

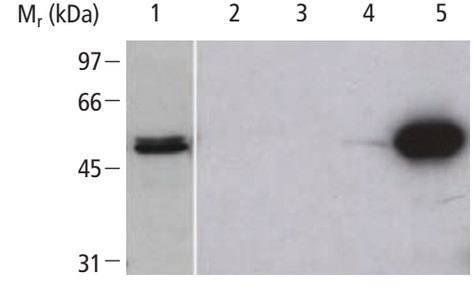


FIGURE 16 **Detection of Rat Homer Protein Isoforms.** Western blot of Homer isoforms probed with anti-Homer [177-366] (AHP737) which detects the control and isoform 1c. **Lane 1**: control, 30 μg rat cortical extract. **Lane 2**: untransfected control. **Lane 3**: Isoform 1a. **Lane 4**: Isoform 2b. **Lane 5**: Isoform 1c.

Demonstrating Antibody Specificity

A simple way to demonstrate that the band seen on a Western blot is specific to the interaction between a target protein and a properly matched antibody is by blocking the antibody from binding to the target epitope through competition with a peptide that matches the sequence of the epitope. AbD Serotec offers a range of blocking peptides for this purpose.

The experiment is carried out during the antibody incubation stage and compares antibody alone to antibody treated with the blocking peptide. If the peptide sequence correctly corresponds to the epitope then the antibody will bind to the peptide and will not be available to bind to the target protein on the blot. This technique would only be used with monoclonal antibodies, since they are directed against a single epitope, and when the epitope is known. In the example below, lane 2 has no signal because the peptide has effectively blocked the antibody from binding, whereas in lane 1 the band is visible as expected.

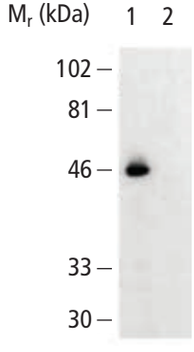


FIGURE 17 **Peptide Blocks Binding of Anti-CX3CR1 (AHP566) Antibody.** **Lane 1:** THP-1 cell lysate (LYS008). **Lane 2:** THP-1 cell lysate + peptide to aa175-189 of human CX3CR1.

Alternate Forms of a Protein – Prion Disease

Prion disease is a rare, progressive, neurodegenerative disorder that affects humans (vCJD, CJD), cows (BSE), sheep (Scrapie), and elk/deer (CWD). It leads to characteristic brain lesions and a rapid loss in neurological functions after a long latency period. The accumulation of an altered isoform of a normal cellular protein appears to be instrumental in the development of prion disease. There are several ways to identify the presence of the abnormal prion protein - a bioassay, immunohistochemistry on diseased tissue samples, and with a faster, well-characterized, and sensitive Western blot.

The Western blot below shows Scrapie infected sheep brain lysates (Lanes 3&4) compared to normal sheep brain lysates (Lanes 1&2). The key to this particular Western assay is the use of Proteinase (PK) treatment on parallel sets of samples. Since the Scrapie associated form of the prion protein is resistant to digestion, it is possible to distinguish between normal cellular forms and abnormal prion protein in a sample based upon their sensitivity to PK. Thus, it is possible to discriminate diseased samples from normal ones quickly and unambiguously on a Western blot. The absence of bands in lane 2 indicates that the normal form is present as compared to the bands visible in lane 4, which indicate the presence of the pathogenic form.

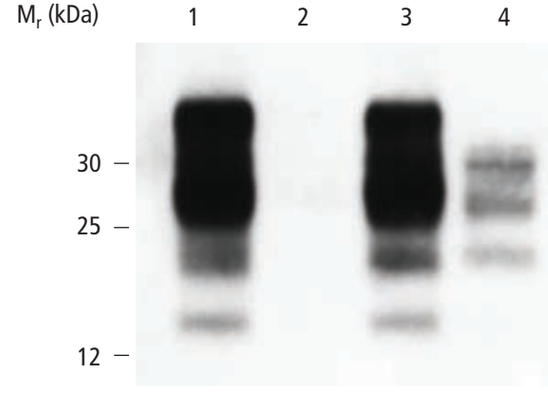


FIGURE 18 **Detection of Scrapie Infected Sheep Brain.** Bands in lane 4 indicate that the protease resistant form of the prion protein is present in the sample as compared to normal tissue in lane 2. **Lanes 1&2**: Uninfected sheep brain homogenate. **Lanes 3&4**: Scrapie-infected sheep brain homogenate. **Lanes 1&3:** No PK added. **Lanes 2&4**: Digested with PK.

Confirmation of HIV

In the diagnosis of HIV infection in patients, an ELISA is used first because it demonstrates 99.5% specificity and is quick and easy to perform on a large number of samples. Western blotting is used as a supplementary assay because the ELISA is subject to false positives. In order to perform this assay, patient serum samples are used as the source of antibodies for immunodetection, and blots containing HIV antigens, proteins, viral lysates, or peptides are used as the source of target antigen on the Western blot. If the HIV target proteins on the blot are detected by the patient serum then it indicates that the patient sample is a true positive for HIV infection, since an immune response has been mounted by the patient. A similar strategy is also used in tests for Lyme disease and autoimmune disease.

Buffers and Protocols

Chapter 5

Overview

The following buffers and methods provide a general starting point for use with the majority of AbD Serotec reagents in Western blotting. When specific recommendations are provided on product datasheets, those instructions should always be used instead of the general guidance offered here. With specialized laboratory equipment, such as for gel electrophoresis and electroblotting, it is advisable to follow the manufacturer's instructions. If difficulties arise with any of our antibodies or related products, please feel free to contact **tech.uk@abdserotec.com**, as we may have additional information on file and can assist with troubleshooting.

Buffers

N.B. Azide should not be added to any buffers that will be used with HRP-labeled antibodies because it will inactivate the HRP enzyme.

PBS

8.0 g NaCl

0.2 g KCl

1.15 g Na_2HPO_4

0.2 g KH_2PO_4

Dissolve in 800 ml distilled water, adjust pH to 7.4, and then add more dH_20 to a final volume of 1 liter. Sterilize by autoclaving and store at room temperature (RT).

TBS

8.0 g NaCl

0.2 g KCl

3.0 g Tris base

Dissolve in 800 ml distilled water, adjust pH to 8.0 with 1 M HCl, and then add more dH_20 to a final volume of 1 liter. Sterilize by autoclaving and store at RT.

10x TBS Stock:

500 mM Tris-HCl, pH 7.4

1.5 M NaCl

Cell Lysis Buffers

NP-40 Lysis Buffer

50 mM Tris, pH 8.0

150 mM NaCl

1% NP-40 (or Triton® X-100)

+ fresh protease inhibitors, see below

RIPA (Radio Immuno Precipitation Assay) Buffer

50 mM Tris, pH 8.0

150 mM NaCl

1% NP-40 (or Triton® X-100)

0.5% Sodium deoxycholate

0.1% SDS

+ fresh protease inhibitors, see below

Proteases Inhibitors

Various proteases inhibitors are often added to lysis buffer to maintain the integrity of the target protein when cells are disrupted. The following table provides some recommended working concentrations:

Protease Inhibitors

Inhibitor	Working Concentration	Protease Inhibited
Aprotinin	1-2 μg/ml	Trypsin, Chymotrypsin, Plasmin, Kallikrein
EDTA	1-2 mM	Metalloproteases (Mg^{2+} and Mn^{2+})
EGTA	1 mM	Metalloproteases (Ca^{2+})
Leupeptin	0.5-2 μg/ml	Plasmin, Trypsin, Papain, Cathepsin B
Pepstatin A	1 μg/ml	Pepsin, Cathepsin D
PMSF (highly toxic)	50-100 μg/ml	Serine and Cysteine proteases

Electrophoresis and Transfer Buffers

Laemmli 2x Sample Buffer

4% SDS

20% Glycerol

125 mM Tris, pH 6.8

0.02% Bromophenol blue

200 mM DTT or 10% βME

For best results DTT or βME is added fresh, just before use.

Gel Electrophoresis Running Buffer

25 mM Tris base

190 mM Glycine

0.1% SDS

Transfer Buffer

50 mM Tris base

380 mM Glycine

0.1% SDS

20% Methanol

Ponceau S Stock Solution

2% Ponceau S

30% Trichloroacetic acid

30% Sulfosalicylic acid

Dilute 10-fold in distilled water prior to use.

Washing Buffers

PBST

PBS with 0.1% Tween® 20

TBST

TBS with 0.1% Tween® 20

Block Ace Wash Buffer (BUF029)

Reconstitute each 4 g vial in 100 ml distilled water. Dilute the reconstituted solution 10-fold, and add Tween® 20 to a final concentration of 0.05-0.2% v/v.

Blocking Buffers

*Use BSA or Block Ace **(BUF029)** to block when probing with anti-phosphoprotein antibodies, or for biotinylated primary antibodies detected with an anti-biotin secondary.

5% Nonfat Dried Milk in PBST or TBST (Blotto/BLOTTO)

Add 5 g nonfat dried milk powder to 100 ml PBST or TBST. Dissolve with gentle stirring. Store at 4°C.

3% BSA in PBST or TBST

Dissolve 3 g of BSA Fraction V in 100 ml PBST or TBST with gentle stirring. Store at 4°C.

Block Ace (BUF029)

Reconstitute each 4 g vial in 100 ml distilled water, and use undiluted.

Protocols

Cell Lysis - Mammalian Cells

*For yeast and bacterial cell lysis consult *Sambrook et al.*, or *Harlow and Lane.*

Adherent cells:

1. Wash cells directly in the tissue culture flask or dish by adding cold PBS and rocking gently. Aspirate PBS and repeat. Keep tissue culture dish on ice throughout.
2. Add appropriate volume ice cold lysis buffer (with fresh protease inhibitors), to the flask, approximately 1ml for a 100 mm tissue culture dish. (Alternatively, cells can be removed from the flask with Trypsin-EDTA and then prepared using the suspension cell instructions below).
3. Incubate for 20 minutes on ice, and then scrape cells from the surface using a rubber spatula.
4. Transfer to a microfuge tube and clarify the lysate by spinning for 10 minutes at 12,000 RPM, at 4°C.
5. Transfer supernatant to a fresh tube and store on ice or frozen at -20°C or -80°C.

Suspension cells:

1. Pipet cells into a fresh conical tube and place on ice.
2. Spin cells on low speed at 4°C, and aspirate off media.
3. Add 10 ml ice cold PBS, and gently invert tube to wash cells.
4. Spin cells on low speed, and aspirate off supernatant.
5. Repeat wash and aspiration. Resuspend cells in 5 ml ice cold PBS.
6. Count cells, and centrifuge on low speed at 4°C to form a cell pellet. Aspirate off liquid.
7. Gently resuspend the cell pellet in ice cold cell lysis buffer (with fresh protease inhibitors), use 1 ml buffer for 10^7 cells.
8. Incubate cells for 30 minutes on ice. If needed, sonicate the lysates on ice for 15-30 seconds to disrupt genomic DNA and cellular components.
9. Transfer to a microfuge tube and clarify the lysate by spinning at 4°C, for 10 minutes at 12,000 RPM.

10. Decant the supernatant to a fresh tube, and discard cell pellet. Store on ice for immediate use, or at -20°C or -80°C until needed.
11. Measure protein concentration with BCA, Lowry, or Bradford assays, or by absorbance, prior to loading on a gel.

Tissue samples:

1. Tissue samples are rinsed in cold PBS and placed in a tube on ice immediately following dissection.
2. For small samples (100 mg) chop into pieces with clean dissecting instruments. Add tissue and 1-2 ml ice cold lysis buffer to dounce homogenizer, or sonicate in small tube. Homogenize or sonicate on ice.
3. Clarify the lysate with a high speed spin in a microfuge at 4°C, for 10 minutes at 12,000 RPM.
4. Transfer supernatant to a fresh tube and discard cell pellet. Store on ice for immediate use, or at -20°C or -80°C until needed.
5. Measure protein concentration with BCA, Lowry, or Bradford assays, or by absorbance, prior to loading on a gel.

Gel Electrophoresis & Protein Transfer by Electroblotting

* Detailed instructions for making gels in-house can be found in *Sambrook et al.*

1. For standard denaturing and reducing conditions, mix sample 1:1 by volume with Laemmli 2x sample buffer.
2. Heat to 100°C for 5 minutes or 70°C for 10 minutes.
3. Load 20-50 μg sample per lane, along with suitable positive and negative controls.
4. Run the gel and transfer to PVDF or nitrocellulose membrane according to the manufacturer's instructions for the equipment and materials in use.
5. Confirm protein transfer by staining for 1 minute with Ponceau S, followed by complete destain in distilled water.
6. Mark position of molecular weight standards if they are unlabeled.

(For our in-house Western blotting, we use NuPAGE® Novex® precast 4-12% gradient gels run in Tris-Acetate buffer and transferred to nitrocellulose using the Xcell SureLock™ II blot module from Invitrogen, as per the manufacturer's instructions.)

Immunodetection – Indirect

1. Place membrane into blocking solution for at least 2 hours at RT or overnight at 4°C. Use a sufficient volume to keep the blot fully covered, with gentle agitation throughout.
2. Rinse the membrane briefly in washing buffer.
3. Incubate with primary antibody diluted in wash buffer or blocking buffer (an antibody concentration of 1-10 μg/ml is generally acceptable). Incubate overnight at 4°C, or for 2 hours at room temperature. Use a sufficient volume to keep the blot fully covered, with gentle agitation throughout.
4. Rinse the membrane in wash buffer (3x10 minutes), with gentle agitation and a sufficient volume to keep membrane well covered.
5. Add appropriate enzyme conjugated secondary antibody diluted in wash buffer or blocking agent, and incubate for 1 hour at RT with gentle agitation in a sufficient volume to ensure coverage. (* No azide in the buffers if the secondary is labeled with HRP)
6. Incubate the membrane with gentle agitation as follows: 4x5 minutes in wash buffer, followed by 2x5 minutes in PBS or TBS.
7. Drain excess PBS/TBS from the membrane and transfer to appropriate enzyme substrate solution and incubate for time period recommended by manufacturer to visualize protein bands.

Immunodetection – Direct

1. Place membrane into blocking solution for at least 2 hours at RT, or overnight at 4°C. Use a sufficient volume to keep the blot fully covered, with gentle agitation throughout.
2. Rinse the membrane briefly in washing buffer and then incubate with labeled primary antibody diluted in wash buffer or blocking buffer (a concentration of 1-10 μg/ml is generally acceptable). Incubate overnight at 4°C, or for 2 hours at room temperature. Use a sufficient volume to keep the blot fully covered, with gentle agitation throughout.
3. Wash the membrane in a sufficient volume and with gentle agitation as follows: 4x5 minutes in wash buffer and 2x5 minutes in PBS or TBS.
4. Drain excess PBS/TBS from the membrane and transfer to appropriate enzyme substrate solution and incubate for time period recommended by manufacturer to visualize protein bands.

Troubleshooting

Chapter 6

Overview

While Western blotting is a relatively simple and straightforward technique, it does not always yield results that meet with expectations. When this occurs, it is useful to be able to quickly isolate the possible causes and to formulate an effective solution by troubleshooting the experiment. In this chapter, suggestions are offered to assist in identifying and resolving some frequently encountered problems that arise during the course of Western blotting. Many of these problems can be avoided with careful attention to experimental protocols, and with optimization at key stages throughout the procedure. As mentioned at the end of Chapter 3, the proper antibody concentration can be determined with the use of dot blots and slot blots, significantly reducing the need for future troubleshooting. Additionally, the use of negative and positive controls are of great help in assessing where things have gone wrong, and eliminating some possible sources of error under consideration.

Since one issue that commonly arises during Western blotting is the presence of unusual or unexpected bands on the blot, our troubleshooting section begins with a table describing some common reasons and potential solutions for addressing and evaluating this type of problem.

In most cases, the rest of the troubleshooting issues can be grouped into three major types: no bands, faint bands, and signal on Western blots that interferes with bands. Therefore, the potential causes and solutions have been organized in this manner, and also with regard to potential problem sources, i.e. antibody, antigen, technique, or buffer related where applicable. For further assistance when using our products in Western blotting, contact **tech.uk@abdserotec.com.**

Unusual or Unexpected Bands

Difference seen	Possible Cause	Action/Solution
Band(s) at lower molecular weight than expected	▪ Target protein has been cleaved or digested ▪ Splice variants exist ▪ Another protein bearing the same/similar epitope has been detected by antibody	▪ Use a fresh sample which has been kept on ice ▪ Add fresh protease inhibitors to the lysis buffer ▪ Try alternate antibody
Band(s) at slightly higher molecular weight than expected, and may be blurred	Protein may be glycosylated or otherwise modified at one or more amino acid residues	▪ Use enzymes to remove suspected modification returning molecular weight closer to expected ▪ Check amino acid sequence and literature
Band(s) at significantly higher molecular weight than expected	Dimers, multimers, or protein-protein interactions may be occurring because samples have not been fully reduced and denatured	▪ Add fresh DTT or βME to samples and reheat before repeating experiment ▪ Prepare new samples with fresh loading buffer
Multiple bands at various molecular weights	Primary antibody concentration may be too high, or there is a cross-reactivity with similar epitopes on other proteins	▪ Use an affinity-purified primary antibody ▪ Optimize primary antibody concentration ▪ Try another antibody ▪ Check antibody specificity with blocking peptide
	Secondary antibody concentration is too high leading to non-specific binding	▪ Decrease/optimize the concentration of the secondary antibody ▪ Use an affinity-purified secondary antibody ▪ Repeat immunodetection with secondary antibody alone to check for non-specific binding
	Protein exists in several different isoforms	▪ Check literature
Bands are blurry	Gel was run at too high a voltage	▪ Repeat gel at lower voltage
	Incorrect running buffer composition	▪ Prepare fresh running buffer
	Trapped air bubble present during transfer	▪ Carefully remove air bubbles between the gel and the membrane before protein transfer
Bands are smile shaped, not flat	Running conditions were too fast so gel became over heated	▪ Check and optimize gel electrophoresis conditions ▪ Run gel at 4°C
White (negative) bands on the film when using ECL detection	Too much protein has been loaded	▪ Load less sample ▪ Repeat with dilution series of sample
	Antibody concentration is too high	▪ Reduce/optimize the antibody concentrations

No Bands

Source of Problem	Possible Cause	Action/Solution
Antibody related	Inappropriate secondary antibody used	▪ Retrace steps to check compatibility between primary and secondary antibodies ▪ Reprobe with correct secondary or strip blot and reprobe if necessary ▪ Repeat experiment with the correct antibody combination
	Wrong concentration of antibody or low affinity to the target protein	▪ Increase the antibody concentration 2-4 fold higher than initially recommended ▪ Increase length of incubation ▪ Test/optimize antibody on dot blots ▪ Try another antibody
	Antibody not suitable for Western blotting	▪ Check datasheet for recommended conditions ▪ Test/optimize antibody on dot blots ▪ Try alternate antibody
	Antibody has lost activity due to long term or improper storage	▪ Test on a dot blot at several concentrations ▪ Use fresh aliquot of antibody that has been stored at -20°C or below
Antigen related	Antigen not expressed in the source material	▪ Use another source of target protein
	Not enough antigen loaded on the gel	▪ Check concentration of sample ▪ Increase the amount of source material ▪ Immunoprecipitate, fractionate, or concentrate the sample
Technique related	Transfer did not work properly	▪ Confirm protein transfer by staining the membrane with Ponceau S and/or the gel with Coomassie dye. ▪ Note how well any prestained molecular weight markers have transferred onto the blot ▪ Optimize/check transfer conditions and set up (especially orientation to electrodes) ▪ Repeat using two membranes in case protein has transferred through the first
	Washes are too stringent	▪ Use fewer washers ▪ Reduce washing time
Buffer related	Blocking agent is interfering with signal	▪ Try lower concentration ▪ Try alternate blocking agent
	Buffers may contain sodium azide which inactivates HRP	▪ Use azide free buffers
	Peroxide may be inactive reducing activity of peroxidase	▪ Add fresh peroxide to substrate buffer
	ECL detection reagents have been contaminated	▪ Use fresh detection reagents

Faint Bands or Weak Signal

Source of Problem	Possible Cause	Action/Solution
Antibody related	Primary or secondary antibody concentrations were too low	▪ Repeat using higher concentration of antibody ▪ Optimize antibody concentration with dot blots
	Low antigen-antibody binding affinity	▪ Reduce the number of wash steps to a minimum ▪ Increase the antibody concentration 2-4 fold higher than the recommended starting dilution
Antigen related	Insufficient sample loaded on the gel	▪ Check concentration of sample ▪ Increase the amount of source material ▪ Immunoprecipitate, fractionate, or concentrate sample
	Blot has been stripped and reprobed	▪ Redo blot since antigen may have been stripped off or damaged by stripping process
Technique related	Low transfer efficiency	▪ Confirm transfer with Ponceau S staining ▪ Optimize transfer conditions for target protein size ▪ Optimize transfer buffers for methanol and SDS concentrations
	Film exposure time (ECL detection) was too short	▪ Test different exposure times
Buffer related	Non-fat dry milk may mask some antigens	▪ Decrease % of milk in the blocking and antibody solutions ▪ Try alternate blocking solution

High Background Signal on the Blot

Source of Problem	Possible Cause	Action/Solution
Antibody related	Concentration of primary and/or secondary antibody too high	▪ Reduce/optimize the antibody concentrations ▪ Reduce/optimize incubation times ▪ Use affinity purified antibodies
	Primary and/or secondary binds to the blocking agent	▪ Use alternate blocking solution
	Antibody has reduced activity due to long term or improper storage	▪ Use a fresh aliquot of antibody that has been stored at -20°C or below
	Phospho-specific antibody has reacted with casein (a phosphoprotein) in nonfat milk blocking agent	▪ Use alternate blocking solution- BSA or Block Ace (AbD Serotec BUF029)
	Secondary antibody is binding to blocking agent	▪ Try another blocking agent ▪ Reduce protein concentration of blocking agent ▪ Test secondary antibody alone on blot ▪ Try alternate form of secondary, such as $F(ab')_2$
Antigen related	Non-specific interaction with genomic DNA in samples	▪ Sonicate the lysates ▪ Add DNAse to lysis buffer
Technique related	Membrane blocking is not sufficiant	▪ Confirm concentration matches that recommended in the protocol ▪ Try other blocking agents ▪ Try alternate incubation temperatures, including room temperature
	Membrane has dried out	▪ Repeat procedure taking care that the blot does not dry out during any step by using sufficient volumes and agitation throughout
	PVDF membrane has higher background	▪ Try nitrocellulose instead
	Inadequate washing between incubations	▪ Increase the length of washing steps ▪ Use a larger volume of wash buffer
	Incubation temperature too high	▪ Try alternate incubation temperatures such as 4°C
	Film over-exposed or blot developed for too long	▪ Wait 5-10 minutes and then re-expose blot to film ▪ Reduce exposure and/or development times
Buffer related	Insufficient concentration of detergent in the buffers	▪ Use TBS containing >0.1% Tween® 20 ▪ Try stronger detergent, such as NP-40
	Transfer, incubation, or blocking solutions are contaminated	▪ Use fresh buffers

Patchy or Uneven Spots on the Blot

Source of Problem	Possible Cause	Action/Solution
Antibody related	HRP-linked secondary antibody has aggregated	▪ Spin secondary antibody and filter to remove aggregates
Technique related	Not enough solution used during incubation and/or washing	▪ Make sure the membrane is fully immersed and agitated throughout incubations
	Trapped air bubbles	▪ Carefully remove air bubbles between the gel and the membrane before protein transfer
	Uneven agitation during incubation	▪ Use a shaker for all incubations
Buffer related	Antibodies are binding to the blocking agent	▪ Filter the blocking agent ▪ Try another blocking agent

References and Further Reading

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