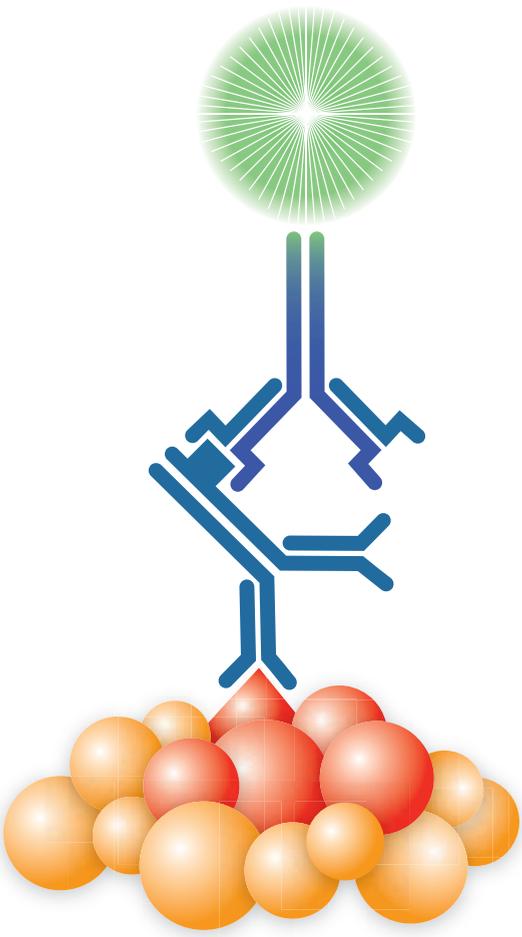# Western Blotting

Principles and Methods

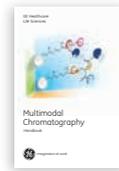# Handbooks from GE Healthcare Life Sciences

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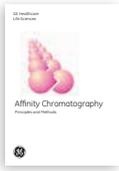
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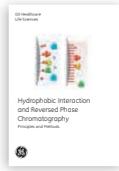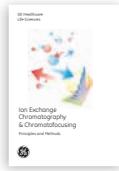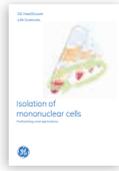
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Western Blotting
Principles and Methods

Contents

Chapter 1
Introduction

Western blotting, also known as immunoblotting, is a well established and widely used technique for the detection and analysis of proteins. The method is based on building an antibody:protein complex via specific binding of antibodies to proteins immobilized on a membrane and detecting the bound antibody with one of several detection methods. The Western blotting method was first described in 1979 (1) and has since become one of the most commonly used methods in life science research.

The aim of this handbook is to guide and inspire beginners as well as experts toward successful Western blotting. It provides expertise and support, taking you through the complete Western blotting workflow, from sample preparation to detection and analysis. Chapters 2 to 8 follow the Western blotting workflow, step-by-step, and describe theoretical as well as practical aspects of the technique together with useful hints and tips. Examples of typical applications, as well as some new approaches to Western blotting, are described in Chapter 9. Moreover, protocols and recipes are provided in Chapter 11, including a recommended standard Western blotting procedure to help you design and run your experiments. Over the past decade, improved detection methods and software have brought quantitative analysis to Western blotting and this handbook provides examples and protocols to help you adapt your methods and obtain more quantitative data from your Western blots. Finally, a troubleshooting guide is provided in Chapter 10, just in case!

A series of methodology handbooks is available from GE Healthcare, many of which cover topics closely connected to Western blotting, such as sample preparation or 2-D gel electrophoresis. Where appropiate, we refer to these handbooks in the text and provide links to the PDF versions in the on-line version of this handbook.

The Western blotting workflow

Although the details of Western blotting protocols may vary from application to application, with adaptations to suit specific protein characteristics and the level of information required, they all follow some common basic steps.

Sample preparation → Gel electrophoresis → Transfer → Antibody probing → Detection → Imaging → Analysis

The sample of interest, for example, must usually undergo some degree of preliminary treatment before continuing to separation by electrophoresis. A sample can consist of a complex protein mixture such as a cell or tissue extract, but it can also be a sample of purified proteins, such as a fraction from a purification procedure.

Gel electrophoresis is applied to the sample for protein separation and the proteins are then immobilized on a membrane following electrotransfer from the gel. Areas on the membrane where no binding of protein has occurred are blocked to prevent nonspecific binding of primary antibodies in the next step. The membrane is incubated with a primary antibody that specifically binds to the protein of interest. Unbound antibodies are removed by washing and a secondary antibody conjugated to an enzyme, a fluorophore, or an isotope is used for detection. The detected signal from the protein:antibody:antibody complex is proportional to the amount of protein on the membrane.

The most commonly used method for detection is chemiluminescence, based on secondary antibodies conjugated with horseradish peroxidase enzyme. On the addition of a peroxide-based reagent, the enzyme catalyses the oxidation of luminol resulting in the emission of light. The light signal can be captured either using a charge-coupled device (CCD) camera-based imager or by exposure to X-ray film.

A more recent detection method is fluorescence. The secondary antibodies are labeled with a fluorophore such as a CyDye™. A fluorescent light signal can be detected directly using a laser scanner or a CCD camera-based imager equipped with appropriate light sources and filters. Regardless of the detection method, the signal intensity correlates with the amount of protein and can be visually estimated as well as quantitated using analysis software.

GE Healthcare and Western blotting

Since the introduction of the first enhanced chemiluminescent (ECL™) detection reagent for Western blotting—Amersham™ ECL—in 1990, the portfolio of products offered by GE Healthcare has been improved and optimized across all Western blotting requirements from electrophoresis and transfer equipment to highly sensitive detection systems and software. By selecting the optimal equipment, gels, markers, blockers, secondary antibodies, detection reagents, imaging systems and software, the portfolio of GE Healthcare products for Western blotting enables you to easily achieve excellent results.

Sample
preparation

Gel
electrophoresis

Transfer

Antibody
probing

Detection

Imaging

Analysis

Amersham ECL detection reagents

Chemiluminescence	Fluorescence
Amersham ECL Amersham ECL Prime Amersham ECL Select™	Amersham ECL Plex™

Chemiluminescence

Chemiluminescence is defined as light emission produced in a multistep reaction whereby peroxidase catalyzes the oxidation of luminol. In the presence of chemical enhancers and catalysts, the light intensity and the duration of light emission is greatly increased, in a process known as enhanced chemiluminescence, ECL. The light emission produced in the ECL reaction peaks after 5 to 20 min and decays slowly, depending on the characteristics of the detection reagent. The maximum light emission occurs at a wavelength of 425 nm and can be detected by a CCD camera or by exposure to X-ray film.

ECL based on horseradish peroxidase (HRP)-conjugated secondary antibodies has become the most commonly used detection method for Western blotting. It is a sensitive detection method, where the light emission is proportional to protein quantity. Minute quantities of proteins can be detected and quantitated.

Amersham ECL

Amersham ECL is a sensitive chemiluminescence detection reagent suitable for a wide range of Western blotting applications for detection of moderate levels of proteins. The light signal can be captured on X-ray film, or a CCD camera-based imager. It is an excellent choice for confirmatory Western blotting applications when the protein amount is not limited such as in protein purification processes or detection of the expression of recombinant proteins.

Amersham ECL Prime

Amersham ECL Prime is a chemiluminescence detection reagent, designed to provide very high sensitivity and long signal duration. The light is intense, which makes it optimal for CCD camera-based imagers, such as Amersham Imager 600, although X-ray film can be used as well. Moreover, the high signal intensity allows the use of low antibody concentrations with sustained performance. Minute levels of protein can be detected and a linear signal response across a wide range of protein levels allows high-precision quantitative analysis. Amersham ECL Prime is useful in all kinds of applications, but is especially recommended where high sensitivity and quantitation are required from a Western blotting experiment.

Amersham ECL Select

Amersham ECL Select is a highly sensitive reagent for chemiluminescent Western blotting detection. It is the most sensitive chemiluminescent detection reagent in the Amersham ECL product family. The dynamic range of Amersham ECL Select is complementary to that of Amersham ECL and Amersham ECL Prime. The outstanding signal intensity of Amersham ECL Select makes it suitable for the most demanding Western blotting applications including the detection of minute protein quantities. Signals can be detected with CCD camera or X-ray film.

Fluorescence

Fluorescence is produced upon excitation of a fluorophore at a specific wavelength which then emits light at a distinct wavelength. Excitation of the fluorophore and detection of the light emission is done using an imager equipped with appropriate excitation light sources and emission filters.

Fluorescence detection is a relatively recent method for Western blotting and has some advantages over chemiluminescence detection. The fluorescent signal is detected directly from fluorophore-labeled secondary antibodies without the addition of any further reagents. The signal is stable and does not decay significantly. It is a very sensitive detection method where the signal is proportional to protein quantity. Moreover, it is possible to detect more than one protein at the same time by using secondary antibodies labeled with different fluorophores. These factors make fluorescence detection an excellent choice for quantitative, multiplexed analysis.

Amersham ECL Plex

Amersham ECL Plex fluorescence detection systems provide high sensitivity, as well as a broad linear dynamic range and are well adapted to quantitative Western blotting. The fluorescent signal is stable on the membrane for up to 3 mo and is captured using a multichannel fluorescent imager or a CCD camera-based imager equipped with appropriate light sources and emission filters.

The emission of light of different wavelengths enables multiplexed detection – the simultaneous detection of up to three proteins on the same membrane. This makes Amersham ECL Plex optimal for quantitative applications where a target protein and an internal standard such as a housekeeping protein can be detected at the same time without stripping and reprobing. In addition, two proteins of similar molecular weight (henceforth referred to as relative molecular mass, M_r), such as a phosphorylated protein and the corresponding unmodified protein, can be simultaneously detected.

Symbols

This symbol indicates general advice on how to improve procedures or recommends measures to take in specific situations

This symbol indicates where special care should be taken

Highlights chemicals, buffers, and equipment

Outline of experimental protocols

Reference

1.   Towbin, H. *et al.* Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U S A.* **76,** 4350–4354 (1979).

Chapter 2
Sample preparation

| Sample preparation | Gel electrophoresis | Transfer | Antibody probing | Detection | Imaging | Analysis |

The importance of good sample preparation cannot be stressed too highly. By understanding the nature of your starting sample and having a clear picture of the information you wish to derive from your Western blotting experiments, you increase your chances of a successful analysis. The emphasis of this chapter, therefore, is on the ground rules of good practice in sample preparation, helping ensure that you get it right from the start. More detailed information on sample preparation can be found in the handbook, Protein Sample Preparation from GE Healthcare (1). This chapter is focused on those issues that impact most significantly on Western blotting.

2.1 Introduction

In principle, all sources of protein, from single cells to whole tissues as well as extracellular matrices, biological fluids, and proteins secreted *in vitro*, are open to analysis by Western blotting. Whereas sources such as mammalian cells in suspension are easily disrupted under mild conditions and readily release their proteins, it is more difficult to extract proteins from cells deeply embedded in intact tissues or within solid tumors. Extraction of the proteins from plants, bacteria, and fungi are further complicated by the presence of the rigid, carbohydrate-rich cell wall that surrounds and protects the living cell.

Regardless of the source and protein of interest, however, the aim must be to devise an extraction procedure aggressive enough to access and disrupt the cells without irreversibly altering the very proteins of interest, while at the same time, obtaining a sufficient yield of material at an acceptable level of purity.

☞   Sample preparation – Be gentle! Stay cool!

- Use extraction procedures that are as mild as possible. Over-vigorous cell or tissue disruption might directly denature the target molecule, form permanent protein complexes, cause chemical modifications, or lead to the release of compartmentalized proteolytic enzymes.

- Extract proteins quickly, on ice if possible, in the presence of a suitable buffer to maintain pH, ionic strength, and stability in order to prevent protein degradation. Prechill equipment and keep samples on ice at all times.

Biological matrices are complex. The target protein is likely to be one among many thousands present in the sample, in addition to nucleic acids, polysaccharides, and lipids, all of which might interfere with the analysis. The efforts invested in extraction and purification depend on the end goal; if the aim is to detect a low-abundant protein, for example, it is advisable to affinity isolate that specific protein from the sample using a technique such as immunoprecipitation. On the other hand, the analysis of robust and abundant proteins can be satisfactorily accomplished using virtually native samples.

The choice of extraction method depends primarily on the sample and whether the analysis is targeting all the proteins in a cell or only a component from a particular subcellular fraction.

In addition, any endogenous proteases liberated upon cell disruption might degrade the target molecule. To avoid these uncontrolled protein losses, the sample should be protected during cell disruption and subsequent purification by the use of a cocktail of protease inhibitors.

Numerous methods are available for disrupting cells and preparing their contents for analysis by Western blotting. Table 2.1 lists some of the most popular extraction methods and indicates their applicability to the treatment of specific cell or tissue sources. In general, gentle methods are employed when the sample consists of easily lysed cultured cells or blood cells. More vigorous methods are employed for the disruption of more robust bacterial or plant cells, or mammalian cells embedded in connective tissue.

Table 2.1. Overview of extraction options for different cells and tissues

Sample	Typical lysis options
Tissue culture	Detergent lysis
Cell suspensions	Ultrasonication
Most plant and animal tissues	Mechanical homogenization (e.g., Waring™ blender or Polytron™)
Soft animal tissues and cells	Dounce (manual) and/or Potter-Elvehjem (mechanical) homogenization
Bacterial and mammalian cells	Freeze/thaw lysis
Bacteria, erythrocytes, cultured cells	Osmotic shock lysis
Solid tissues and plant cells	Manual grinding with mortar and pestle
Cell suspensions, yeast cells	Grinding with abrasive component (e.g., sand, glass beads, alumina)
Bacteria, yeast, plant tissues, fungal cells	Enzymatic digestion
Bacteria, yeast, plant cells	Explosive decompression (nitrogen cavitation)
Microorganisms with cell walls	French press
Plant tissues, fungal cells	Glass bead milling

2.2   Protein extraction options

2.2.1   Detergent-based lysis

Detergent lysis is most frequently the method of choice for the treatment of mammalian cells. Cell suspensions are gently centrifuged and resuspended in lysis solution containing detergent. The membranes are solubilized, lysing cells and liberating their contents. Adherent cells such as fibroblasts may be directly solubilized on the tissue culture surface by addition of lysis solution, or alternatively may firstly be scraped from the surface in the presence of a nonlytic buffer using a rubber scalpel, centrifuged, and treated as cell suspensions. The use of a mild, nonionic detergent such as Triton™ X-100, nonyl phenoxypolyethoxylethanol (NP40) or a zwitterionic detergent such as 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), minimizes denaturation of target proteins.

2.2.2   Freeze/thaw lysis

This method is applicable to suspensions of mammalian or bacterial cells. The major attractions of freeze/thaw lysis are simplicity and low cost. Cells are disrupted by the repeated formation of ice crystals and the method is usually combined with enzymatic lysis. The cell suspension may be rapidly frozen using liquid nitrogen. The sample is then thawed, and resuspended by pipetting or gentle vortexing in lysis buffer at room temperature and the process is repeated several times. Between cycles, the sample is centrifuged, and the supernatant is retained.

2.2.3   Osmotic shock

This is a very gentle method that is sufficient for the lysis of suspended mammalian or bacterial cells without the use of a detergent. The method, often combined with mechanical disruption, relies on changing from high- to low-osmotic medium, and is well-suited to applications in which the lysate is to be subsequently fractionated into subcellular components.

2.2.4   Ultrasonication

This method of protein extraction is most frequently applied to cell suspensions. Cells are disrupted by high-frequency sound waves (typically 20 to 50 kHz) via a probe inserted in the sample. The sound waves generate a region of low pressure, causing disruption of the membranes of cells in the vicinity of the probe tip. Cell suspensions should be sonicated in short bursts to avoid heating and samples should be cooled on ice between bursts. This is suitable for small-scale sample preparation. Aggregates of proteins (inclusion bodies) must be resolubilized. Although relatively simple, ultrasonication is a stringent method of sample preparation, where generated heat must be continually kept under control and sensitive target molecules can be vulnerable to shearing forces.

☞ For protein preparations, the release of DNA can lead to highly viscous samples that are difficult to process. Viscosity is reduced by adding DNase.

2.2.5   Mechanical methods

Proteins can be extracted from cells and tissues using a number of crude but effective crushing and grinding measures. For example, cell membranes can be disrupted by **liquid shear forces** as the sample is forced through a narrow gap; the tighter the gap, the greater the shearing force. Liquid shear forces can be achieved manually by dounce homogenization or mechanically by Potter-Elvehjem homogenization. This mild method is excellent for small volumes and cultured cells.

Homogenization of tissues, prepared by chopping or mincing in chilled buffer, may be achieved using a Waring blender or Polytron. The Polytron differs from the Waring blender in that it draws the tissue into a long shaft that contains rotating blades. Different capacity shafts are available, allowing sample sizes as small as 1 ml.

Mortar and pestle: Tissues or cells are normally frozen in liquid nitrogen and ground to a fine powder. The addition of alumina or sand aids grinding. Cell walls are disrupted by mechanical force.

Glass bead milling: Rapid agitation of cells with fine glass beads disrupts cell walls. Bead milling will lyse most Gram positive and Gram negative bacteria, including mycobacteria.

2.2.6   Enzymatic digestion

Enzymatic methods are frequently used when extracting proteins from bacteria, yeast, or other organisms with cell membranes surrounded by a robust protective structure. The enzymes dissolve cell walls, coats, capsules, capsids, or other structures not easily sheared by mechanical methods alone. Enzymatic digestion is often followed by homogenization, sonication, or vigorous vortexing in a lysis buffer. Enzymatic methods are most commonly used for bacteria and yeast. Enzymatic methods are also used for the extraction of proteins from eukaryotic cells embedded in fibrous tissues, where, for example, collagenase can enhance the breakdown of fibrillar collagen. Table 2.2 provides a summary of enzymes and their uses.

Table 2.2. Enzymatic digestion of bacteria and yeast cells

Enzyme	Description	Comments
Lysozyme	Also known as muramidase or N-acetylmuramide glycanhydrolase. Lysozyme is one of a family of enzymes that damage bacterial cell walls by catalyzing hydrolysis of 1, 4- -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins	Used primarily with bacterial cells
Zymolyase™	The main enzymatic activities are β-1, 3 glucanase and β-1, 3-glucan laminaripentaohydrolase, which hydrolyze glucose polymers at the β-1, 3-glucan linkages releasing laminaripentaose as the principal product	Used primarily with yeast
Lysostaphin A	*Staphylococcus simulans* metalloendopeptidase, specific for the cell wall peptidoglycan of staphylococci	Used primarily with staphylococci Can function as an extremely potent anti-staphylococcal agent

2.2.7   Explosive decompression

This technique is most usually applied to bacteria, yeast, plant cells or other robust samples. Cells are equilibrated with an inert gas, such as nitrogen at high pressure (typically 5500 kPA/800 psi). Using a French press, this method works via a rapid pressure drop when the sample is transferred from a chamber at high pressure through an orifice into a chamber at low pressure. This is a fast and efficient method, suitable for large volumes.

2.2.8   Premade lysis buffers and sample preparation kits

GE Healthcare offers a range of products for protein extraction from mammalian cells, yeast, bacteria, and animal tissues.

Sample Grinding Kit may be used to disrupt small tissue and cell samples for protein extraction. Up to 100 mg of sample per tube is treated in about 10 min. The kit consists of microcentrifuge tubes, each containing a small quantity of abrasive grinding resin suspended in water, and disposable pestles. The tube is first centrifuged to pellet the resin and water is removed. Then extraction solution of choice and the sample are added to the tube, and the pestle is used to grind the sample. After centrifugation, cellular debris and grinding resin are firmly lodged in the conical bottom of the tube, and the supernatant is easily removed.

illustra™ triplePrep™ Kit is designed for the rapid isolation and purification of high yield genomic DNA, total RNA, and total denatured proteins from undivided samples of animal tissues and mammalian cells. The workflow reduces the overall number of steps, enabling the preparation of all three analytes in less than 1 h. The buffer, columns, and protocol ensure high recovery from limited samples such as biopsies, archived tissues, and tumors.

Mammalian Protein Extraction Buffer is designed for the efficient and gentle extraction of biologically active, total soluble proteins from mammalian cultured cells. This buffer is based on organic buffering agents and can be used for cell suspensions as well as adherent cells.

Yeast Protein Extraction Buffer Kit is useful for the extraction of soluble proteins from yeast cells, and is a proprietary improvement on Zymolyase based spheroplast preparation and extraction of soluble proteins from yeast cells. This kit is provided with a protocol to make spheroplasts and remove the lytic enzyme, Zymolyase, prior to lysis and extraction of yeast proteins. The buffer is based on organic buffering agents containing mild nonionic detergents, and a proprietary combination of various salts and agents to enhance extraction and stability of

proteins. A ready-to-use Zymolyase preparation is also provided. Depending on the application, additional agents such as reducing agents, chelating agents, and protease inhibitors may be added. The Yeast Protein Extraction Buffer Kit eliminates the need for laborious glass-bead lysis of yeast cells.

2-D Fractionation Kit simplifies analysis of complex protein mixtures by reducing the amount and number of protein species loaded into the gel matrix. Fractionation makes it possible to isolate groups of proteins, or fractions from the total proteome. This allows for improved resolution when an individual fraction is analyzed, provides less crowded 2-D maps, simplifies analysis and interpretation, and increases the chances of discovering novel proteins of diagnostic or therapeutic interest. These scalable kits help ensure high sample recovery and are compatible with downstream separation techniques, such as 2-D gel electrophoresis.

2.2.9   Protecting your samples

Protease inhibitors must be included in lysis buffers to prevent degradation of proteins following the release of endogenous proteases during the process of cell lysis.

Protease Inhibitor Mix: Sample preparation often requires the inhibition of protease activity. GE Healthcare offers this unique combination of competitive and noncompetitive protease inhibitors, which protect proteins from proteolysis during purification from animal tissues, plant tissues, yeast, and bacteria. The cocktail, containing inhibitors of serine, cysteine, and calpain proteases, effectively inhibits over 95% of the protease activity and has been specifically developed for sample preparation in 2-D gel electrophoresis studies. Optionally, ethylenediaminetetraacetic acid (EDTA) may be added to inhibit metalloproteases.

While it is important to maintain proteases in an inactive state during protein extraction (Table 2.3), other potentially compromising contaminants should also be considered. For example, if the objective of your Western blot is to detect phosphorylated proteins, it is important to protect your sample from the dephosphorylating action of phosphatases liberated into the lysate during sample preparation. One way to protect your sample is by adding a phosphatase inhibitor, such as sodium vanadate to your lysis buffer. It may also be necessary to protect your proteins against unwanted modifications, such as acetylation, ubiquitinylation, or glycosylation.

Table 2.3. Protease inhibitors in lysis buffers

Inhibitor	Target	Notes
Aprotinin	Serine proteases	Also inhibits related proteolytic enzymes
Chymostatin	Chymotrypsin, chymotrypsin-like serine proteinases, chymases, and lysosomal cysteine proteases	Common cocktail constituent for plant extracts
Leupeptin	Cysteine, serine, and threonine proteases	Common cocktail constituent
Pefabloc™	Serine proteases, such as chymotrypsin, kallikrein, plasmin, thrombin, and trypsin	Irreversible inhibitor. Specificity similar to phenylmethylsulfonyl fluoride (PMSF), but more stable at low pH
Pepstatin	Aspartyl proteases	Inhibits nearly all acid proteases with high potency Common cocktail constituent
PMSF	Serine and thiol proteases	Very rapidly degraded in water. Stock solutions are usually made in a solvent, such as dimethylsulfoxide (DMSO) Inactivated by reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol

2.3 Sample cleanup

It is not usually necessary to treat samples prior to 1-D gel electrophoresis, but it is very important in 2-D gel electrophoresis applications. However, if you experience problems with separation, such as blurred bands, sample cleanup improves performance by removing potentially interfering compounds such as nucleic acids, polysaccharides, and salts. The addition of DNase, for example, is used to counter problems with viscosity caused by the release of nucleic acids. Table 2.4 provides a list of common contaminants and options for dealing with them.

Table 2.4. Contaminants that affect downstream analyses

Contaminant	Reason for removal	Technique
Endogenous small ionic molecules, such as nucleotides, metabolites, phospholipids	These substances are often negatively charged and can disturb some downstream analyses	Trichloroacetic acid (TCA)/acetone precipitation Precipitate the sample in TCA, ammonium sulfate[1], or phenol/ammonium acetate, then centrifuge Solubilize sample in sodium dodecylsulfate (SDS) or at high pH[2]
Insoluble material	Insoluble material in the sample can block the pores of gels	Centrifugation or filtration
Ionic detergents	Ionic detergents, such as SDS are often used during protein extraction and solubilization, but can strongly interfere with some downstream analyses	Dilute into a solution containing a zwitterionic or nonionic detergent, such as CHAPS, Triton X-100, or NP40 Acetone precipitation of the protein will partially remove SDS - precipitation at room temperature will maximize removal of SDS, but protein precipitation is more complete at -20°C Precipitate the sample in TCA, ammonium sulfate, or phenol/ammonium acetate, then centrifuge[1] Solubilize at high pH[2]
Lipids	Many proteins, particularly membrane proteins, are complexed with lipids - this reduces their solubility and can affect both the isoelectric point (pI) and molecular weight Lipids form insoluble complexes with detergents, reducing the effectiveness of the detergent as a protein solubilizing agent When extracts of lipid-rich tissues are centrifuged, there is often a lipid layer that can be difficult to remove	Strongly denaturing conditions and detergents minimize protein-lipid interactions - excess detergent may be necessary Precipitation with acetone removes some lipid Precipitate the sample in TCA, ammonium sulfate, or phenol/ammonium acetate, then centrifuge[1] Solubilize sample in SDS or at high pH[2]

Contaminant	Reason for removal	Technique
Phenolic compounds	Phenolic compounds are present in many plant tissues and can modify proteins through an enzyme-catalyzed oxidative reaction	The presence of a reducing agent, such as DTT or β-mercaptoethanol during extraction reduces phenolic oxidation
		Rapidly separate proteins from phenolic compounds by precipitation
		Inactivate polyphenol oxidase with inhibitors such as diethyldithiocarbamic acid or thiourea
		Remove phenolic compounds by adsorption to polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP)
Polysaccharides	Polysaccharides can block the pores of gels	Precipitate the sample in TCA, ammonium sulfate, or phenol/ammonium acetate, then centrifuge[1]
	Some polysaccharides are negatively charged and can complex with proteins by electrostatic interactions	Ultracentrifugation will remove high molecular weight polysaccharides
		Solubilize sample in SDS or at high pH[2]
Salts, residual buffers, and other charged small molecules carried over from sample preparation	Salts disturb some downstream analyses	Dialysis
		Spin dialysis
		Gel filtration
		Precipitation/resuspension
Nucleic acids	Disturbs migration and clogs the wells	Add DNase

[1] The use of ammonium sulfate precipitation requires a subsequent desalting step.
[2] For 2-D gel electrophoresis, SDS must be removed.

2.3.1　Sample cleanup products

SDS-PAGE Clean-Up Kit is designed for the preparation of samples that are difficult to analyze due to the presence of salts or a low protein concentration (Fig 2.1). This kit uses a combination of a precipitant and coprecipitant to quantitatively precipitate the sample proteins while leaving interfering substances such as detergents, salts, lipids, phenolics, and nucleic acids in solution. Proteins are pelleted by centrifugation. The pellet is washed further to remove nonprotein contaminants and centrifuged again. The resultant pellet is resuspended, mixed with SDS sample buffer for polyacrylamide gel electrophoresis (PAGE), and heated. The sample is then ready for SDS-PAGE. The procedure can be completed in under 2 h.
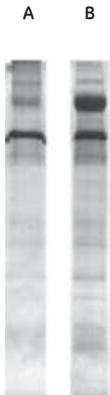
Fig 2.1. Comparison of SDS-PAGE Clean-Up Kit with ethanol precipitation. (A) Urinary protein precipitated with 10 volumes of ethanol. (B) Urinary protein precipitated with SDS-PAGE Clean-Up Kit. Gel: 8 × 9 cm, 12.5% acrylamide, 0.1% SDS, run on SE 260 Mini-Vertical Unit. Stain: Coomassie™ Blue R-250.

2-D Clean-Up Kit is designed to prepare samples for 2-D gel electrophoresis (Fig 2.2), but can also be used in Western Blotting applications. The reagents quantitatively precipitate proteins while leaving interfering substances, such as detergents, salts, lipids, phenolics, and nucleic acids, in solution. Treatment of the sample with 2-D Clean-Up Kit greatly improves the quality of 2-D gel electrophoresis results, reducing streaking, background staining, and other artefacts. For more information on 2-D Clean-Up Kit, see the 2-D Electrophoresis, Principles and Methods Handbook from GE Healthcare (2).
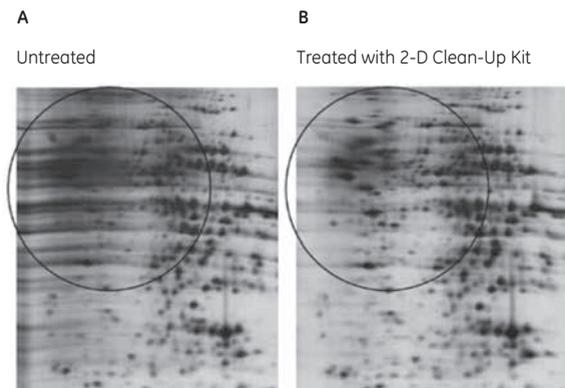
Fig 2.2. 2-D Clean-Up Kit eliminates most of the horizontal streaking caused by residual SDS. Sample: Rat liver extracted with 4% SDS, 40 mM Tris base. First dimension: Approximately 20 µg of rat liver protein, Immobiline™ DryStrip (pH 4–7, 7 cm). Ettan™ IPGphor™ 3 Isoelectric Focusing Unit unit 17.5 kVh. Second dimension: SDS-PAGE (12.5%), run on SE 260 Mini-Vertical Unit (8 × 9 cm gel). Stain: PlusOne Silver Staining Kit, Protein.

2.3.2 Depletion of high-abundance protein from serum or plasma samples

When investigating plasma or serum by Western blotting, abundant plasma proteins, such as albumin and IgG can obscure the signals of less abundant proteins. Prepacked columns, such as HiTrap™ Albumin & IgG Depletion are designed to deplete samples of these potentially problematic proteins, removing > 95% albumin and > 90% IgG, respectively.

HiTrap Albumin & IgG Depletion 1 ml column is designed for the depletion of albumin and IgG from sample volumes of approximately 150 µl of undiluted human plasma or serum, containing normal levels of albumin (~ 40 mg /ml) and IgG (~ 15 mg/ml). The depletion procedure takes approximately 35 min, and can be performed using a liquid chromatography system from the ÄKTA™ design platform, a peristaltic pump, or manually with a syringe. When working with smaller volumes, Albumin & IgG Depletion SpinTrap™, designed for volumes of ~ 50 µl of human plasma or serum, is recommended.

2.3.3 Desalting and concentrating samples

Before applying your sample to an electrophoresis gel, it is important that the solvent does not contain an excessive concentration of salts or other low molecular weight contaminants. High salt levels in samples cause the proteins to migrate in inconsistent and unpredictable patterns. Desalting is achieved in a single step based on gel filtration, and at the same time transferring the sample into the desired buffer. However, desalting and buffer exchange procedures often result in sample dilution. In electrophoresis applications, a relatively high sample concentration is needed for good results and sample concentration might be necessary. A sample can be concentrated efficiently and easily by membrane ultrafiltration.

Some desalting and concentrating products provided by GE Healthcare are summarized in Table 2.5.

Table 2.5. Desalting columns from GE Healthcare

Product	Sample volume (ml)	Desalting capacity (%)	Recovery (%)	Exclusion limit (M_r)	Chemical stability
Disposable PD-10 Desalting Columns	1.0 to 2.5	> 90	70 to > 95	5000	All commonly used buffers
PD MidiTrap™ G-25/G-10	0.5 to 1.0 (G-25) 0.4 to 1.0 (G-10)	> 90	70 to 90	5000 (G-25) 700 (G-10)	All commonly used buffers
PD MiniTrap™ G-25/G-10	0.1 to 0.5 (G-25) 0.1 to 0.3 (G-10)	> 90	70 to 90	5000 (G-25) 700 (G-10)	All commonly used buffers
HiTrap Desalting Column	0.25 to 1.5	> 99	95	5000	All commonly used buffers
Vivaspin™	0.1 to 20	[1]	> 95	3000 to 100 000	All commonly used buffers

[1] Vivaspin columns are designed for sample concentration but they can also be used for buffer exchange.

2.4 Determination of total protein concentration

When comparing the amount of protein from samples run in different lanes within the same gel or between gels, it is very important that all the lanes have been loaded with the same total amount of protein. If there is a significant difference of total protein between adjacent lanes, the actual difference in expression of protein of interest can be masked, and therefore misleading.

Several spectrophotometric methods are routinely used to determine the concentration of protein in a solution (3). These include measurement of the intrinsic ultraviolet (UV) absorbance of the protein as well as methods based on a protein-dependent color change, such as the classic, copper-based Lowry assay (4), the Smith copper/bicinchoninic assay (BCA) (5) and the Bradford dye assay (6). Although widely used, none of the these procedures are particularly convenient.

UV absorbance, for example, requires access to a pure protein of a known extinction coefficient, in a solution free of interfering (UV absorbing) substances. The approximate concentration of a protein in solution (assuming the use of a cuvette with a path length of 1 cm) can be estimated by using either of the following equations;

$$A_{280} = 1 \text{ } A^* \text{ (ml/cm mg)} \times [\text{Conc.}^\dagger] \text{ (mg/ml)} \times 1 \text{ (cm)}$$

$$A_{205} = 31 \text{ } A^* \text{ (ml/cm mg)} \times [\text{Conc.}^\ddagger] \text{ (mg/ml)} \times 1 \text{ (cm)}$$

* A_{280} represents light absorbed by proteins at 280 nm, primarily a result of the presence of ringed amino acids tyrosine and tryptophan. A_{205} represents light absorbed by proteins at 205 nm, primarily the result of peptide bonds between amino acids.

† Protein concentration (mg/ml) = $A_{280}/1$

‡ Protein concentration (mg/ml) $A_{205}/31$

Different proteins, however, have widely different extinction coefficients at both 280 and 205 nm, and concentration estimates obtained in this way are at best a rough estimate. UV absorbance requires that the protein solution is free of other UV-absorbing substances, such as nucleic acids, and that the measurements are carried out using a quartz cuvette.

Copper/BCA assays are based on reduction of Cu^{2+} to Cu^+ by amides. Although quite accurate, these assays require freshly prepared reagent solutions, which must be carefully measured and mixed during the assay. This is followed by lengthy, precisely timed incubations at closely controlled, elevated temperatures, and then immediate absorbance measurements. Both assays can be affected by other substances frequently present in biochemical solutions, including detergents, lipids, buffers, and reducing agents (3). With copper/BCA assays, it is therefore advisable to include a series of standard solutions, each with a different, known concentration of protein, but otherwise having the same composition as the sample solutions.

The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable in its double protonated form (red). Upon binding to protein, however, it is most stable in an unprotonated form (blue).

In comparison with the other assays described above, the Bradford dye assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response. The assay is prone, however, to influence from nonprotein sources, particularly detergents, and becomes progressively less linear at the high end of its useful protein concentration range. The response also varies with the structure of the protein. These limitations make it necessary to use protein standard solutions in this assay.

The Bradford dye reagent reacts primarily with arginine residues and, to a lesser extent, with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The assay is thus less accurate for basic or acidic proteins and is more sensitive to bovine serum albumin than "average" proteins, by about a factor of two. IgG is the preferred protein standard for the Bradford dye assay.

2.4.1 Products for determination of total protein concentration

The use of UV/visible spectrophotometers is widespread in protein analysis. The Ultrospec spectrophotometer series from GE Healthcare, for example, provides modules for protein determination, enzyme activity kinetics, DNA and RNA quantitation, and fraction analysis. These units are compatible with the traditional protein determination methods described above. The instruments are equipped with an eight-position sample changer and are optimal for protein concentration determination with 2-D Quant Kit.

In addition, the Novaspec Plus visible spectrophotometer has stored methods for protein concentration determination by Bradford dye assay, BCA, Biuret, and Lowry assays, as well as basic modes of absorbance, transmittance, optical density at 600 nm wavelength (OD_{600}), and concentration.

2-D Quant Kit, despite its name, can be used in many different applications including the accurate determination of protein concentration in samples. The procedure works by quantitatively precipitating proteins while leaving interfering substances behind. The assay is based on the specific binding of cupric ions to the polypeptide backbone of any protein present. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The absorbance at 480 nm is inversely related to the protein concentration. The assay has a linear response to protein concentrations in the range of 0 to 50 µg/ml, using a recommended sample volume of 1 to 50 µl. In addition, 2-D Quant Kit is compatible with most reagents employed in the many techniques described for sample preparation, such as SDS.

2.5 References

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Chapter 3
Gel electrophoresis

| Sample preparation | Gel electrophoresis | Transfer | Antibody probing | Detection | Imaging | Analysis |

Electrophoresis is a commonly used method for separating proteins on the basis of size, shape and/or charge. The aim of this chapter is to help you to select the conditions that will enable you to most effectively get the information you require from your specific analysis.

Although proteins may be separated and detected within gels by staining following electrophoresis, or may be subjected to the specialized process of 2-D gel electrophoresis for proteomics applications, this chapter will focus primarily on 1-D gel electrophoresis prior to transfer from gel to membrane for Western blotting.

We will consider some of the most important variables when planning electrophoretic separations, such as whether to use native or denaturing conditions, the choice of the most appropriate gel density (acrylamide percentage) as well as recommendations for the most appropriate buffer system.

3.1 Electrophoresis

Electrophoresis is a separation technique based on the mobility of charged molecules in an electric field. It is used mainly for the analysis and purification of large molecules such as proteins or nucleic acids.

Electrophoresis is normally carried out by loading a sample containing the molecules of interest into a well in a porous matrix to which a voltage is then applied. Differently sized, shaped, and charged molecules in the sample move through the matrix at different velocities. At the end of the separation, the molecules are detected as bands at different positions in the matrix (Fig 3.1). The matrix can be composed of a number of different materials, including paper, cellulose acetate, or gels made of polyacrylamide, agarose, or starch. In polyacrylamide and agarose gels, the matrix can also act as a size-selective sieve in the separation.
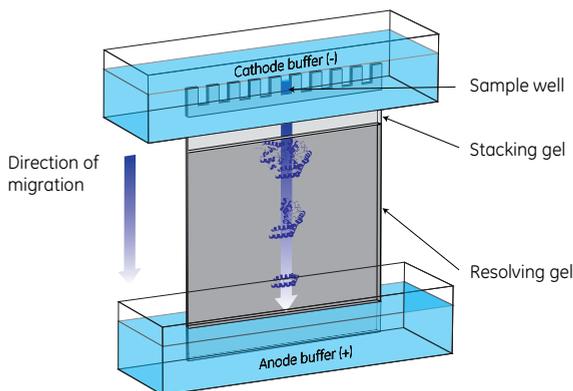
Fig 3.1. A vertical electrophoresis apparatus is set up with cathode (-) buffer in an upper chamber and anode (+) buffer in a lower chamber. Molecules in sample wells migrate toward the anode (+) in an electric field. After a set amount of time, the molecules will have migrated to positions based on their size, shape, and charge. As an alternative to vertical electrophoresis systems, horizontal systems are also available.

Polyacrylamide and agarose gels are the most commonly used matrices in research laboratories for separation of proteins and nucleic acids, respectively. The size of the pores of these gels is similar to the sizes of many proteins and nucleic acids. As molecules are forced through the gel by an applied voltage, larger molecules are retarded in their migration more than smaller molecules. For any particular gel, molecules significantly smaller than the pores in the matrix are not retarded at all; they migrate almost as if in free solution. At the other extreme, molecules larger than the pores cannot enter the gel at all.

In most electrophoresis units, the gel is mounted between two buffer chambers in such a way that the only electrical connection between the anode (+) and cathode (-) chambers is through the gel. With various options in gel density as well as sample well number, gels come in many sizes, precast or hand made, with the optimal combination depending on the distance required for satisfactory separation and the amount of sample. Both horizontal and vertical system have been developed for electrophoresis.

3.1.1   Polyacrylamide gels

Polyacrylamide gels (Fig 3.2) are inert, cross-linked structures. The pore sizes in these gels are similar to the molecular radius of many proteins. As molecules are forced through the gel in an electric field, larger molecules are retarded by the gel more than smaller molecules.
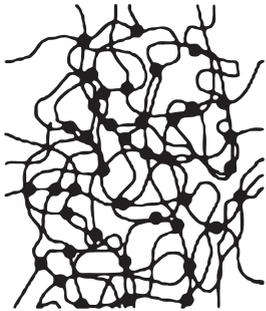
Fig 3.2. Polyacrylamide gels have covalent cross-links formed by bisacrylamide (•) between strands of acrylamide polymers.

The gels (Table 3.1) are formed by the addition of a chemical initiator and catalyst (e.g., ammonium persulfate [APS] and TEMED) to a solution of acrylamide and bisacrylamide monomers to propagate the cross-linking chain reaction. Alternatively, cross-linking can be induced via a photochemical method where riboflavin and longwave ultraviolet (UV) light are the initiators. Polyacrylamide gels are ideal for electrophoretic applications for many reasons; polyacrylamide is a thermostable medium, transparent, strong, and relatively chemically inert. Its versatility, however, lies in the fact that it can be prepared with a wide range of pore sizes, which is the intrinsic characteristic of the gel that most critically determines how proteins of different sizes will migrate. The pore size of a gel can be controlled by the user and is determined by the concentrations of both acrylamide monomer and bisacrylamide cross-linker.

The average pore size is determined by the percentage of the amount of cross-linker and total amount of acrylamide used. Polyacrylamide is used to separate most proteins, ranging in molecular weight (M_r) between 5000 and 200 000.

Table 3.1. Chemicals used in polyacrylamide gels

Chemical	Function in polyacrylamide gel electrophoresis (PAGE)
Acrylamide	The monomeric unit of the gel matrix
APS	Polymerization initiator
Bisacrylamide (N,N'-methylenebisacrylamide)	Cross-linking agent for the formation of polyacrylamide
TEMED (N, N, N', N'-tetramethylethylenediamine)	Polymerization catalyst
Tris ($C_4H_{11}NO_3$, molecular weight 121.14)	Commonly used as the solvent when preparing gels. With pKa of 8.3 at ambient temperature, it has good buffering capacity in a pH range from 7 to 9
Butanol (water-saturated) or isopropanol	Used to overlay the resolving gel immediately after casting but prior to polymerization, preventing the formation of an uneven upper edge After polymerization, the butanol/isopropanol layer is decanted, leaving a flat surface
Glycine	Source of trailing ions, with pKa of 9.7

A typical gel consists of two sections of different densities, cast between two glass plates. The first section to be cast, known as the resolving or separating gel, is prepared from a high-concentration solution of acrylamide and bisacrylamide. When this layer has set, a second gel known as the stacking or spacer gel, prepared from a lower concentration solution of acrylamide and bisacrylamide is cast above the resolving gel. The height of the stacking gel should be at least double that of the sample in each well. A comb is inserted between the glass plates into the unpolymerized stacking gel to create the wells into which the samples will be loaded. The comb is then carefully removed after the gel has set and the wells are rinsed by flushing with running buffer using a pipette or syringe.

By initially running the samples through a lower density stacking gel, proteins are concentrated in a matter of minutes into a thin starting zone by the time the sample contents reach the resolving gel; this process is known as isotachophoresis. The interface between the two gel densities can therefore be regarded as the starting line for all the proteins in each well and on entering the resolving gel, the proteins begin to separate according to size.

The density (pore size) of the gel is an important factor affecting the separation profile of proteins. In a gel of any given density, rapidly migrating small proteins will resolve into more discrete bands than slowly migrating larger proteins that barely penetrate the gel. Where separation is desired over a wide range of molecular weights, a gradient gel should be used, in which the polyacrylamide mesh increases in density toward the anode (+). In such a gel, over a given time, small proteins will reach dense regions of the gel while larger proteins will migrate within less dense regions (Table 3.2 and Fig 3.3). The resolution of band positions is thus sufficient to enable a precise measurement of protein sizes across a wide molecular weight range.

If the sizes of the proteins of interest in the sample are known, the density of the resolving gel may be chosen for optimal separation of proteins around specific molecular weights, with lower density matrices providing better resolution of larger proteins. If, on the other hand, the sizes of the proteins in a sample are not known, it may be necessary to test several acrylamide concentrations to optimize separation conditions. In Table 3.2, the acrylamide concentrations giving a linear separation of proteins within different molecular weight ranges are shown.

Although proteins of sizes outside the indicated ranges also migrate in the gels, their mobility will not conform to the linear migration pattern.

Table 3.2. Recommended acrylamide content in SDS-containing polyacrylamide gels for linear separation of target proteins within defined size ranges

Target protein size range (M_r)	Recommended acrylamide concentration (%)
36 000 to 205 000	5
24 000 to 205 000	7.5
14 000 to 205 000	10
14 000 to 66 000	12.5
14 000 to 45 000	15

Depending on the application, however, it is not necessarily important to achieve strictly linear separation of all proteins of interest. In a Western blotting application involving two target proteins, for example, it is more important to select a gel with a polyacrylamide concentration that most discretely resolves these two proteins. Figure 3.3 presents the migration patterns of several proteins in homogenous and gradient precast Amersham ECL Gel.
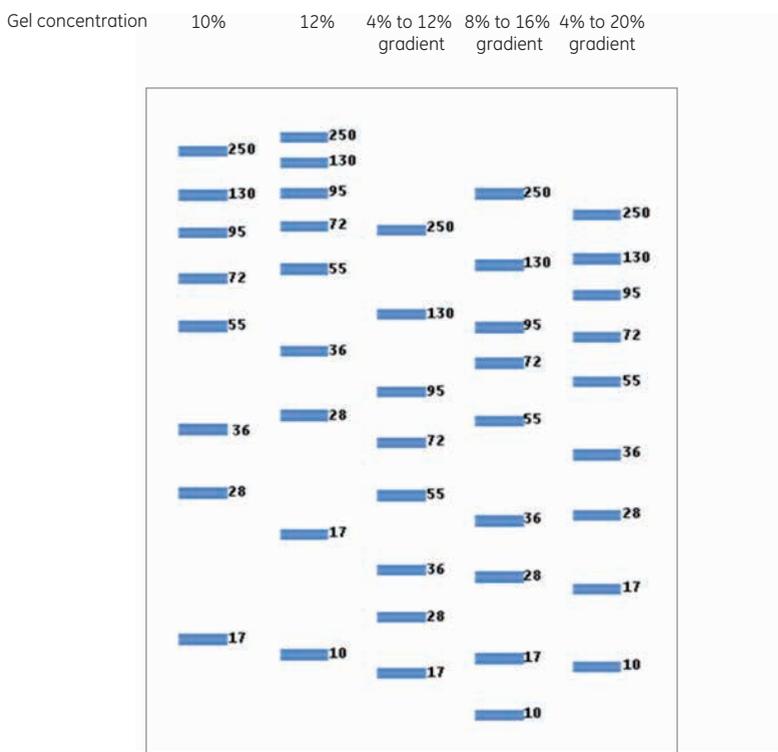
Fig 3.3. When selecting a gel, it is important to use an acrylamide concentration that will allow optimal separation of the proteins in your sample. High molecular weight proteins will be optimally resolved in gels containing a lower acrylamide content, while smaller proteins should ideally be run in more acrylamide-dense gels. The image shows the separation pattern for nine different proteins for each acrylamide concentration.

3.1.2   Buffer systems and pH

Proteins are amphoteric (or zwitterionic) compounds. They are therefore either positively or negatively charged, because they contain both acidic and basic amino acid residues. Most of the charge on a protein comes from the pH-dependent ionization of carboxyl and amino groups on the amino acid side chains. As these groups can be titrated over normal electrophoresis pH ranges, the net charge of a protein is determined by the pH of the surrounding medium and the number and types of amino acids carrying amino or carboxyl groups. Post-translational modifications (PTMs), such as sulfhydryl cross-links, and blocking amino or carboxyl termini might also affect the overall charge on a protein.

For each type of protein, there is a pH at which the molecule has no net charge. At this pH, called the isoelectric point (pI), the weak acids and bases are titrated to the point that there is an equal number of positive and negative charges on the molecule. Each protein has a characteristic pI. In a solution of pH above the pI, a protein has a net negative charge and migrates toward the anode (+) in an electric field. When in a solution of pH below the pI, the protein has a net positive charge and migrates toward the cathode (-). For electrophoretic separation based on protein mobility, the pH of the solution must be kept constant to maintain the charge and, hence, the mobilities of the proteins. As the electrolysis of water generates $1/2O_2$ at the anode (+) and $2H^+$ at the cathode (-), the solutions used in electrophoresis must be buffered.

Two types of buffer systems—continuous and discontinuous systems—are used in protein gel electrophoresis. A continuous system uses the same buffer for both the tanks and the gel. In a discontinuous system, two gel layers are each made with a different buffer, and the tank buffers differ from the gel buffers. Continuous systems are slightly easier to set up than discontinuous systems and suffer from fewer problems related to sample precipitation and aggregation. However, discontinuous systems provide improved resolution and are more widely used for protein electrophoresis in research applications.

Laemmli (Tris-glycine) buffering systems are the most commonly used and are comprised of a stacking gel of pH 6.8 and a resolving gel of between pH 8 and 9. One potential drawback of this popular system is that disulfide bonds tend to form between cysteine residues at this relatively high pH, although this problem can be alleviated by the addition of a reducing agent to the sample. Alternatively, this problem can be solved by using a buffer that resolves proteins at a lower pH. For example, Tricine, a zwitterionic amino acid with a useful buffering range of pH 7.4 to 8.8, has been used as the trailing ion in a discontinuous system for the separation of polypeptides of M_r below 10 000 (1).

The inclusion of counter ions in buffer systems has been shown to be advantageous to the electrophoretic separation of proteins of M_r 1000 to 100 000 (2). This buffer system uses bicine and sulfate as trailing and leading ions, respectively, and Bis-tris and Tris as counter ions in the stacking and resolving phases, respectively. This counter ion principle enables the separation of a wider range of rapidly migrating proteins than would be possible using the more commonly used Laemmli system.

Other variants of Tris-based running buffers include Tris-acetate and Tris-tricine buffers, which are optimal for the precast ExcelGel™ and PhastGel™ systems from GE Healthcare.

3.1.3   Denaturing gels: SDS-PAGE

Gel electrophoresis, as a means to separate proteins prior to Western blotting, is usually performed under denaturing conditions imparted by the presence of the detergent, SDS, both in the sample and as a constituent of the gel and running buffer. 1.4 g of SDS will bind to each gram of protein, so that any inherent charge on the protein is masked by the coating of negatively charged detergent micelles.

Denaturing gels can be run under nonreducing conditions (no sample boiling and no added reducing agent) when it is important to maintain the native structure of proteins for further analysis. Alternatively, denaturing gels can be run under reducing conditions, where a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol is added to the sample buffer and heated. These reagents act by cleaving disulfide bonds between cysteine residues to disrupt the quaternary and tertiary structure of the proteins, creating linear chains of polypeptides. Proteins treated in this way migrate at rates that are a linear function of the logarithm of their molecular weights.

In addition, it is desirable to standardize proteins in terms of three-dimensional structure. Much of the function of proteins depends on how polypeptide chains are folded into specific shapes to form the clefts, pockets, and tunnels needed for recognition and interaction with binding partners. Differently folded proteins take up different volumes; it is possible that a short peptide, if folded in a particular way, will occupy more space than a larger, but tightly folded polypeptide. A short polypeptide, under these circumstances, migrates more slowly than a larger polypeptide through a gel, giving the incorrect impression of having the higher molecular weight of the two.

SDS and a disulfide reducing agent in the sample will thus ensure that your proteins are separated solely on the basis of size and not on charge or three-dimensional structure. Before samples are added to the wells of a gel, they should be mixed with sample loading buffer in order to help visualize the samples for loading and to enable the user to monitor protein migration during electrophoresis. Table 3.3 presents a summary of the chemicals commonly used in sample loading buffer.

Table 3.3. Supplementary chemicals used in sample loading buffer

Chemical	Function
Bromophenol blue	Enables the user to monitor the migration through the gel of the leading dye front (and hence the smallest proteins), indicating when it is appropriate to turn off the current and end the gel run
Glycerol	Increases density of the samples, which enables loading and helps anchor the sample in the sample wells until an electric field is applied to the gel **(this is critical; without glycerol the sample rapidly mixes with the running buffer and will be irretrievably lost)** This reagent is not included for horizontal systems such as Multiphor™ II electrophoresis system or PhastSystem™ electrophoresis unit, because glycerol has been shown to affect the migration of proteins
Reducing agent	Breaks any inter- and intrachain disulfide bonds, linearizing polypeptides and disrupting quaternary and tertiary protein structures
SDS	Detergent that binds proteins so that any inherent charge is masked by the coating of negatively charged detergent micelles - proteins thus migrate at a rate that is a linear function of the logarithm of their molecular weights, and independently of native charge

3.1.4 Native gels: PAGE

Native or nondenaturing gel electrophoresis is run in the absence of SDS. Whereas in SDS-PAGE, the electrophoretic mobility of proteins depends primarily on molecular mass, mobility in native PAGE depends on both charge and hydrodynamic size.

The intrinsic charge of a protein at the pH of the running buffer depends on the amino acid composition of the protein as well as PTMs, such as addition of sialic acids. Since the protein retains its folded conformation when run under native conditions, its hydrodynamic size and mobility on the gel will also vary with the nature of this conformation (higher mobility for more compact conformations, lower for larger structures). If native PAGE is carried out near neutral pH to avoid acid or alkaline denaturation, then it can be used to study conformation, self-association or aggregation, and the binding of other proteins or compounds.

Native gels can thus be sensitive to any process that alters either the charge or the conformation of a protein, making them excellent tools for applications such as:

- Changes in charge due to chemical degradation (e.g., deamidation)

- Changes in conformation due to folding/unfolding

- Aggregation (both covalent and noncovalent)

- Binding events (protein-protein or protein-ligand)

Native gels are excellent for analyzing accelerated stability samples, demonstrating comparability of different lots or processes, or examining the effects of excipients. Another advantage of native gels is that it is possible to recover proteins in their native state after the separation. Recovery of active biological materials might, however, need to be performed prior to any fixing or staining.

3.1.5 Two-dimensional (2-D) gel electrophoresis

In this handbook, we are primarily concerned with blotting following electrophoresis in one dimension, in which proteins are separated on the basis of size. The increasing power of analytical techniques in the field of proteomics, however, demands further resolution of proteins in the gel and to this end, 2-D gel electrophoresis is widely used. It can also be used as the separation step prior to Western blotting.

Proteins are separated first by isoelectric focusing (IEF), which is an electrophoretic method that separates proteins according to pI. Proteins are amphoteric molecules, meaning that they carry a positive, negative, or zero net charge depending on amino acid composition and the pH of the surrounding medium. The pI is the specific pH at which the net charge of the protein is zero. In IEF, a pH gradient is used and under the influence of an electric field a protein will move to the position in the gradient where its net charge is zero. The resolution of separation is determined by the strength of the electric field, and IEF is therefore performed at high voltage (typically in excess of 1000 V). When proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically below 1 mA).

IEF can be run under either native or denaturing conditions in a matrix formed as a strip or a rod. Native conditions are preferred when proteins are required to be in their native states after separation, for example if activity staining is to be employed. The use of native IEF, however, is often limited by the fact that many proteins are not soluble at low ionic strength or are only partially soluble at pH close to their pI. In these cases, denaturing IEF should be employed. Urea is the denaturing agent of choice, as this uncharged compound can solubilize many proteins that are otherwise insoluble under IEF conditions.

IEF is best performed using a horizontal electrophoresis apparatus, as this allows very efficient cooling, which is necessary to counter the effects of the high voltages.

After this first dimension separation, the resulting strip or rod is equilibrated in a solution of SDS and then applied to SDS-PAGE where the proteins are separated according to molecular weight. As the proteins are thus separated according to two distinct properties—pI and size—the power of separation in 2-D gel electrophoresis is much greater than that of its one-dimensional counterpart.

The power of 2-D gel electrophoresis as a biochemical separation technique has been recognized virtually since its introduction. Its application, however, has become increasingly significant as a result of rapid developments in the field of proteomics, due to its outstanding ability to separate thousands of proteins simultaneously. The technique also enables detection of PTMs and cotranslational modifications that cannot be predicted from the genome sequence.

Besides proteomics, applications of 2-D gel electrophoresis include cell differentiation, detection of disease markers, therapy monitoring, drug discovery, cancer research, purity checks, and microscale protein purification. In addition, 2-D Western blotting is a very useful technique for the study of PTMs and examples of this kind of application are given in Chapter 9.

Detailed information on 2-D gel electrophoresis can be found in the handbook, 2-D Electrophoresis, Principles and Methods, from GE Healthcare (3).

3.1.6   Electrophoresis equipment from GE Healthcare

There are many equipment options available for running polyacrylamide gels, each with characteristics particularly adapted to a set of applications. Choices include gel size and thickness, vertical or horizontal orientation, precast or lab-cast gels, speed and resolution requirements, application target, and cost considerations. Table 3.4 lists the instruments available from GE Healthcare.

Separations are performed in either vertical or horizontal systems (Fig 3.4). Vertical systems are widely used and offer a great deal of flexibility with accessories. With simple casting units, gels can be poured with a choice of buffers in a variety of thicknesses to accommodate various sample types and sizes in both mini-gel and standard-gel formats.

Fig 3.4. PAGE is run using either vertical or horizontal formats. SE 400 Vertical Unit (left) and Amersham ECL Gel system (right) are shown as examples. Both systems, and others, are available from GE Healthcare.

Horizontal systems, such as Multiphor II electrophoresis system use ultrathin gels polymerized on support films. These precast gels and buffer strips offer advantages over vertical systems, such as simple sample loading, more secure gel handling, good cooling efficiency, automation, and the possibility to wash, dry, and rehydrate the gels. Ready-made gels and buffer strips also effectively reduce the volumes of buffer used in electrophoresis.

GE Healthcare also offers the neutral pH, horizontal precast Amersham ECL Gel electrophoresis system. This convenient system is very easy to load, run and handle, with a running time of approximately 1 h and a shelf life for the gels of up to 12 mo.

Table 3.4. Electrophoresis equipment available from GE Healthcare

Horizontal systems

Product	Gel dimensions (cm)	Capacity	Electrophoresis run time (h)	Notes
Amersham ECL Gel system	8 × 7.5	2, 10, or 15 samples/gel	1	Precast gels and horizontal gel box
				Available with acrylamide concentrations of 10%, 12%, 4% to 12%, 4% to 20%, and 8% to 16%
Multiphor II	25 × 11 25 × 18	1 gel/25 to 48 samples per gel	1.5	Temperature control with ceramic heat exchanger in conjunction with a recirculating water bath
				High resolution separations at high voltages
				Wide range of precast gels available
				Precast buffer strips eliminate need for large volumes of buffer
PhastSystem	4.3 × 5.0	1 or 2 gels/6, 8, or 12 samples per gel	1.5 (including staining time)	Programmable power and Peltier-controlled temperature conditions for separation and staining
				Precast gels available: Denaturing; native; and IEF

Vertical systems

Product	Gel dimensions (cm)	Capacity	Electrophoresis run time (h)	Notes
miniVE Vertical Electrophoresis System	8 × 7 8 × 9	1 or 2 gels/5 to 15 samples per gel	1 to 2	One piece to both cast and run gels Blotting module can be used in the same unit
Mini-Vertical Units SE 250 and SE 260	8 × 7 8 × 9.5	1 or 2 gels/5 to 15 samples per gel	1 to 2	Temperature control with built-in heat exchanger in conjunction with a recirculating water bath
SE 600 Ruby and SE 660 Dual Cooled Vertical Units	14 × 16 14 × 24	1 to 4 gels/10 to 28 samples per gel	3 to 5	Temperature control with built-in heat exchanger in conjugation with a recirculating water bath
SE 400 Vertical Unit	14 × 15	1 or 2 gels/10 to 28 samples per gel	3 to 5	

3.2 Molecular weight markers

Molecular weight markers are used to define the size of proteins run in a gel. Markers are composed of different proteins of known size and the distances migrated over the time course of the run provide a logarithmic scale by which to estimate the size of unknown proteins. For most runs, it is convenient to reserve at least one separate lane on the gel to run the molecular weight markers. In addition to size estimation, the inclusion of visible molecular weight markers allows the progress of proteins to be monitored throughout electrophoresis as well as to assess the efficiency of transfer of proteins from gel to membrane.

A wide selection of prestained and unstained molecular weight markers from GE Healthcare allows you to estimate the molecular weight of blotted proteins from M_r 3500 to 669 000 (Table 3.5).

Table 3.5. Overview of molecular weight markers for PAGE and Western blotting

Product	Feature
Full-, High-,and Low-Range Rainbow™ Molecular Weight Markers	Visible, colored bands in gels and on blots
Amersham ECL DualVue™ Western Blotting Markers	Combined marker for ECL applications; includes a prestained marker to monitor migration and transfer, and tagged proteins for detection via chemiluminescnce that can be detected on film or via a charge-coupled device (CCD) camera-based imager
Amersham ECL Plex Fluorescent Rainbow Markers	Visible, colored bands in gels and on blots. Fluorescence detected in Cy™5 and Cy3 channels in a fluorescence-based imager (laser scanner) such as Typhoon™
Protein Molecular Weight Markers	Unlabeled protein markers with multiple staining options. Bands can be visualized using Coomassie Brilliant Blue, or silver staining

3.2.1   Rainbow Molecular Weight Markers

Rainbow Molecular Weight Markers (Fig 3.5) enable fast and simple identification of proteins by SDS-PAGE. These ready-to-load markers provide sharp, intense bands on gels and blots and discrete band spacing enables accurate molecular weight determination. The bright, distinctive colors of the markers allow confirmation of transfer to blotting membranes and orientation. The markers comprise a mixture of individually colored proteins that are combined to produce bands of equal color intensity. Rainbow Molecular Weight Markers are available in three size ranges: Full-Range (10 proteins, M_r 12 000 to 225 000); High-Range (eight proteins, M_r 12 000 to 225 000); or Low-Range (seven proteins, M_r 3500 to 38 000).
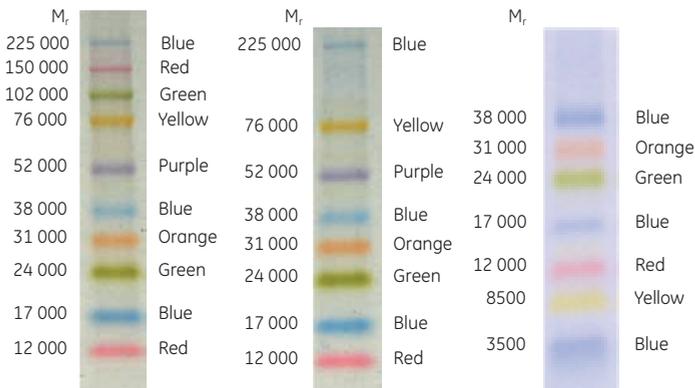
Fig 3.5. Rainbow Molecular Weight Markers are available in three size ranges for use with SDS-PAGE. Full-, High-, and Low-Range (left to right).

3.2.2   Amersham ECL DualVue Western Blotting Markers

Amersham ECL DualVue Western Blotting Markers (Fig 3.6) perform two key functions. First, prestained marker proteins allow you to monitor electrophoresis and confirm protein transfer from gel to membrane, in addition to clearly defining blot orientation. Second, recombinant tagged proteins are detectable on film or using CCD camera-based imaging in conjunction with target proteins, enabling highly accurate molecular weight determination. The tagged markers are detected easily and specifically by means of a specific conjugate, which eliminates any cross-contamination between target proteins and markers.

The markers contain a mixture of three prestained colored markers of defined molecular weight (M_r 15 000, 16 000, and 100 000) and seven tagged recombinant proteins (M_r 15 000 to 150 000). The product is compatible with any HRP substrate as well as polyvinylidene fluoride (PVDF) and nitrocellulose membranes. Amersham ECL DualVue Western Blotting Markers are recommended for chemiluminescence detection using reagents such as Amersham ECL, Amersham ECL Prime, and Amersham ECL Select.
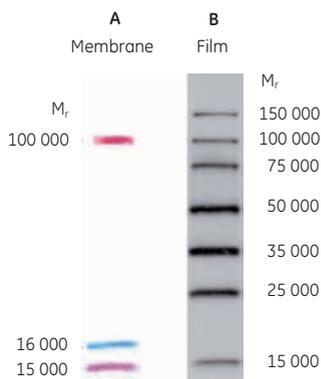
Fig 3.6. Amersham ECL DualVue Western Blotting Markers after electrophoresis on a 4% to 20% SDS gel and transfer onto an Amersham Hybond™ ECL nitrocellulose membrane (now replaced by Amersham Protran™ Premium 0.45 nitrocellulose blotting membrane). Prestained indicator proteins are shown as a photograph of the membrane (A), together with tagged recombinant proteins after detection with Amersham ECL as a photograph of the film, following 1 min exposure (B).

3.2.3   Amersham ECL Plex Fluorescent Rainbow Markers

These protein molecular weight markers are optimized for use with the Amersham ECL Plex Western Blotting Detection System, providing visible marker bands on gels and membranes, as well as images using Cy3 and Cy5 channels of fluorescence-based imagers, such as Typhoon.

☞ Minimal volumes of 1.5 to 3 µl of Amersham ECL Plex Fluorescent Rainbow Markers (Fig 3.7) should be applied per lane of a 10 × 10 cm mini gel. Overloading can disturb the analysis of samples in the adjacent lane. If low-abundance proteins are to be detected, only 1.5 µl of the markers should be loaded, or alternatively, load one lane with sample loading buffer between sample and markers.
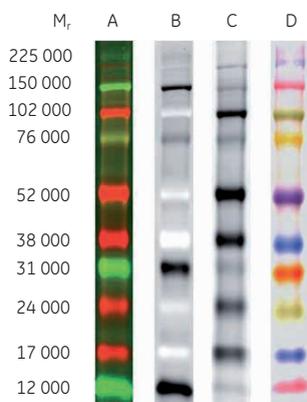
Fig 3.7. Amersham ECL Plex Fluorescent Rainbow Markers imaged on a Typhoon scanner. From left to right: (A) Full-color Cy3 and Cy5; (B) Cy3 channel; (C) Cy5 channel; and (D) visible spectrum.

3.2.4   Unlabeled Protein Molecular Weight Markers

Unlabeled molecular weight markers can be visualized after electrophoresis by using total protein stains such as SYPRO™ Ruby, Coomassie Brilliant Blue, or silver staining. Markers are available as Low Molecular Weight (LMW)-SDS Marker Kit (M_r 14 000 to 97 000), High Molecular Weight (HMW)-SDS Marker Kit (M_r 53 000 to 220 000), and HMW Native Marker Kit (M_r 66 000 to 669 000).

3.3 *Determining the molecular weight of unknown proteins from molecular weight markers*

As SDS-treated proteins migrate through a polyacrylamide gel, there is a linear relationship between the logarithm of the molecular weight and distance travelled.

The molecular weight of an unknown protein can be calculated by plotting the relative distance of migration (Rf) of the markers against the logarithm of their molecular weights (Fig 3.8). From this calibration plot, the M_r of proteins in the samples can be calculated.
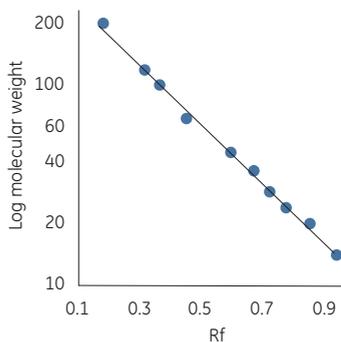
Fig 3.8. Plot showing the relationship between distance migrated and molecular size.

☞   Size determination can easily be performed automatically using ImageQuant™ TL software (see Chapter 8.3).

3.4 *Total protein stains*

As proteins are not directly visible in the gel, the gel must be stained. Proteins are usually stained with a dye such as Coomassie Blue or are silver stained. After staining the gel, a permanent record can be made by imaging the gel with a suitable instrument (see Chapter 7). The captured image can be used for image analysis with appropriate software programs.

Staining the gel post-transfer ensures that the proteins have successfully migrated from the gel to the membrane. Continuing to the blotting step will not be necessary if the proteins have not migrated onto the membrane. For some applications, it is sufficient to analyze the whole protein pattern in the gel directly after electrophoresis, for example, to check the quality of protein purification.

Silver: Silver staining (Fig 3.9) is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels. In silver staining, the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by protein. Silver-stained proteins can be imaged using systems such as Amersham Imager 600.
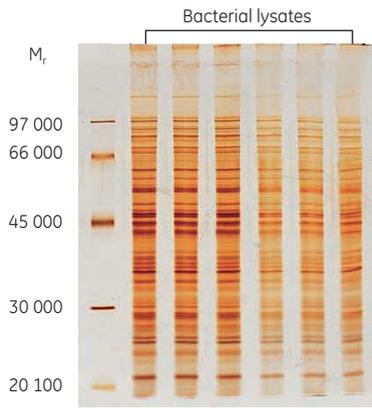
Fig 3.9. An *E. coli* extract was separated on a vertical SDS-polyacrylamide gel and the gel was stained using PlusOne Silver Staining Kit, Protein. The first lane contains Low Molecular Weight Markers.

Coomassie Blue: Coomassie Blue staining is based on the binding of the dye, Coomassie Brilliant Blue, which binds nonspecifically to virtually all proteins. Although less sensitive than silver staining, it is widely used due to its convenience; the gel is simply soaked in a solution of the dye and any unbound dye diffuses out of the gel during the destaining steps. Coomassie Blue-stained protein can be imaged using systems such as Typhoon FLA 7000, Typhoon FLA 9500, or Amersham Imager 600.

Properties of some of the more common protein stains and labels are summarized in Table 3.6.

Table 3.6. Properties of common protein stains and labels

Protein stain or label	Detection method	Detection limit	Dynamic range
Silver	Densitometry, Fluorescence Imager	~ 1 ng	~10
Coomassie Blue	Densitometry, Fluorescence Imager	~ 10 ng	~10^2
SYPRO Ruby	Fluorescence Imager	~ 1 to 2 ng	~$10^{2.5}$
CyDye	Fluorescence Imager	~ 0.25 ng	~10^4

3.5 General polyacrylamide gel electrophoresis (PAGE) protocol

1. Prepare your samples by adding sample loading buffer and heating at 95°C for 5 min.

☞ For PAGE under denaturing and reducing conditions: Add SDS and reducing agent to sample loading buffer.

☞ For PAGE under denaturing conditions: Add SDS to sample loading buffer.

☞ For PAGE under native conditions: **No SDS, no reducing agent, no heating!**

☞ Samples may be boiled in sample loading buffer, aliquoted, and stored at -20°C for 3 to 4 w or at 4°C for 1 w. Warm the sample to 37°C for a few minutes before analysis to resolubilize precipitated SDS.

☞ Always check the protein concentration before loading, as overloading might result in "vertical streaking".

2. Place the gel in the electrophoresis equipment and add the appropriate running buffer.

3. Remove the comb and rinse out the wells with running buffer.

☞ Washing removes unpolymerized acrylamide that can disturb sample loading.

4. Load your samples and molecular weight markers in the wells.

☞ Centrifuge all samples in a microfuge tube at 12 000 × g for 2 to 5 min prior to loading to remove any aggregates.

☞ To make it easier to load samples, use thin tips or, alternatively, a Hamilton syringe with a long thin needle.

☞ Remember to load any empty wells (no sample or markers) with sample loading buffer.

☞ If it is difficult to see the wells when using a vertical setup; hold a piece of paper behind the tank when you are loading your samples to improve the contrast.

5. Place the safety lid on the unit and plug the color-coded leads into the jacks in the power supply.

6. Run the gel under appropriate conditions.

7. When the tracking dye reaches the bottom of the gel, turn off the power supply, disconnect the leads, and remove the safety lid.

8. Release the gel cassette from the electrophoresis apparatus and proceed to the next step in your application.

3.6 References

1.   Schägger, H. and von Jagow, G. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-100 kDalton. *Anal. Biochem.* **166**, 368–379 (1987).

2.   Wiltfang, J. *et al*. A new multiphasic buffer system for sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins and peptides with molecular masses 100,000-1000, and their detection with picomolar sensitivity. *Electrophoresis* **12**, 352–366 (1991).

3.   *2-D Electrophoresis, Principles and Methods,* GE Healthcare, 80-6429-60, Edition AD (2010).

Chapter 4
Transfer

| Sample preparation | Gel electrophoresis | Transfer | Antibody probing | Detection | Imaging | Analysis |

The method of immobilizing and detecting proteins on a solid support, following separation by electrophoresis on a gel, originated in the laboratory of George Stark at Stanford University (1). The term, "Western blotting", was applied specifically to the transfer of proteins and their detection by antibodies (2) and was presumably coined to indicate its relationship to a similar technique used for the detection of DNA; this method was called Southern blotting and was named after its inventor. This family of related techniques has continually expanded to include Northern blotting (RNA), Eastern blotting (post-translational modifications), Far Western blotting (protein:protein interactions), and Far Eastern blotting (lipid detection). Here, we are concerned exclusively with Western blotting and will describe the different options used to transfer proteins from gels to membranes. The focus, however, is placed on electrotransfer, as this is the most commonly used contemporary method in Western blotting workflows.

4.1 Protein transfer

On completion of the separation of proteins by polyacrylamide gel electrophoresis (PAGE), the next step is to transfer the proteins from the gel to a solid support membrane, usually made of a chemically inert substance, such as nitrocellulose or PVDF. Blotting makes it possible to detect the proteins on the membrane using specific antibodies. The proteins transferred from the gels are immobilized at their respective relative migration positions at the time point when the electric current of the gel run was stopped.

4.1.1   Electrotransfer

Electrotransfer is almost exclusively the contemporary transfer method of choice (3) due to its speed, uniformity of transfer, and transfer efficiency. Electrotransfer relies on the same electromobility principles that drive the migration of proteins during separation in PAGE. The gel, membrane, and electrodes are assembled in a sandwich so that proteins move from gel to membrane where they are captured in a pattern that perfectly mirrors their migration positions in the gel (Fig 4.1).

Transfer setup

Blotting stack on the cathode (-) side of the gel

Blotting stack on the anode (+) side of the membrane

Cathode (-)

Anode (+)
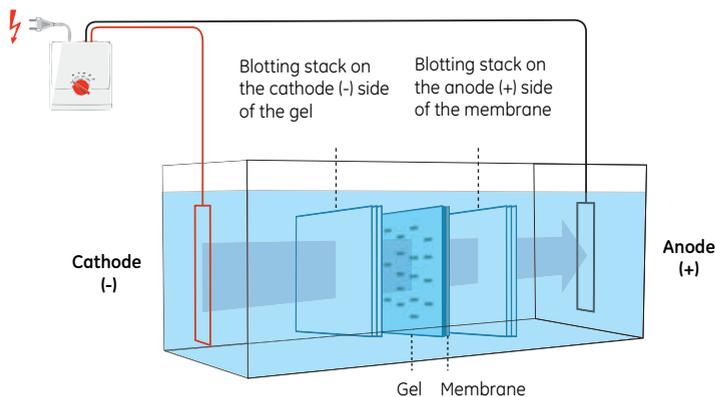
Gel   Membrane

Post-transfer

Gel: No protein bands remain after effective electrotransfer

Membrane with copy of band pattern from gel
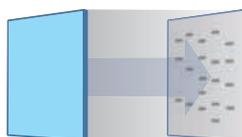
Fig 4.1. Transfer of proteins from gel to membrane. The gel is placed in contact with the membrane and the proteins then migrate toward the positively charged anode (+) in an electric field.

It is important that transfer is as uniform as possible across the entire area of the gel:membrane sandwich in order to ensure that large and small proteins are transferred with similar efficiency. The use of gradient gels can optimize uniformity of transfer but the disadvantage is that it is more difficult to transfer the proteins, as they are more strongly "locked" into the polyacrylamide matrix. This is a particularly important consideration for the quantitative analysis of proteins.

Electrotransfer is fast, efficient, and simple to perform using any of the many commercially available transfer systems available on the market. In addition, it is simple to regulate and standardize the transfer conditions once the parameters such as transfer time and applied current are optimized. Two types of electrotransfer, known as wet transfer and semidry transfer, are in common usage in laboratories today. GE Healthcare provides both tank blotting instruments for high performance wet transfer as well as semidry blotting instruments. The latter semidry blotting instruments require low voltages and minimal buffer volumes and do not require cooling (Table 4.1).

Table 4.1. Instruments from GE Healthcare for wet and semidry transfer

	miniVE	TE 22	TE 62	TE 70	TE 77	Multiphor II NovaBlot Kit
Transfer type	Wet	Wet	Wet	Semidry	Semidry	Semidry
Heat exchanger (cooling)	Passive	Built-in	Built-in	N/A	N/A	N/A
Buffer volume (l)	0.3	1.0	5.0	0.2	0.2	0.2
Maximum gel size (cm)	9 × 10	9 × 10	15 × 21	14 × 16	21 × 26	20 × 25
Number of gels	4	4	4	1	2	1[1]
Recommended power supply	EPS 301	EPS 2A201	EPS 2A201	EPS 301	EPS 2A201	EPS 301

[1] *Up to four 10 × 8 cm gels can be blotted simultaneously.*

miniVE Vertical Electrophoresis System: Electrophoresis and electrotransfer in one compact instrument, with only four major parts: A gel module; a blot module (optional); a common lower buffer chamber; and a safety lid. You can use two gel modules or two blot modules at one time.

TE 22 Mini Tank Transfer Unit: Electrotransfer of proteins from four mini-gels simultaneously to membranes using only one liter of buffer. The instrument contains a heat exchanger with a magnetic stirrer, which circulates the buffer, ensuring uniform temperature during transfer. Cooling via the heat exchanger is only effective if used in conjunction with a recirculating water bath, such as a MultiTemp™ Thermostatic Circulator (also applies to the TE 62 Transfer Unit.

TE 62 Transfer Unit: Uses transverse electrophoresis to transfer proteins from polyacrylamide slab gels onto nitrocellulose, PVDF, or nylon membranes. The unit transfers four 15 × 21 cm gels or up to sixteen 7 × 10 cm mini-gels at one time.

TE 70/TE 70 PWR and TE 77/TE 77 PWR Semidry Transfer Units: Designed for electrotransfer using low current and voltage with a minimal amount of buffer. The TE 70 PWR/TE 77 PWR units come with a built-in power supply and offer a novel automatic stopping feature; when the buffer becomes depleted, the transfer is stopped automatically, avoiding overheating. The units are limited to 30 V. TE 70 is suitable for gels up to 14 × 16 cm, while TE 77 accepts gels up to 21 × 26 cm or four mini-gels, side-by-side. Transfer is complete in less than 1 h.

MultiPhor II NovaBlot Kit: An accessory for the Multiphor II electrophoresis unit for fast, even and efficient semidry transfer of proteins. Transfer is normally complete in about 1 h. Gels up to 20 × 25 cm, or up to four smaller gels (10 × 8 cm) can be transferred. Novel graphite electrodes provide an even distribution of current across the entire surface, ensuring uniform electrotransfer.

4.1.1.1 Wet transfer

In this choice of transfer, the gel and membrane are both fully immersed in transfer buffer and a current is applied in the direction of the gel to the membrane. Generally, wet transfer requires cooling of the unit and internal recirculation of the transfer buffer by the presence of a stirring magnet. Wet transfer is recommended for large proteins, but it is a relatively slow technique, requiring large volumes of buffer. Wet transfer should be applied in preference to semidry transfer when it is important to obtain blots of the highest quality in terms of distinct, sharp bands and efficient transfer. During wet transfer, most buffers become heated, increasing in temperature to a point where proteins might be irreversibly damaged. It is therefore important to start the transfer process using cooled buffer and to maintain a low temperature. For this reason, many commercial wet transfer systems are fitted with enhanced designs to allow

efficient cooling, such as cooling coils or ceramic heat exchange plates, when connected to an external temperature-controlled recirculating water bath. Another common procedure is to perform the entire wet transfer in a 4°C environment, such as a cold room.

Western blotting: Wet transfer

Assembly of the sandwich is the step in Western blotting where most mistakes are made. At this stage, it is important to consider the position of the gel in relation to the membrane and the anode (+) to ensure that the proteins end up on the membrane. If the membrane lies between the gel and the cathode (-), the negatively charged proteins (from the SDS coating) will migrate to the anode (+) and will not be transferred to the membrane. To avoid this, try to assemble the sandwich exactly the same way with the membrane between the gel and the anode (+) every time until it becomes a routine that you can easily perform.

1. Cut away the stacking gel and cut one corner from the resolving gel. Note which corner you have cut, for example, bottom right. This will enable you to correctly orientate the gel if it flips over during equilibration.

2. Equilibrate the gel in transfer buffer.

3. Prewet and equilibrate the membrane in transfer buffer.

☞ PVDF membranes need to be prewetted in methanol and water before equilibration in transfer buffer.

☞ Always wear gloves when handling membranes to avoid fingerprints, which will negatively affect results.

4. Place the blotting cassette in a tray filled to a depth of 3 cm with chilled transfer buffer. Assemble the transfer stack so that proteins migrate toward the membrane. For negatively charged proteins, build the stack on the half of the cassette that will face the anode (+).

5. Prewet a sponge and place it on the submerged part of the cassette. Press gently until all air is expelled.

6. Place two prewetted blotting papers on to the sponge.

7. Place the membrane on top of the blotting papers.

8. Place the gel on top of the membrane.

9. Place two additional prewetted blotting papers on the gel.

10. Finally place a prewetted sponge on top of the stack and close the cassette, after gently pressing to remove air bubbles.

☞ The assembled cassette should hold the gel in firm contact with the membrane without squeezing the gel. If the stack seems loose, add sheets of blotting paper; if the stack seems tight, replace the top sponge (above the gel) with a sheet of blotting paper. If you remove the bottom sponge (below the membrane), substitute at least two sheets of blotting paper to create space between the membrane and the cassette panel.

11. Add prechilled transfer buffer to the transfer tank.

☞ A stirring magnet can be used to circulate the buffer during transfer.

✋ Do not drop the stirring magnet into the tank - place it gently in the transfer tank to avoid damaging the ceramic heat exchanger!

12. Place the cassette in the transfer tank.

☞ Again, think about the orientation! The membrane should be closest to the anode (+) (red lead).

☞ Work quickly when moving the assembled cassette to the tank to avoid draining the sponges.

☞ Place the tray holding the cassette near the tank and slide it into a set of vertical slots.

13. Connect the transfer tank to the power supply and transfer according to the recommendations of the manufacturer. Ensure that the transfer tank is filled to the correct height with transfer buffer.

The setup of a wet transfer is illustrated in Figure 4.2.

Sponge
Upper filter paper stack
Gel
Membrane
Lower filter paper stack
Sponge

Fig 4.2. Wet transfer system. Assembly of the transfer sandwich is best performed in a tank filled with transfer buffer to a depth of at least 3 cm. The sandwich is built on the side of the transfer cassette facing the anode (+) and starts with a sponge, followed by two wetted filter papers, the membrane of choice, the gel, two additional wetted filter papers, and, finally a second sponge. Take care to avoid wrinkles, folds, or air bubbles between the different layers of the sandwich. This construct is then securely fixed in the transfer cassette and submerged in an electrotransfer tank containing transfer buffer. The orientation of the construct must be so that the membrane is on the anode (+) side of the gel.

4.1.1.2   Semidry transfer

Semidry transfer is faster than wet transfer and, in addition, consumes less buffer. The membrane is placed in direct contact with the gel and several layers of filter paper soaked in transfer buffer are placed above and below the gel and membrane. The filter paper:gel:membrane:filter paper layers are then sandwiched between two plates that form an anode (+) and a cathode (-) when an electric field is applied. Semidry transfer is usually less efficient than wet transfer, especially for large proteins (Fig 4.3). Heating is less of a problem with semidry transfer for normal transfer times, as the electrode plates adsorb heat, but semidry systems should be avoided for extended transfer times as this might lead to overheating and gel drying due to buffer depletion.

The filter papers and the membrane should be carefully cut to be a few millimeters smaller than the length and breadth of the gels. This ensures that the the papers and membranes do not overlap the gel, forming a potential short cut for the current, leading to inefficient or uneven transfer of proteins.

☞   The use of a plastic manifold with an opening cut to exactly the same size as the gel can be used to limit the chance of current bypassing the stack.

Wet transfer   **Semidry transfer**

Fig 4.3. Comparison of blots following wet and semidry transfer. A two-fold dilution series of transferrin starting at 5 µg was transferred to Amersham™ Hybond P (now replaced by Amersham Hybond P 0.45) membranes (PVDF). Semidry transfer is less efficient for this particular protein than wet transfer; less protein throughout the dilution series is transferred, leading to reduced sensitivity.

Western blotting: Semidry transfer

The procedure described below is for the semidry transfer of negatively charged proteins.

1. Cut away the stacking gel and cut one corner from the resolving gel. Note which corner you have cut, for example, bottom right. This will enable you to correctly orientate the gel if it flips over during equilibration.

2. Equilibrate gel in transfer buffer.

3. Cut at least six pieces of blotting paper the same size as the gel or slightly smaller.

   - Estimate the amount of buffer required according to the thickness or number of blotting paper layers. Each 21 × 26 cm piece of blotting paper will absorb approximately 50 ml of transfer buffer. Each 14 × 16 cm blot paper will absorb approximately 20 ml.

4. Saturate at least three pieces of blotting paper with transfer buffer. One by one, center each sheet on the lower electrode (the anode [+]) and remove all trapped air by rolling a clean pipette or roller from the center of the papers toward the edges.

5. Cut one membrane the same size as the gel or slightly smaller. Prewet and equilibrate the membrane in transfer buffer.

   ☞ PVDF membranes need to be prewetted in methanol and water before equilibration in transfer buffer.

   ☞ Nitrocellulose membranes should be prewetted in distilled water before equilibration in transfer buffer.

   ✋ Always wear gloves when handling the membranes to avoid fingerprints.

6. Place the prewetted membrane onto the stack of wetted blotting paper.

   ☞ The blotting paper and membrane must be the same size as the gel or 1 to 2 mm smaller in length and breadth. If the blotting paper or membrane are larger that the gel, the overlap will provide an electrical path for the current to bypass the gel, resulting in poor, uneven or a complete lack of transfer of proteins from gel to membrane.

7. Place the gel on the membrane.

   ☞ Proteins bind to the membrane as soon as contact occurs, so it is important to place the gel correctly.

8. Cover the gel with three layers of saturated blotting paper.

   ☞ Stack each layer with care, with edges parallel. As each layer is added, remove any trapped air by rolling a clean pipette from the center to the edges. Add a few drops of buffer to local areas of the stack; this makes it easier to press out any stubborn air pockets.

9. Connect color coded leads to the power supply.

   ☞ Before connecting the leads to the power supply, ensure that the power is switched off and that both the current and voltage controls are set at zero.

10. Set the power supply current and timer according to the recommendations of the manufacture. Start the transfer.

The setup of a semidry transfer is illustrated in Figure 4.4.

Cathode (-)

Upper filter paper stack

Gel

Membrane

Lower filter paper stack
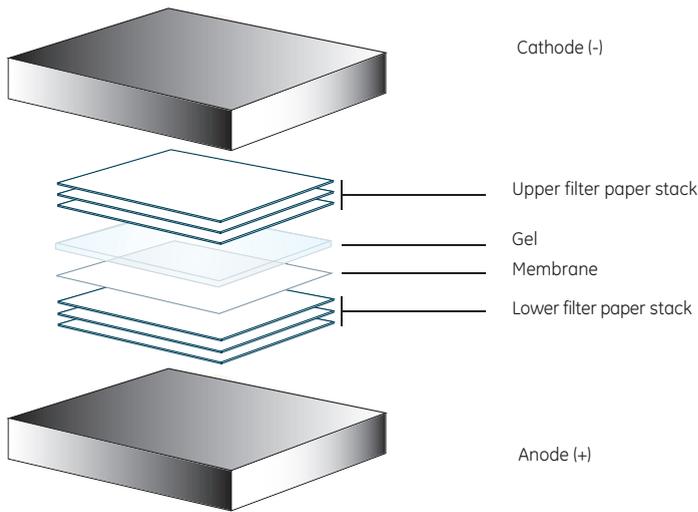
Anode (+)

Fig 4.4. Semidry transfer system. The sandwich is built on the lower electrode (anode [+]). The first layer consists of at least three pieces of wetted filter papers, followed by the membrane, the gel and, finally, three additional wetted filter papers. As each layer is added, take care to avoid wrinkles, folds or trapped air bubbles. This construct is placed and compressed between two flat conduction plates that also serve as cathode (-) and anode (+). The orientation of the construct must be so that the membrane is on the anode (+) side of the gel.

☞ When performing electrotransfer, ensure that no air bubbles form when applying the gel to the membrane. Bubbles will cause blank spots on the membrane where no protein transfer occurs.

☞ Use precooled transfer buffer to counter the generation of potentially damaging heat.

☞ Apply a membrane to both sides of your gel to avoid losing your proteins if you set up the stack incorrectly, or connect the power supply incorrectly. Colored markers will help you determine to which of the membranes the proteins have migrated!

☞ Use two membranes if working with small proteins as they can transfer through the membrane.

4.1.2   Diffusion transfer

This method of transfer has been shown to lead to bands of high resolution when transferring DNA-binding proteins (4). Diffusion transfer is a nonelectrophoretic transfer technique and its main application, although markedly less efficient than electrotransfer, is to obtain multiple blots from a single gel. Up to 12 blots containing proteins from M_r 60 000 to 240 000 have been obtained using diffusion transfer (5).

An additional benefit of diffusion transfer is that it is mild and can be considered where retention of protein function is critical. Diffusion transfer should also be considered if the process of electrotransfer is suspected of altering the antigenicity of the blotted proteins and render them less susceptible to detection with antibodies.

With diffusion blotting, it is possible to analyze only a limited proportion of the transferred proteins on the membrane as this type of transfer is at best 30% efficient. The advantage of the technique, however, is that only a small amount of protein needs to be present on the membrane to be detected by the very sensitive techniques used for blots. The protein remaining in the gel can then be used for detection using less sensitive staining techniques, or excised for manipulations such as analysis by mass spectrometry.

4.2 Transfer buffers and running conditions

Several alternative recipes for transfer buffers are described in the literature, and the optimal choice depends on the application. Transfer buffer should act as an electrically conducting medium in which proteins are soluble and it should not interfere with binding of the proteins to the membrane. Depending on the pH of the buffer, transfer can be directed either toward the cathode (-) or the anode (+). Further, the degree to which molecules bind to the membrane is affected by buffer characteristics such as salt type and concentration, methanol concentration, and the presence of detergents such as SDS.

☞   If the transfer buffer and electrophoresis buffer systems differ, the gel should be equilibrated with transfer buffer before the act of transfer to ensure that any swelling or shrinking occurs before the gel contacts the membrane. The omission of this step may lead to band distortion or loss of band resolution.

Most transfer buffers contain methanol. The addition of **methanol** is necessary to achieve efficient binding to the membrane, particularly nitrocellulose membranes (6). The improved binding is partly a result of the removal of protein-bound SDS. However, methanol might cause a gel to shrink, resulting in a decreased rate of protein elution. This effect is more pronounced with the transfer of large proteins. A low methanol concentration, in combination with a longer equilibration period prior to transfer, is thus recommended for the transfer of large proteins.

☞   Buffers containing methanol can deteriorate if stored for long periods - add methanol just prior to transfer.

☞   Methanol should be analytical grade - low grade methanol contains metallic contaminants that can be deposited on the electrodes.

SDS is detrimental to the binding of proteins to membranes, and the general rule is that all excess SDS should be removed from the gel prior to transfer by equilibration for 15 to 30 min in transfer buffer. However, it may be necessary to include low amounts of SDS (0.02% to 0.1%) in the transfer buffer if the proteins are only partially insoluble due to high molecular weight or if they contain a surplus of hydrophobic amino acids.

☞   If the sample elution rate is slow, a longer transfer period will be required. If sample binding is inadequate, try different buffer conditions.

☞   Ethanol may be used in place of methanol, and is more environmentally friendly.

The most widely used buffer system for protein transfer is the classical Towbin buffer (192 mM glycine, 25 mM Tris, 20% methanol (V/V)). This low ionic strength buffer has a pH of 8.3, which is higher than the isoelectric point (pI) of most proteins, resulting in migration of the negatively charged proteins toward the anode (+). One alternative, recommended for sequencing applications, is a buffer containing N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) at pH 11. The use of this buffer reduces the problem of high background sometimes generated by the glycine in Towbin buffer when used in combination with the Edman chemistry of sequencing reactions (7).

A general guideline is to use Towbin buffer in a continuous buffer system, but as recommendations vary between manufacturers, the instructions provided with each system should be followed. A discontinuous buffer system used together with semidry transfer can improve results. One example of a discontinuous buffer system is based on the isotachophoresis theory (8). Here, two Tris buffers at different concentrations, but with identical pH, are used as the anode (+) buffer. The cathode (-) buffer has a lower pH and contains acid. The function of the anode (+) buffer is to neutralize excess protons generated on the surface of the anode (+) plate. The acid in the cathode (-) buffer migrates through the gel toward the anode (+) during transfer and serves as a trailing ion.

The pH of Towbin and Laemmli buffers, usually around pH 8.3, should never be adjusted with acid or base. This will lead to conductivity issues which can seriously disrupt the experiment and severely damage the apparatus.

4.2.1   Notes on transfer of large and small proteins

The relative content of SDS and methanol in the transfer buffer, protein size, and the percentage of acrylamide in the gel can all affect transfer efficiency. The following steps can be followed to help optimize transfer efficiency:

For large proteins (M_r > 100 000)

Use low acrylamide concentration in gels
Transfer of proteins from gel to membrane can be slow, just as proteins can run slowly within the gel during separation. If blotting a large protein, be sure to run your samples in a gel with a low concentration of polyacrylamide. Low density gels are fragile, and must be handled carefully.

A little more SDS, a little less methanol
Large proteins tend to precipitate in gels, hindering transfer. Adding SDS to a final concentration of 0.1% in the transfer buffer will discourage this. In addition, methanol weakens the interaction between SDS and proteins, so reducing methanol to 10% or less will reduce the risk of precipitation.

Choose wet transfer
…and do it slowly, for example, by lowering the voltage or current, and running the transfer for longer. In addition, performing transfer at 4°C will help counter any unwanted effects of generated heat, such as gel distortion.

For small proteins (M_r < 100 000)

Remove SDS from transfer buffer
All proteins are hindered from binding to membranes by SDS, but this is especially true for small proteins.

Keep the methanol content at 20%
This will help remove as much SDS as possible and improve transfer efficiency.

4.2.2   Current and transfer time

Current and transfer time are important parameters. Insufficient current (or voltage) as well as insufficient transfer time can lead to incomplete protein transfer, whereas excessive transfer time can lead to protein loss - particularly for smaller proteins that pass straight through the membrane without binding (a phenomenon known as "blow through"). Excessive current (or voltage) will lead to the potentially problematic generation of heat.

If long transfer times are required, wet transfer should be used as it will be necessary to counter the inevitable increase in temperature by proactive cooling. Semidry transfer should not be used for extended transfer as the small quantities of buffer present between the plates will eventually dry out, leading to cessation of transfer and potentially irreversible damage to both the blot itself and the blotting apparatus. In general, wet transfer is performed at constant voltage and the resultant increase in current during the course of transfer leads to heating. To avoid heating, use prechilled transfer buffer and run the transfer in a cold room. Some transfer equipment has cooling elements or can be used with ice blocks. Stirring will help evenly distribute the regulated temperature throughout the transfer buffer volume. Semidry transfer is run at a constant current of 0.8 mA/cm^2. A higher current will risk damage to the blot and apparatus.

4.2.3   Monitoring and optimizing novel blotting protocols

The standard procedures for wet transfer and semidry transfer described above work well for most blots but if you experience problems with your particular protein, there are a number of steps you can try:

☞ To transfer buffer, add 0.1% SDS to enhance and maintain the solubility of larger proteins and 20% methanol to enhance adsorption to the membrane.

☞ Try biphasic transfer (for wet transfer only). This means running a first transfer at low current (1 mA/cm^2) for 1 h to reduce the rate of transfer, allowing longer residence time of the proteins in the membrane. This might improve the retention of smaller proteins. To transfer larger proteins, a second period of higher current (3.5 to 7.5 mA/cm^2) may be applied.

☞ Stain the gel after transfer to verify that all proteins are completely eluted. By including a lane with prestained markers, transfer efficiency to the membrane can be monitored. Additionally, the transfer can be verified by treating the membrane with a reversible stain, such as Ponceau S.

☞ Place a membrane on the cathode (-) side of the gel to detect transfer of proteins, such as histones and ribosomal proteins that are positively charged in the transfer buffer. This is only appropriate in the absence of SDS and requires an extended period of equilibration in transfer buffer or, alternatively, use of a native gel. The method is applicable when transferring proteins that have a pI higher than the pH of the buffer.

☞ Place two membranes in sequence to capture smaller proteins that have passed through the first membrane (blow-through).

4.3 Membranes

In addition to buffer characteristics such as pH, salt type, salt concentration, and the presence of detergents such as SDS, the degree to which molecules bind to a membrane is influenced by the physical and chemical characteristics of the membrane itself. The properties of a protein affect its ability to bind to membrane surfaces. Finding the right membrane requires testing of your specific protein on different membranes.

In general, membranes are porous materials with pore sizes from 0.05 to 10 µm in diameter. The binding capacity of a membrane depends primarily on the pore size. A membrane with many small pores has a larger binding surface than one with larger pores, and thus generally has a higher binding capacity. The exact mechanism by which biomolecules interact with the membrane is not known, but it is assumed to be a combination of noncovalent and hydrophobic forces. It should be noted that although protein conformation and buffer composition also affect binding capacity, the overall sensitivity of a Western blot depends on the amount of protein immobilized on the membrane and presented to the primary antibody. Note, however, that excessive protein binding does not necessarily lead to improved signals in immunoblotting and can in fact have the opposite effect. This is because proteins concentrated at high density tend to self-associate through weak interactions rather than interacting with the membrane surface. This means that protein:antibody complexes, the formation of which lies at the heart of the detection principle in Western blotting, are easily lost from the membrane during washing steps.

Nitrocellulose and PVDF membranes are the most common types of membranes used for Western blotting, although nylon-based membranes are also sometimes used.

Nitrocellulose membranes are the most frequently used and their main advantage is the low background, no matter the detection method applied. The exact mechanism by which biomolecules interact with the membrane is not known, but it is assumed to be a combination of noncovalent and hydrophobic forces. Note that inclusion of methanol in the transfer buffer improves protein binding to nitrocellulose membranes. Nitrocellulose membranes are not recommended for multiple stripping and reprobing, as they become brittle and difficult to handle when dry. However, mechanical strength has been improved by the incorporation of a polyester support web to membranes such as as Amersham Protran Supported.

PVDF membranes have higher protein binding capacity and mechanical strength and are ideal for Western blotting applications where stripping and reprobing are needed. As PVDF membranes are highly hydrophobic, they need to be prewetted in either methanol or ethanol before use to be compatible with aqueous solutions. Proteins bind to PVDF membranes via a combination of dipole and hydrophobic interactions. PVDF membranes tend to have a higher background due to their higher protein binding capacity.

✋ Always handle membranes with gloves or forceps to avoid contaminating the membrane with proteins from your fingers. The smearing and contamination caused by touching the membrane will interfere with the signal from the proteins of interest.

Nylon-based membranes are a possible alternative to nitrocellulose and PVDF. Nylon-based membranes improve durability, mechanical strength, binding capacity, and sensitivity compared with nitrocellulose and PVDF. However, nylon is rarely chosen for protein transfer due to considerable problems of unwanted background binding. In addition, it is not possible to destain nylon of some of the most commonly used anionic dyes such as Coomassie Brilliant Blue or Ponceau S.

A summary of some of the most important features and benefits of nitrocellulose, PVDF, and nylon membranes are shown in Table 4.2.

Table 4.2. Some important characteristics of membranes commonly used in Western blotting

Membrane	Interaction mode	Optimal immobilization conditions	Staining options	Advantages	Disadvantages
Nitrocellulose	Noncovalent or hydrophobic	High salt/low methanol	Amido black Aniline blue black Ponceau S Colloidal gold Fast green Toluidine blue	Highly versatile Low background	Fragile – limited possibilities to strip and reprobe Not recommended for small proteins due to large average pore size
PVDF	Dipole and hydrophobic interactions	Prewet in methanol before using with aqueous buffers	Amido black India ink Silver Coomassie Brilliant Blue Ponceau S Colloidal gold	Suitable for small proteins High protein-binding capacity Mechanical strength Chemical stability	
Nylon				High protein-binding capacity Mechanical strength	Background Anionic dyes bind irreversibly

4.3.1 Nitrocellulose membranes from GE Healthcare

The Amersham Protran Western blotting transfer membrane family are Schleicher & Schuell™ Inc. legacy products. Amersham Protran family membranes are composed of high-quality nitrocellulose , which is 100% pure. The sheets and rolls are available in three pore sizes with high surface area and excellent uniformity. Amersham Protran membranes have good binding properties for Western blot, dot-blot assays, and other protein or nucleic acid methods.

Amersham Protran nitrocellulose membranes are available in 0.1, 0.2, and 0.45 µm pore sizes in a variety of package sizes containing rolls, precut sheets, and sandwiches for most applications. The Amersham Protran nitrocellulose membrane is a binding matrix for Western blotting with high affinity for proteins, blocking ability, and compatibility with a range of detection methods (e.g., chemiluminescence, chromogenic, fluorescence).

In general, binding affinity for small peptides increases with decreasing pore size. Consequently, both Amersham Protran 0.1 and Amersham Protran 0.2 are good choices for working with small proteins and peptides. The Amersham Protran 0.1 membrane has a minimal pore size of 0.1 µm, exhibits excellent binding affinity for small peptides (M_r < 10 000) and nucleic acids (< 300 bp), and produces very low background in chemiluminescent Western blotting.

Amersham Protran Premium (0.2 and 0.45 µm pore size) membranes give high sensitivity, resolution, and low background for all labeling and detection systems (chemiluminescence, fluorescence, colorimetric, and radioactive). The low background enables a dynamic range for complex samples.

Amersham Protran Supported (0.2 and 0.45 µm pore size) membranes consist of nitrocellulose supported by an inert polyester nonwoven material within the membrane. The support does not affect transfer conditions or results but gives the membrane exceptional handling characteristics, allowing reprobing.

Amersham Protran membranes are compatible with several different staining methods such as Amido black, Aniline blue black, Ponceau S, Colloidal gold, Fast Green, and Toluidine blue.

4.3.2   PVDF membranes from GE Healthcare

Amersham Hybond (0.2 and 0.45 µm pore size) are robust and chemically stable membranes that are an excellent choice for stripping and reprobing. The small pore size of the 0.2 µm membrane eliminates blow-through and increases protein binding over a wide range of molecular weights. This membrane is compatible with a range of solvents used for rapid destaining. Amersham Hybond should be immersed in 100% methanol, and then soaked in ultrapure water before use.

Amersham Hybond LFP (0.2 µm pore size) membrane is a hydrophobic PVDF membrane specifically optimized for fluorescence detection in Western blotting applications. It has low background fluorescence, resulting in high sensitivity. This membrane must be activated with methanol and is particularly recommended in combination with detection using Amersham ECL Plex™.

Amersham Hybond SEQ (0.2 µm pore size) membrane is designed specifically for protein sequencing applications.

☞ In most cases, it is possible to replace methanol with ethanol in the preparation of PVDF membranes.

4.3.4 Membrane selection guide

Table 4.3. Membranes for protein blotting

	Material	Physical strength	Binding capacity ($\mu g/cm^2$)	Pore size (μm)	Minimal protein size (M_r)	Recommended detection
Amersham Protran	Nitrocellulose	-	80 to 150	0.1, 0.2, or 0.45		Colorimetric Chemiluminescence Isotopic Fluorescence
Amersham Protran Premium	Nitrocellulose	-	100	0.2 or 0.45	> 2000 > 7000	Chemiluminescence Fluorescence Colorimetric
Amersham Protran Supported	Supported nitrocellulose	+	75 to 90	0.2 or 0.45	> 2000 > 7000	Colorimetric Chemiluminescence Isotopic
Amersham Hybond 0.2	PVDF	+	> 200	0.2 or 0.45	> 2000	Colorimetric Chemiluminescence Isotopic
Amersham Hybond 0.45	PVDF	+	125	0.45	> 2000 > 7000	Chemiluminescence Chemifluorescence
Amersham Hybond LFP 0.2	PVDF	+	400	0.2		Fluorescence
Amersham Hybond SEQ 0.2	PVDF	+	> 200	0.2	> 7000	Colorimetric Chemiluminescence Isotopic

Table 4.4. Membrane compatibility with different detection methods

	Nitrocellulose membranes			PVDF membranes			
	Amersham Protran	Amersham Protran Premium	Amersham Protran Supported	Amersham Hybond 0.2	Amersham Hybond 0.45	Amersham Hybond LFP 0.2	Amersham Hybond SEQ 0.2
Amersham ECL	++	+++	+	++	+++	+	++
Amersham ECL Prime	++	++	+	++	+++	nt	++
Amersham ECL Select	++	++	+	++	+++	nt	++
Amersham ECL Plex	++	+++	-	-	-	+++	-
Colorimetric	++	+++	+	++	++	nt	++
Radioactive	++	+	++	++	+	nt	++

Suitable = +, Recommended = ++, Highly recommended = +++, Not recommended = -, nt = Not tested

[1] *Amersham Hybond LFP 0.2 is developed for fluorescence and has not been validated for other detection reagents.*

4.4 Confirmation of protein transfer to the membrane

After electrotransfer, it is necessary to confirm that all the proteins in the gel have been completely eluted. This can be achieved by staining the gel using a total protein stain after electrotransfer. To ensure that all the proteins are on the membrane (and have not passed through or migrated in the opposite direction!), the membrane can also be treated with a total protein stain. The use of visible (prestained) molecular weight markers such as Rainbow Molecular Weight Markers or Amersham ECL DualVue Western Blotting Markers is a very simple and convenient way to check that all proteins across the range of molecular weights on the gel have transferred to the membrane, as high and low molecular weight proteins might migrate with different efficiencies under similar electrophoretic conditions. When using Amersham ECL DualVue markers that contain specially tagged proteins that only appear at chemiluminescent detection, the molecular weight of the protein of interest can be assigned.

4.4.1 Total protein stains

In Western blotting, it is often useful to check by staining that the transfer from gel to membrane has worked and that transfer efficiency is evenly distributed over the entire membrane. Total protein stains can also be used to enable quantitative comparison between lanes of specific bands of interest. Some commonly used stains are listed and briefly described below.

Colloidal gold
A highly sensitive stain for blotted proteins, with limit of detection (LOD) down to 1 ng of protein. Colloidal gold be used on both nitrocellulose and PVDF membranes. The low pH of the colloidal gold ensures that the particles bind selectively to proteins on membranes. Signals can be further enhanced using a silver enhancement reagent.

Coomassie Brilliant Blue
Coomassie Brilliant Blue is generally applied to polyacrylamide gels but they are applicable to membranes also. PlusOne Coomassie Blue PhastGel R-350 Tablets from GE Healthcare can detect most proteins at levels of 50 to 100 ng/band. The membranes are stained using 0.1% Coomassie dye dissolved in a mixture of 45% methanol, 45% water, and 10% acetic acid. The background is then destained using a mixture of 25% methanol, 65% water, and 10% acetic acid.

Ponceau S
Offers a quick and easy reversible staining method for Western blots, but sensitivity is poor with an LOD greater than 250 ng of protein. Ponceau S is compatible with both nitrocellulose and PVDF membranes. Ponceau S staining is a quick and easy way to visualize proteins transferred to membranes following PAGE. Ponceau S is easily removed with water and is regarded as a "gentle" treatment that does not interfere with subsequent immunological detection steps. Note that when using PVDF membranes in combination with SDS-PAGE, it is important to ensure that the membrane is washed in 100% methanol after transfer and before incubating in Ponceau S solution, as this stain is incompatible with SDS.

India Ink
A cheap, sensitive staining method and does not interfere with subsequent binding of antibody to the antigen. Block the membrane in PBS-Tween (0.05%) before staining to avoid excessive background. Alternatively, dilute in PBS-Tween (0.05%) with 1% acetic acid as a staining solution.

Amido Black

Designed for rapid staining of protein on membranes. The sensitivity of Amido Black is similar to that of Coomassie Blue, but it stains faster. It is the preferred stain for protein sequencing and *in situ* cleavage of proteins for the determination of internal sequences because the mild staining and destaining conditions minimize the likelihood that any protein will be extracted during treatment. Proteins can be easily destained using 25% isopropanol and 10% acetic acid.

Many stains are available on the market, several of which are supplied by GE Healthcare (Table 4.5).

Table 4.5. Stains used for the detection of proteins on membranes

Stain	Advantages	Disadvantages	Notes
Colloidal gold	Compatible with nitrocellulose and PVDF membranes		Highly sensitive stain for blotted proteins
Coomassie Brilliant Blue	Inexpensive, reusable	Proteins might release the dye during background destaining process	Staining times can be reduced by using hot stain and destain solutions
		Proteins destain at different rates	Adsorbant tissues can be placed in the destaining container, which will absorb the dye, increase the destaining procedure and decrease the time of destaining
		Time-consuming	Sensitivity can be increased by staining the membrane with 0.25% Coomassie Brilliant Blue in 50% trichloroacetic acid
		Relatively narrow dynamic range	
Ponceau S	Rapid, stable in storage	Poor sensitivity	Reversible with water
			Recommended for staining prior to immunodetection
India Ink	Rapid, inexpensive staining solution stable for up to 1 mo	Difficult to record photographically due to lack of contrast	Irreversible but does not interfere with immunodetection
Amido Black	Rapid, stable in storage	Risk of membrane distortion	Irreversible
			Not compatible with immunodetection

4.5 References

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Chapter 5
Antibody probing

Sample preparation	Gel electrophoresis	Transfer	Antibody probing	Detection	Imaging	Analysis

Once your protein samples are separated and transferred onto a membrane, the protein of interest is detected and localized using a specific antibody. Usually, Western blotting protocols utilize an unlabeled primary antibody directed against the target protein and a species-specific, labeled secondary antibody directed against the constant region of the primary antibody. The secondary antibody serves not only as a carrier of the label but is also a mechanism to amplify the emitted signals, as many secondary antibodies can theoretically bind simultaneously to the primary antibody (Fig 5.1). This is one of the most effective ways to maximize the potential sensitivity of the assay. For this reason, secondary antibodies are most often polyclonal and can target epitopes on the framework regions of the primary antibody; specificity is thus limited to species and immunoglobulin isotype. The signal emitted by the labeled secondary antibody is then measured and is proportional to the quantity of protein of interest present on the membrane.

With this highly specific immunodetection process, it is possible to reveal the presence of a very low quantity of a specific protein in a complex sample.

Single primary antibody bound to antigen on a membrane

Multiple, labeled secondary antibodies bound to several epitopes on the primary antibody results in an amplification of signal
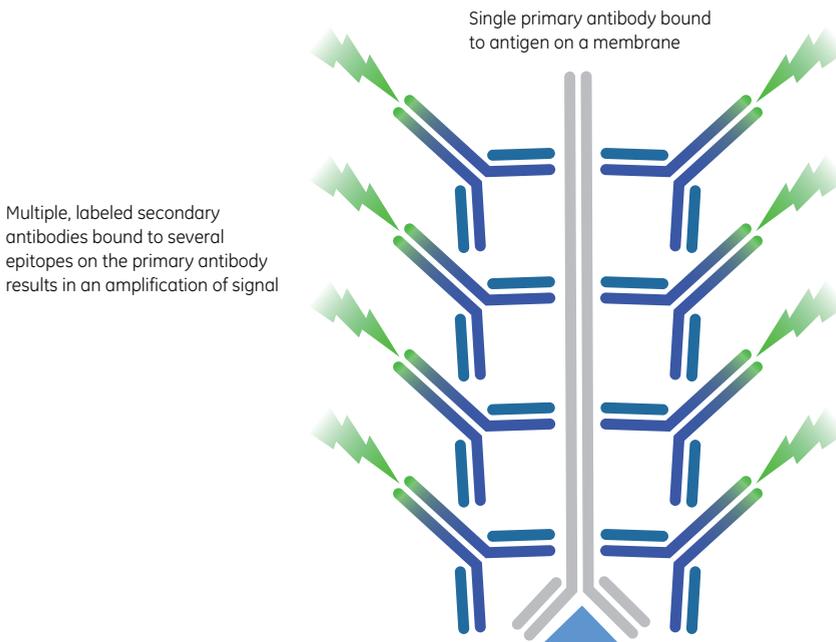
Fig 5.1. As multiple secondary antibodies conjugated to an enzyme or a fluorophore can bind to a single, specific primary antibody, the signal can be greatly amplified, contributing to the sensitivity of Western blotting.

5.1 Blocking

Western blotting involves the immobilization of biomolecules on a membrane via hydrophobic interactions. As nonspecific binding of antibodies to the membrane is detrimental to the specificity and sensitivity of the assay, it is essential to "block" spaces not already occupied by proteins. Your choice of blocking strategy will be guided by your samples and the antibodies used. In order to optimize the blocking step, and depending on your system, you should consider several options among blocking agents and buffers. As a starting point for choosing blocking agents, buffers and optimal conditions, begin by using those recommended by the manufacturers of the detection reagents.

Two main classes of blocking agents (proteins and nonionic detergents) are commonly used for Western blotting.

5.1.1   Proteins as blocking agents

Proteins generally inhibit several interactions: Nonspecific interactions between proteins and the membrane; and interactions between proteins. Proteins are permanent blocking agents as they form a physical attachment to the membrane. It is critical that the blocking agent has a greater affinity for the membrane than the antibodies used in subsequent steps. In addition, the blocking agent should fill all unoccupied binding sites without disrupting binding interactions between transferred proteins and the membrane.

The most common permanent blocking agents include bovine serum albumin (BSA), non-fat milk, normal goat serum, casein, and fish gelatin (see Table 5.1).

Table 5.1. Proteins used as blocking agents in Western blotting

Protein	Recommended concentration	Buffers	Membrane compatibility
BSA	0.2% to 5% (W/V)	Tris-buffered saline (TBS)/phosphate-buffered saline (PBS)	Nitrocellulose Polyvinylidine difluoride (PVDF)
Non-fat milk	3% to 5% (W/V)	TBS, PBS	Nitrocellulose PVDF
Amersham ECL Prime Blocking Reagent	2% to 5% (W/V)	TBS, PBS	Nitrocellulose PVDF
Casein	5% (W/V)	TBS, PBS	Nitrocellulose PVDF
Fish gelatin	2% to 10% (W/V)	TBS, PBS	Nitrocellulose PVDF
Serum	1% to 5% (V/V)	TBS, PBS	Nitrocellulose PVDF

☞　Be aware that the content and quality of dried milk can vary between vendors and also between batches.

As each antibody:antigen pair has unique characteristics, no single blocking agent is ideal for every Western blotting process. Determining the optimal blocking agent and concentration are key steps for the success of immunodetection and it is well worth the effort to spend some time developing this stage of the process in order to optimize the signal-to-noise ratio. This ratio is measured by comparing the signal obtained with a sample containing the target protein to that obtained with a sample containing no target protein. Low concentrations of blocking agent might result in high background and a reduced signal-to-noise ratio. On the other hand, excessive concentrations of blocking agent can mask antibody:antigen interactions, and lead

to much the same effect. If you have not had the opportunity to test for the most effective concentration of blocking agent for your application, you should begin with the concentration recommended by the manufacturer.

If using biotinylated or Concanavalin-conjugated antibodies, non-fat milk should not be used as a blocking agent as milk contains both glyocoproteins and biotin, leading to a decreased signal.

It should also be noted that crude protein preparations can contain phosphatases (which dephosphorylate proteins on specific amino acid residues), making them inappropriate as blocking agents when detecting phosphorylated proteins using phosphospecific antibodies. Where phosphorylated proteins are the targets of interest, phosphatase inhibitors should be added to the blocking solution (1). Additionally, TBS is recommended as a buffer to dilute the blocking agent, as phosphate in PBS can interfere with and therefore reduce phosphospecific antibody binding (see 5.1.3). Non-fat milk should not be used as a blocking agent in the detection of phosphorylated proteins as milk contains casein (and therefore phosphoepitopes), which can interfere with antiphosphotyrosine antibodies and lead to an increase in background signal.

The following list of protein-based blocking solutions may be used as a guide for the optimization of enhanced chemiluminescence (ECL) based detection in Western blotting.

Milk/Tween-20: Combinations of dried milk and Tween-20 are inexpensive and are commonly prepared as 5% dried milk in PBS containing 0.1% Tween-20. Very clean backgrounds can be achieved but care must be taken to avoid disguising some antigens. This preparation is a common blocking agent for ECL based detection in Western blotting.

Dried milk powder: A common blocking agent. Dried milk powder is inexpensive and often gives a very clean background. Dried milk solutions are usually prepared at a concentration of 5% in PBS or TBS. The solution deteriorates rapidly and it can disguise some antigens. Reduction to a concentration of 1% can improve the detected signal.

Fish gelatin: Fish gelatin has fewer hydrogen binding amino groups than gelatin from mammalian sources and hence tends to give lower backgrounds. Fish gelatin is usually used at a concentration of 2%, is easy to dissolve and can be used at 40°C without gelling. It can mask some proteins, however, and contains some competitive reactants, such as biotin. Fish gelatin is also relatively expensive.

BSA: BSA is relatively inexpensive and can allow the generation of optimal signals from specific detected target proteins. It is usually used at a concentration of 0.3% to 3% in PBS or TBS with or without 0.1% Tween-20. A concentration of 2% BSA is recommended if probing with antibodies to phosphotyrosine.

Serum: Horse or fetal calf serum is frequently used at a concentration of 10% in a solution containing 0.02% sodium azide. This blocking solution is expensive and might contain potentially cross-reactive immunoglobulins.

5.1.2   Detergents as blocking agents

Detergents inhibit nonspecific hydrophobic binding of proteins to membranes (Table 5.2). They are considered nonpermanent blocking agents since they do not attach to the membrane and can be removed in a simple washing step. A solution of Tween-20 is commonly used, but alternatives may be considered, such as Triton X-100, sodium dodecyl sulfate (SDS) or NP40, which are especially useful to counter strong background signals. Detergent blocking agents are usually used in conjunction with protein blocking agents.

Table 5.2. Detergents used as blocking agents in Western blotting

Detergent	Recommended concentration	Buffers	Membrane compatibility
SDS	0.02% to 0.05% (W/V)	TBS, PBS	Nitrocellulose PVDF
Triton X-100	1% (V/V)	TBS, PBS	Nitrocellulose PVDF
Tween-20	0.05% to 0.1% (V/V)	TBS, PBS	Nitrocellulose PVDF
NP40	0.02% to 0.05% (V/V)	TBS, PBS	Nitrocellulose PVDF

5.1.3 Buffers for blocking agents

PBS or TBS are commonly used as buffers for blocking agents. Not all blocking buffers are compatible with every blocking agent. For example, for applications using a secondary antibody conjugated to alkaline phosphatase (AP), a blocking agent in TBS should be selected as PBS interferes with the activity of the enzyme. TBS buffers are also preferable to PBS for the detection of phosphorylated target proteins, as the primary antibody can compete for phosphate on the target protein and in the buffer.

5.1.4 Timing and temperature

It is important to soak the blotted membrane in freshly prepared blocking agent for 30 min to 2 h at room temperature with constant agitation. Alternatively, soaking the membrane for 1 h at 37°C or overnight at 4°C can help solve some persistent background issues. A blocking time of 2 h at room temperature should not be exceeded due to the risk of staining artifacts and high background. If longer blocking times are required, this should be performed at 4°C.

5.2 Primary and secondary antibody probing

Following the blocking step, the protein of interest can be detected using antibodies. The primary antibody, which is specific for the target protein, can be labeled or unlabeled. To maximize sensitivity and signal-to-noise ratio, most Western blotting procedures use an unlabeled primary antibody that is specifically recognized by a labeled secondary antibody. Depending on the type of detection system used, the secondary antibody will generate a signal that can be quantitated by chemiluminescent, chemifluorescent, chromogenic, fluorescent, or radiolabeling methods.

5.2.1 Primary antibodies

Although it is sometimes necessary to produce your own primary antibody, for example against a newly discovered or rare target, a vast range of antibodies are commercially available and there are probably several options specific to your protein of interest.

When selecting a primary antibody, several factors should be considered. The choice of antibody depends on how the protein of interest is folded, as different epitopes will be exposed under different conditions. An animal immunized with a denatured antigen can generate antibodies that recognize internal epitopes concealed within the structure of the protein. In a Western blotting system using denatured proteins, the best results are likely if this type of antibody is selected.

In contrast, if the protein of interest is in its native form, epitopes located on the surface of the protein must be recognized. In this type of application, therefore, excellent results are possible if a native antigen is used for immunization.

☞ If you purchase a commercially available primary antibody, ensure that it has been validated for Western blotting applications according to the manufacturer.

☞ You can save money by reusing the primary antibody solution, but be careful how you store it so that it retains its activity. Use clean tubes and store refrigerated when not in use and use clean boxes when incubating the membrane.

☞ You can consume less primary antibody by incubating the membrane in a smaller box. If the protein of interest is well characterized, that is, if you know its molecular weight and where it is expected to migrate on the membrane, cut the membrane to the appropriate smaller size after transfer.

☞ Blotting manifolds that allow incubation of individual lanes on a membrane (instead of cutting strips) are useful for screening antibodies, and require a smaller volume.

Both monoclonal and polyclonal antibodies can be used for Western blotting analysis (Fig 5.2). Both types possess advantages and disadvantages. It should be noted that while polyclonal antibodies tend to be more sensitive, they are less specific than monoclonal antibodies. Monoclonal antibodies, on the other hand, tend to be more specific but less sensitive. Polyclonal antibodies are usually chosen for their relatively lower price and because they are less time-consuming to produce. Monoclonal antibodies bind to only one epitope and are typically highly specific, pure and consistent in performance, and generally give rise to low backgrounds. Crude antibody preparations, such as serum (polyclonal antibodies) or ascitic fluid (monoclonal antibodies) are sometimes used for Western blotting, but impurities can increase background. For improving the signal-to-noise ratio, antibodies can be affinity-purified using immobilized antigen or immobilized protein A or protein G. The basis for antibody affinity purification is the high affinity and specificity of protein A and G for the Fc region of IgG from a variety of species. Protein A and G have been immobilized to several different matrices resulting in an excellent means of isolating IgG and IgG subclasses from ascitic fluid, cell culture supernatants, and serum.

GE Healthcare provides chromatography media for antibody purification, including ready-to-use HiTrap Protein A/HiTrap Protein G HP columns. The columns are prepacked with Protein A- or Protein G Sepharose™ High Performance chromatography media, respectively. These media are designed for rapid and convenient purification of antibodies from various sources using a syringe, pump, or chromatography system.

Purified or unpurified monoclonal antibody   Unpurified polyclonal
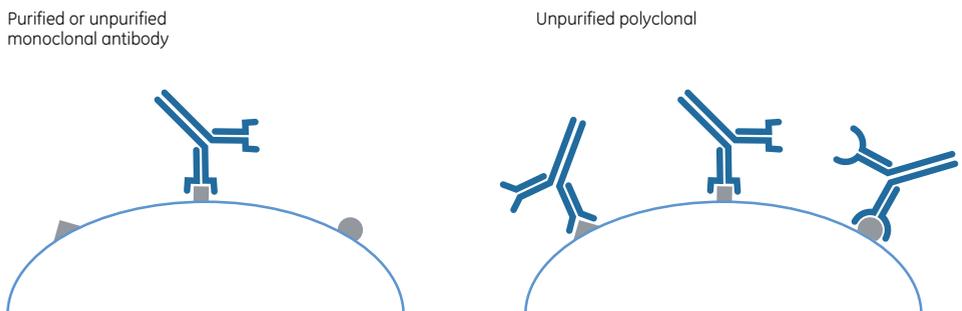
Fig 5.2. While monoclonal antibodies only bind to a single specific epitope on an antigen, polyclonal antibodies tend to be comprised of a mixture of specificities to several epitopes. The specificity of polyclonal antibodies can be improved by affinity purification against fragments of the target protein. Alternatively, the specificity of the antibody response can be influenced at the immunization stage via the use of engineered or truncated immunogens, where only the desired target epitopes are exposed.

How specific does a primary antibody need to be? The appropriate level of specificity of the primary antibody is determined by the part of the protein one wishes to detect. The detection of growth factor-binding domains of a transmembrane receptor, for example, requires an antibody raised against the extracellular domain of that protein. On the other hand, the study of conformation-dependent signal transduction from receptors to secondary messengers demands an antibody directed against a specific sequence of the intracellular domain of the receptor. In addition, high specificity is critical in applications such as the detection of defined, phosphorylated amino acid residues and will probably demand a monoclonal primary antibody.

Additionally, some knowledge of protein similarity across different species will help determine whether a primary antibody can be raised against proteins from species other than the one under study. Highly conserved proteins such as insulin are detected in human samples using antibodies directed against the same protein from other mammals. Moreover, many mouse and rat proteins are highly similar. In general, however, and wherever practically possible, primary antibodies should be raised against proteins from the same species as the one under study. In addition, pay attention to the species in which the antibody was produced. This is particularly important when another primary antibody is used on the same membrane to bind a different protein in multiplexed detection. In such a situation, the primary antibodies must be produced in different animal species in order to be recognized individually by species-specific secondary antibodies.

☞ Primary antibodies should be raised in species as distinct as possible from the sample species. It is better to raise a primary antibody against a mouse protein in a rabbit, for example, rather than a rat.

When the primary antibody is selected, it is important to optimize the concentration to obtain the best results. High primary antibody concentration is a common reason for poor results, such as high background, nonspecific bands or excessive signal intensity. In general, highly sensitive chemiluminescence systems such as Amersham ECL Prime and Amersham ECL Select require a lower concentration of antibodies. Less consumption of primary antibodies reduces costs and is particularly advantageous if antibodies are scarce or expensive. Generally, a good starting point for primary antibody concentration is to follow the manufacturers' recommendations. There is usually little significant variation in performance between different antibody batches, and it should normally be necessary to perform only one titration series. However, concentrations of polyclonal antibodies in sera can vary from animal to animal or from one blood sampling to the next. In these circumstances, or when you notice a change in the results, another titration series should be performed.

Another important factor is the incubation temperature of the primary antibody. Higher temperatures are associated with higher binding, both specific- (increasing the signal) and nonspecific (increasing the background). A general recommendation is 1 h at room temperature or at 4°C overnight. Antibody dilutions are typically made in washing buffer (PBS or TBS). If you experience high background problems, blocking agents such as BSA or non-fat milk and low concentrations of detergent, such as 0.05% to 0.1% V/V Tween-20 or SDS can be included in the antibody solution. When selecting a solution to dilute your antibodies, it is essential to select one that preserves the biological activity of the antibody: Follow manufacturers' recommendations!

More guidelines on how to improve your Western blotting are provided in Appendix A.

5.2.1.1 Washing steps

After primary antibody probing, it is necessary to wash the membrane in order to remove excess antibody that could cause high background and, consequently, a low signal-to-noise ratio. A low concentration detergent solution, such as 0.05% to 0.1% Tween-20 in PBS or TBS is commonly used, especially after incubation with highly concentrated antibody solutions or crude extracts. PBS-Tween is suitable for most washing applications. However, TBS-Tween is preferable where the target proteins are phosphorylated, as the phosphate in PBS might interfere with antibody binding.

Be aware that too much detergent can elute the protein of interest from the membrane, decreasing or obliterating the signal. For monoclonal or highly purified polyclonal antibodies, detergent-free washing buffer is preferable. The number of washes required to optimize the signal-to-noise ratio should be determined empirically. Insufficient washing will lead to excessive background, while excessive washing might elute the antibodies and reduce the signal.

Washing steps should be performed according to the recommendations of the manfacturer of the detection reagent used in the procedure. As a general guide, washing should be performed at least three times (5 min/wash) in a volume of approximately 4 ml of washing buffer/cm^2 of membrane and with constant agitation. A persistent background can be reduced by adding up to 0.5 M NaCl and up to 0.2% SDS to the washing buffer and extending the wash time to 2 h.

5.2.2 Secondary antibodies

A wide variety of secondary antibodies are commercially available and your choice will depend firstly on the species in which the primary antibody was produced. If, for example, the primary antibody was of the IgG isotype and produced in goat, the secondary antibody must be an anti-goat IgG antibody produced in another species as it will bind to the Fc region of the primary antibody. Although there is no strict rule, secondary antibodies raised in certain host species can lead to high background levels. In such a situation, changing the species of origin of the secondary antibody might be necessary. Another way to reduce the background is to absorb the secondary antibody with nonimmune serum from the primary antibody species. This will remove antibodies that have the potential to cross-react with serum proteins, including antibodies, from those species.

The procedures for incubation of the secondary antibody solution and the membrane are essentially similar to those described for the primary antibody.

It is important to follow manufacturers' instructions for the dilutions, which can vary from 1:100 to 1:500 000. To achieve good quality results, optimization of secondary antibody concentrations is recommended. Highly sensitive chemiluminescence systems, such as Amersham ECL Prime and Amersham ECL Select generally require less antibody compared to other detection systems (see Appendix A).

5.2.2.1 Choice of labeled antibodies

The choice of the detection system that best fits your Western blotting application has to be considered when it comes to selecting the secondary antibody. You can choose to label your own secondary antibody using a kit. This has the advantage that your secondary antibody can be used for different detection methods depending on the Western blotting system you are developing. Another solution is to use commercially available labeled secondary antibodies optimized to cover most applications.

☞ Fab or F(ab')$_2$ fragments of antibodies are also available in labeled or unlabeled forms. These fragments are especially useful in assays where binding between the Fc portions of antibodies and Fc receptors present in the protein sample complex must be eliminated.

Enzymes: Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two most commonly used enzymes for protein detection in Western blotting. Both can be used with either chemiluminescent, chemifluorescent, or chromogenic substrates. The advantage of AP is that its reaction rate remains linear, allowing sensitivity to be improved by prolonging incubation time with substrate. Increasing the incubation time, however, often leads to high background, resulting in a low signal-to-noise ratio. The background is generally lower with HRP due to very high substrate specificity. Of the two most common choices of enzymes, HRP is preferred, due to its high activity rate, stability, low, and wider range of substrates (Table 5.3).

Table 5.3. Some key characteristics of AP and HRP as secondary antibody conjugates

	AP	HRP
Molecular weight (M_r)	140 000	40 000
Price	High	Low
Stability	Unstable below 0°C	Stable below 0°C
Number of substrates	Few	Many
Kinetics	Slow	Fast
pH optimum	8 to 10	5 to 7

Fluorophores: Due to the high sensitivity of digital imaging techniques, the use of newly available fluorophores in Western blotting systems is appealing. Fluorescence detection has advantages such as high sensitivity, detection across a wide linear dynamic range of protein quantities, and signal stability over time, making it a suitable approach to quantitative detection. Fluorescence detection opens the possibility for multiplexing and improved quantitative analysis.

Due to spectrally resolved fluorophores, detection using Amersham ECL Plex introduces possibilities of multiplexed detection (the simultaneous detection of more than one protein). Multiplexing enables detection of the protein of interest at the same time as, for example, a housekeeping protein, without the need to strip and reprobe the membrane. This results in more reliable quantitation. In addition, the signal stability of up to 3 mo enables larger experimental series and more scope for extensive and precise quantitative analysis. The multiplexing capacity of Amersham ECL Plex is particularly useful for detecting target proteins of similar molecular weight. An example is the detection of phosphorylated and nonphosphorylated isoforms of the same protein, where the signals can be quantitated, regardless of relative intensities, due to minimal cross-talk between the detection channels (see 6.2.1).

Biotinylated secondary antibodies: By using biotinylated antibodies, a two-step biotin/ streptavidin system may be used to increase the signal intensity in Western blotting, allowing detection of low-abundance targets. In this three layer system, a biotin-labeled secondary antibody is applied. Subsequently, streptavidin/avidin labeled with dyes, fluorophores, radioisotopes, or enzymes is applied, resulting in an irreversible interaction between biotin and streptavidin/avidin (Fig 5.3). In addition, multiple biotin molecules can be conjugated to antibodies, which, in turn, allows interactions with multiple streptavidin molecules, amplifying the signal and increasing the sensitivity of detection of the protein of interest.
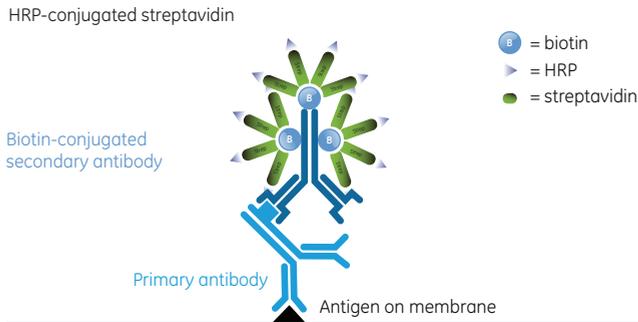

Fig 5.3. Streptavidin binds very strongly to biotin. This interaction can be exploited to detect membrane-immobilized antigens by conjugating the streptavidin to HRP. The amplification effect of multiple streptavidin molecules per biotin is further increased if multiple biotin molecules are attached to each secondary antibody.

Gold-conjugated antibodies: Gold particles can be attached to streptavidin or secondary antibodies. The gold particles are negatively charged and bind the membrane very weakly. The specific labeling of proteins occurs by hydrophobic and ionic interactions. The proteins stain dark red through the accumulation of gold particles. When used together with a silver intensification reagent, a further 10- to 100-fold enhancement can be achieved. AuroProbe is intended for use with negatively charged membranes, constructed of a material such as nitrocellulose or PVDF. Membranes should be handled with gloves and forceps to avoid smears.

Radioisotopes: Radioisotope-conjugated antibodies have been used extensively, but they are expensive, have a limited shelf-life, and require special waste handling and disposal, as well as demanding a safe, specialized working environment. For these reasons, labels such as enzymes and fluorophores are usually preferable.

5.3 Stripping and reprobing membranes

In some applications, it is necessary to detect more than one protein on the same membrane, particularly if you are performing a quantitative analysis. This can be achieved by stripping and reprobing your membrane, but this carries the risk of loss of total protein from the membrane. Stripping and reprobing blots saves time and samples by enabling you to reprobe a single blot with different primary antibodies. However, the stripping conditions can cause the release of proteins from the membrane, resulting in decreased sensitivity. It is therefore critical to use conditions that release antibodies from antigen while minimizing the elution of protein sample bound to the membrane.

By using a combination of detergents, reducing agents, heat and/or low pH, it is usually possible to find the conditions that lead to acceptable results. In the following sections, you will find important points to consider to help you choose the best method for your Western blotting system. Note that reblocking might be required prior to antibody incubation.

☞ An alternative to stripping and reprobing is to use secondary antibodies labeled with fluorophores for simultaneous detection of more than one protein. With Amersham ECL Plex, two proteins can be simultaneously detected on a single blot with minimal cross-reactivity between antibodies or dyes (see 5.2.2.1). Even three target proteins can be detected simultaneously if a third species of primary antibody is used. These features make Amersham ECL Plex an excellent practical method for applications demanding more than one protein signal without the risk of protein loss.

5.3.1   Stripping using heat and detergent

Stripping membranes using heat and detergent (2) has been successfully performed when using ECL based Western blotting detection reagents (for detailed procedure, see Chapter 11). Prewashed membranes are stripped with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8 for 30 min at 70°C. Lower temperatures (50°C to 70°C) might also work well, but should be determined empirically with the antibodies used (see 5.3.2).

5.3.2   A strategy to optimize membrane stripping

The first parameter to consider is temperature. Assuming that you have a sufficient amount of pure protein, the optimal stripping temperature can be determined by testing the conditions using dot-blotting before proceeding further. However, if you have limited quantities of protein, you can perform Western blotting and cut the membrane into strips. It is then recommended to perform your stripping protocol at 50°C before checking the efficiency of the process by reprobing the membrane with secondary antibody (see 5.3.1 and Chapter 11). If stripping is found to be insufficient under these conditions, the procedure may be repeated at increasing temperature increments of 5°C. This optimization step should help you to determine the best conditions for stripping antibodies while minimizing target protein loss from the membrane.

Besides temperature, it is critical to consider antibody affinities and target protein abundance. Four strategies are described in Table 5.4 to help guide you to acceptable results.

Table 5.4. A suggested approach to determine the optimal stripping strategy

	Strategy 1	Strategy 2	Strategy 3	Strategy 4
Experimental circumstance	Two proteins of similar abundance and two antibodies of similar affinity	Two proteins of similar abundance, antibodies of unequal affinity	Two proteins, one high- and the other low-abundance, antibodies of equal affinity	Two proteins, one high- and the other low-abundance antibodies of unequal affinity
Step 1	Detect either protein first	Detect either protein with lowest affinity antibody first	Detect low-abundance protein first	Detect low-abundance protein first
Step 2	Strip	Strip	Strip	Strip
Step 3	Detect remaining protein	Detect protein with highest affinity antibody	Detect high-abundance protein	Detect high-abundance protein

In the first strategy, if both the antibody affinities and the abundance of each protein are approximately equal, then it is not important which protein is detected first.

The second strategy highlights the importance of detecting proteins of similar abundance firstly with the lowest affinity antibody, so that less vigorous stripping is performed first, ensuring that the chance of detecting both proteins before and after stripping is excellent.

The third strategy shows the importance of detecting the lowest abundance protein first if the primary antibodies used are of equal affinity.

The fourth strategy is harder to evaluate but in general, it is advisable to detect the protein of lowest abundance first as target loss is likely to have the greatest effect on the ability to detect protein.

5.3.3   Stripping using low pH

If stripping using heat and detergent do not lead to a satisfactory outcome, an alternative method is to strip your membrane using a solution of 25 mM glycine-HCl, pH 2, supplemented with 1% SDS (see Chapter 11). The membrane should be soaked in stripping solution for about 30 min under constant agitation. The efficiency of the technique should be confirmed in the same way as for stripping using heat and detergent. If stripping is not complete, increasing the incubation time in the stripping solution may help to remove antibodies, at the risk of eluting the protein of interest. For this reason, it is recommended not to exceed 1 h incubation time in acidic stripping solution.

For chemiluminescence applications, you may also try to strip your membrane (Amersham Protran Premium 0.2 and 0.45 nitrocellulose or Amersham Hybond 0.2 and 0.45 PVDF) using a mild protocol of incubation in 0.2 M glycine (pH 2.8) at room temperature for 30 min followed by two washes in TBS-Tween or PBS-Tween for 10 min each. You may then proceed with blocking and immunodetection.

5.3.4   Stripping using high pH

Stripping by soaking the membrane in a solution at high pH has been shown to be a potential remedy if you still have a persistent signal after your first probing. Two incubations in 0.2 M NaOH for 5 min followed by a 5 min wash with water are usually sufficient. If any trace of signal still remains, you can raise the concentration of NaOH to as high as 2 M and the incubation time to 30 min. Reblocking is normally not necessary after stripping using NaOH. However, depending on NaOH concentration and soaking time, you might need to reblock your membrane before reprobing.

5.3.5   Stripping using high salt solution

A high salt concentration has been shown to be another effective solution if you experience problems in stripping your membrane. Soak your membrane in PBS or TBS buffer supplemented with 0.5 M NaCl and 0.2% SDS for between 30 min and 2 h. Then rinse the blot with water. Reblocking is normally not necessary after stripping using salt. However, depending on the soaking time, you might need to reblock your membrane before reprobing.

5.3.6   Hints and tips

As a time-saving alternative to stripping, for quick detection of a second protein of interest on your membrane, sequential labeling is possible with ECL detection. After ECL detection of the first protein of interest, HRP is inactivated (quenched) using hydrogen peroxide (H_2O_2) and the membrane is washed. Labeling and detection of a second protein of interest is then possible without interference from the first label. The concentration of H_2O_2 needed to quench a signal from an HRP-labeled protein will depend on the amount of HRP present and the length of time the membrane is incubated. Usually, 30 min incubation with 15% H_2O_2 in PBS gives good results. The membrane is then washed three times for 5 min in PBS and reprobed for the second run of protein detection.

5.4 Blotting protocol

After transfer it is important to identify the side of the membrane that was in contact with the gel, the "protein side". In every step described below, the protein side must face upwards.

1. Block the membrane with an appropriate blocking agent.

   ✋ Do not use PBS as dilution buffer if the target itself is phosphorylated - use TBS.

   ✋ Do not use non-fat milk as blocking agent if biotinylated or concanavalin-conjugated antibodies are used.

   ✋ Do not use crude protein preparations as blocking agent if the target is phosphorylated.

   ✋ Note that not all blocking agents are compatible with fluorescent Western blotting.

2. Incubate with primary antibody – note that primary antibodies can be re-used.

   ☞ Select a primary antibody raised in a species as distinct as possible from the target species.

   ☞ Ensure that the primary antibody has been validated for Western blotting applications.

3. Wash the membrane.

   - Use TBS-Tween if the target protein is phosphorylated.

4. Incubate with secondary antibody.

   ☞ If probing with fluorophore-conjugated antibodies:

     - Aliquot the antibodies in light-protected tubes.
     - Dilute the antibodies in light-protected containers.
     - Perform secondary antibody probing protected from light.

5. Wash the membrane according to the recommended protocol for the detection system.

5.5 References

1. Sharma. S. K. and Carew, T. J. Inclusion of phosphatase inhibitors during Western blotting enhances signal detection with phospho-specific antibodies. *Anal. Biochem.* **307**, 187–189 (2002).

2. Kaufmann, S. H. *et al.* The erasable Western blot. *Anal. Biochem.* **161**, 89–95 (1987).

Chapter 6
Detection

Sample preparation → Gel electrophoresis → Transfer → Antibody probing → **Detection** → Imaging → Analysis

A variety of detection systems, based on chemiluminescence, chemifluorescence, fluorescence, chromogenic, or radioisotopic detection are available (Fig 6.1). Radioisotopic and chromogenic reagents have been widely used for many years, but have declined in popularity due to safety issues with handling radioactive isotopes and poor sensitivity with chromogenic reagents. As a result of these issues, enzyme-based chemiluminescent and chemifluorescent techniques, as well as direct fluorescence have been extensively developed and are now usually the methods of choice for detection due to their improved sensitivity and wider dynamic range.

Enzymatic detection methods, such as chemiluminescence and chemifluorescence require the addition of a reagent that emits light when it reacts with an enzyme conjugated to a secondary antibody. Fluorescence-based detection, on the other hand, requires no additional reagents after binding of the labeled secondary antibody.

The most commonly used enzymatic detection system is chemiluminescence, based on antibodies conjugated to horseradish peroxidase (HRP) that catalyze the oxidation of luminol in presence of peroxide, resulting in light emission. HRP has several advantages over other enzymes such as alkaline phosphatase (AP) (Table 5.3). HRP can be easily conjugated to antibodies or streptavidin (which binds with extraordinarily high affinity to biotin, a commonly used tag) and can be used with different chemiluminescent reagents. Considerable efforts have been made to develop HRP-based detection reagents to obtain higher detection sensitivity, stronger light intensity and long-lasting signals. This will be further described in this chapter.

Fluorescence-based detection systems use a fluorescent entity, or fluorophore, directly conjugated to an antibody or streptavidin. The fluorophore can be excited using a light source of a specific wavelength causing light emission. Instead of adding a detection reagent, fluorescent signals can be directly detected with equipment, such as laser scanners, fitted with suitable light sources and emission filters.

Western blotting detection methods

Primary antibody

Secondary antibody conjugated to CyDye

Amersham ECL Plex
Fluorescence

Secondary antibody conjugated to HRP

Amersham ECL
Amersham ECL Prime
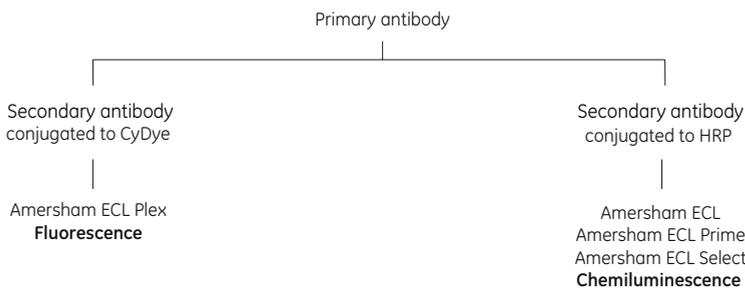Amersham ECL Select
Chemiluminescence

Fig 6.1. The GE Healthcare portfolio of reagents for alternative Western blotting detection methods. Amersham ECL Plex consists of a series of CyDye (Cy2, Cy3, and Cy5)-labeled secondary antibodies. No other detection reagent is required. The fluorescent signal from these antibodies can be directly detected using a laser scanner, such as Typhoon FLA 9500 or Amersham Imager 600. Amersham ECL, Amersham ECL Prime, and Amersham ECL Select are detection reagents for chemiluminescence and require HRP-conjugated secondary antibodies in order to generate a light signal.

6.1 Chemiluminescence

Since the early 1990s, GE Healthcare (as Amersham) has continuously developed chemiluminescence detection systems that are now amongst the most widely used for Western blotting applications. Today, a range of different chemiluminescence detection reagents are available to suit the aim of the experiment.

The HRP-conjugated secondary antibody binds to the primary antibody, specifically bound to the target protein on the membrane. After the addition of a luminol peroxide detection reagent, the HRP enzyme catalyzes the oxidation of luminol in a multistep reaction.

The reaction is accompanied by the emission of low intensity light at 428 nm. In the presence of certain chemicals, the emitted light is enhanced up to 1000-fold, making it easier to detect, and thus increasing the sensitivity of the reaction in a process known as enhanced chemiluminescence (ECL). Several enhancers can be used, but the most effective are modified phenols, especially p-iodophenol, which increases HRP turnover rate and assists in the transfer of electrons from luminol to the enzyme. The intensity of signal is a result of the number of reacting enzyme molecules and is thus proportional to the amount of antibody, which is related in turn to the amount of protein on the blot (Fig 6.2).
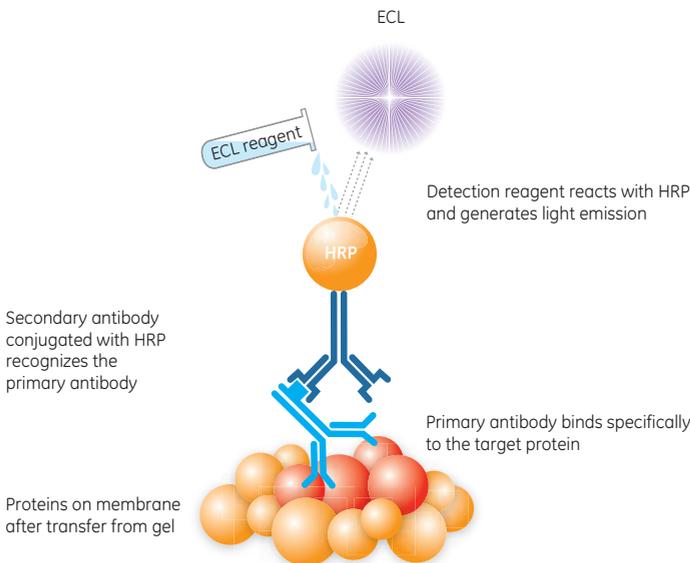

ECL

ECL reagent

Detection reagent reacts with HRP and generates light emission

HRP

Secondary antibody conjugated with HRP recognizes the primary antibody

Primary antibody binds specifically to the target protein

Proteins on membrane after transfer from gel

Fig 6.2. The principle of enhanced chemiluminescence (ECL) detection in Western blotting.

The most appropriate choice of ECL reagent depends on the requirements of the experiment. High-abundance proteins, for example, can be detected quickly and with minimal investment in optimization using Amersham ECL. If, on the other hand, high sensitivity or more accurate quantitation is important, Amersham ECL Prime or Amersham ECL Select are recommended. The signals emitted by Amersham ECL Prime and Amersham ECL Select are very intense, which makes it possible to detect very low levels of proteins as well as to carry out repeated exposures (Table 6.1).

Table 6.1. Amersham ECL, Amersham ECL Prime, and Amersham ECL Select are suited to different Western blotting applications depending on the required sensitivity and availability of primary antibody

	Amersham ECL	Amersham ECL Prime	Amersham ECL Select
Applications	For confirmatory detection and analysis or other applications where the protein of interest is not limited	For high sensitivity and precise quantitation across a wide range of protein levels	For high sensitivity and precise quantitation across a wide range of protein levels
Primary antibody, working range dilution	1:100 to 1:5000	1:1000 to 1:50 000	1:5000 to 1:30 000
Secondary antibody, working range dilution	1:1000 to 1:15 000	1:50 000 to 1:250 000	1:100 000 to 1:300 000
Recommended membrane	Amersham Protran Premium 0.2 and 0.45 nitrocellulose	Amersham Hybond 0.2 and 0.45 PVDF	Amersham Hybond 0.2 and 0.45 PVDF

6.1.1   Amersham ECL

Amersham ECL was the world's first commercially available chemiluminescence detection reagent, capable of detecting protein at quantities as low as 10 pg. This is a highly cited method, and is particularly suitable for applications such as:

• Verification of expression of recombinant proteins

• Verification of highly expressed proteins

• Studies with tagged proteins

• Confirmatory studies

When using antibody-based detection methods, the quality and source of the primary antibody, as well as its affinity for the protein of interest will play a large part in determining the LOD as well as the likelihood of cross-reactivity.

The performance of Amersham ECL in the detection of a dilution series of transferrin is illustrated in Figure 6.3. Amersham ECL is also applicable to quantitative Western blotting, but with less sensitivity than Amersham ECL Prime and Amersham ECL Select.

Amersham ECL

5 ng   39 pg

Fig 6.3. Western blotting detection of transferrin in a two-fold dilution series using Amersham ECL. The image was acquired using an ImageQuant charge-coupled device (CCD) camera-based imager.

Optimal performance of Amersham ECL is achieved using Amersham Hybond and Amersham Protran Premium membranes. These membranes should be blocked using Amersham ECL blocking agent to minimize background. Antibodies should always be used at the concentrations recommended by manufacturers, but as a guideline for Amersham ECL, primary and secondary antibodies can be diluted from 1:100 to 1:5000 and 1:1000 to 1:15 000, respectively. Signals will generally increase with higher antibody concentrations but an excess of antibody might also lead to nonspecific binding to the membrane. It is well worth spending time to test different antibody concentrations (see Appendix A). The emitted signal peaks after 5 to 10 min and then decays slowly, with a half life of approximately 60 min, and can be detected either using Amersham Hyperfilm™ ECL or a CCD camera-based imager (see Chapter 7).

6.1.2   Amersham ECL Prime

Amersham ECL Prime is a highly sensitive detection system and is characterized by extremely stable signal emission, allowing for the possibility of repeated exposures, and making it easier to process several blots in the same experimental run. In addition, the high intensity of the emitted signal means that Amersham ECL Prime is suitable for working with low-abundance proteins, or other applications that utilize the advantages of a highly sensitive CCD camera-based imager. Moreover, highly diluted primary and secondary antibodies can be used, reducing the total cost of experiments as well as minimizing the risk of high background.

The improved performance of Amersham ECL Prime compared with other chemiluminescent reagents is made possible due to the presence of a high-performance enhancer and a catalyst, increasing signal intensity, sensitivity, and duration. Amersham ECL Prime is particularly recommended for use in quantitative analyses such as:

- Applications where high sensitivity is needed
- Changes in protein abundance
- Protein:protein interactions
- Detection of protein isoforms
- Evaluation of protein degradation
- Post-translational modifications (PTM)
- Simultaneous detection of low- and high-abundance proteins

The performance of Amersham ECL Prime is illustrated in Figure 6.4, for the detection of pSTAT3 in a two-fold dilution series of IFNα-treated HeLa cell lysates, from 12.5 µg of total protein extract. Note that the level of sensitivity is dependent on a specific antibody:protein interaction.

Fig 6.4. Detection of pSTAT3 in IFNα-treated HeLa cells using Amersham ECL Prime. Note that protein quantities refer to the total amount of loaded protein extracted from cell lysate. pSTAT3 comprises only a fraction of that total quantity. The control lane contains 6.25 µg of total protein from untreated cells.

6.1.2.1 Sensitivity and precision

The sensitivity and linear dynamic range of Amersham ECL Prime enable detection and precise quantitation of both low- and high-abundant proteins on the same blot after a single exposure if used in combination with a CCD camera-based imager, such as one of the Amersham Imager 600 series (see Chapter 7). When a highly sensitive system is required, for example for the detection of transient protein phosphorylation (a PTM), it can be advantageous to use Amersham ECL Prime.

Another advantage of Amersham ECL Prime is that the strong signal intensity allows the use of highly diluted antibodies with sustained performance (Fig 6.5). This makes Amersham ECL Prime cost-effective if the antibodies used for detection are scarce.
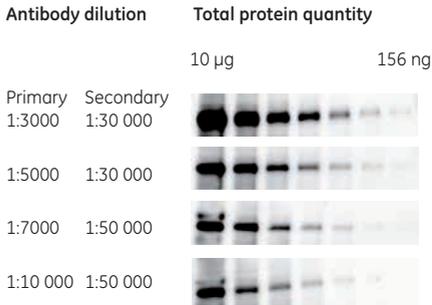
Antibody dilution		Total protein quantity			
		10 µg			156 ng
Primary	Secondary				
1:3000	1:30 000				
1:5000	1:30 000				
1:7000	1:50 000				
1:10 000	1:50 000				

Fig 6.5. Amersham ECL Prime Western blotting detection of β-catenin in NIH 3T3 whole cell lysates in a two-fold dilution series using different dilutions of rabbit anti-β-catenin (primary antibody) and HRP-conjugated anti-rabbit IgG (secondary antibody). Note that the protein quantities refer to the total amount of loaded protein extracted from cell lysates. β-catenin comprises only a fraction of that total quantity.

6.1.2.2 Signal duration

Amersham ECL Prime emits a signal of long duration, with intense signals still being emitted, even for the lowest amounts of protein tested, 3 h after the addition of substrate (Fig 6.6 and Fig 6.7). This enables multiple exposures and a convenient time window between addition of reagent and signal detection.
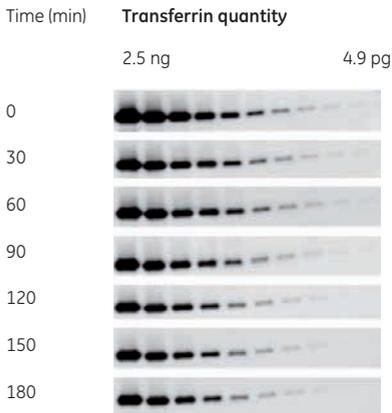
Time (min)	Transferrin quantity		
	2.5 ng		4.9 pg
0			
30			
60			
90			
120			
150			
180			

Fig 6.6. A two-fold dilution series of transferrin from 2.5 ng showing signal intensities at time points up to 3 h after the addition of Amersham ECL Prime reagent. Time 0 corresponds to 5 min post-reagent addition. The signals were detected for 3 min at each time point.
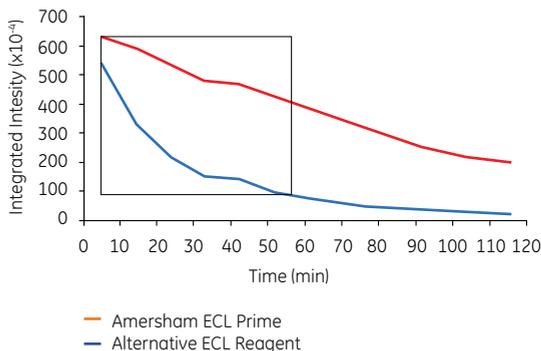

— Amersham ECL Prime
— Alternative ECL Reagent

Fig 6.7. Signal stability over time. The signal duration of Amersham ECL Prime compared to an alternative chemiluminescent detection reagent. The high stability of Amersham ECL Prime provides a broader window of time in which higher sensitivity is maintained.

The performance of Amersham ECL Prime is optimal when used in combination with a CCD camera-based imager and by carrying out an optimization process similar to that applied to Amersham ECL (although blocking is best performed using Amersham ECL Prime blocking reagent). In addition, Amersham Hybond PVDF membranes are recommended due to the high sensitivity made possible by high protein binding capacity. Primary and secondary antibodies should be diluted from 1:1000 to 1:50 000 and 1:50 000 to 1:250 000, respectively. Amersham ECL Prime is also compatible with film, if this is the preferred method of detection.

6.1.3 Amersham ECL Select

Amersham ECL Select is a highly sensitive reagent for chemiluminescent Western blotting detection. It is the most sensitive chemiluminescent detection reagent in the Amersham ECL product family. The dynamic range of Amersham ECL Select is complementary to that of Amersham ECL Prime. The outstanding signal intensity of Amersham ECL Select makes it suitable for the most demanding Western blotting applications including the detection of minute protein quantities. Since the light output is proportional to the amount of protein detected, this results in precise quantitation across a wide range of protein levels on a single blot.

Amersham ECL Select is typically used at the detection stage of the Western blotting workflow, but it also impacts on upstream procedures. The primary antibodies used as probes on the blotted membranes, for example, may be highly diluted if you use Amersham ECL Select for detection. This reduces cost and avoids levels of background signals that can quench the weaker, specific interactions of interest.

Amersham ECL Select is particularly recommended for use in quantitative analyses such as:

- Applications where high sensitivity is needed
- Changes in protein abundance
- Protein:protein interactions
- Detection of protein isoforms
- Evaluation of protein degradation
- Post-translational modifications (PTMs)
- Simultaneous detection of low- and high-abundance proteins

The performance of Amersham ECL Select is illustrated in Figure 6.8. Endogenous TAB 1 was detected in a two-fold dilution series of 293 T-cell lysate, starting at 2 µg of total protein extract. Note that the level of sensitivity is dependent on a specific antibody:protein interaction.
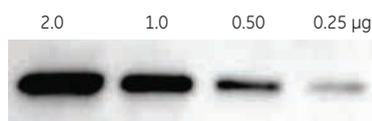

Fig 6.8. Western blotting detection of endogenous TAB 1 in 293 T-cell lysate. The high signal intensity of Amersham ECL Select gives bright bands and highly sensitive detection.

6.1.3.1 Sensitivity and precision

The high sensitivity and signal intensity together with the broad linear dynamic range makes Amersham ECL Select suitable for qualitative as well as quantitative analysis. Amersham ECL Select produces very high light output and this allows you to use highly diluted antibodies in your experiments.

The Western blotting detection of PP2A in a two-fold-dilution series of NIH/T3T cell lysate shown in Figure 6.9 demonstrates that minute levels of protein can be detected using a wide range of primary antibody dilutions with Amersham ECL Select.
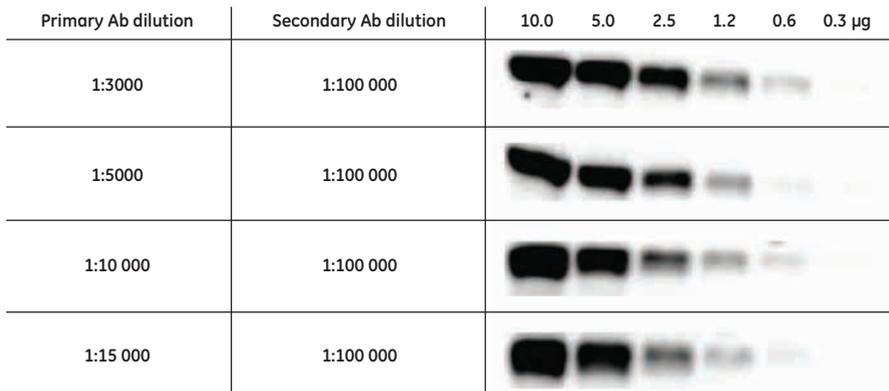
Primary Ab dilution	Secondary Ab dilution	10.0	5.0	2.5	1.2	0.6	0.3 µg
1:3000	1:100 000						
1:5000	1:100 000						
1:10 000	1:100 000						
1:15 000	1:100 000						

Fig 6.9. Amersham ECL Select provides high sensitivity using a wide dilution range of antibodies.

6.1.3.2 Signal duration

When the signal duration of Amersham ECL Select was evaluated in a Western blotting model system detecting ERK 1/2 in HeLa cell lysate, a high signal output with sufficient signals remaining 2 h after addition of the reagent was obtained, even for the lowest amount of protein tested. This results in a convenient time window between the end of an experiment and the beginning of analysis thus allowing for multiple exposures.
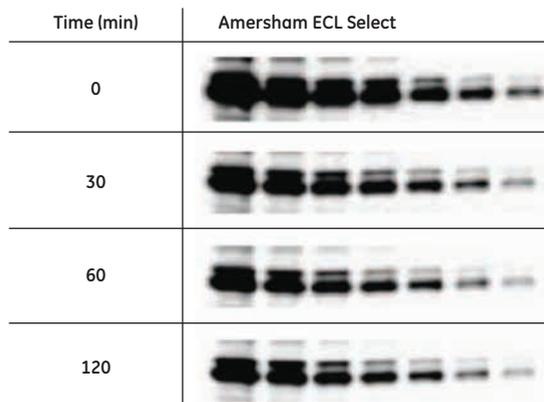
Time (min)	Amersham ECL Select
0	
30	
60	
120	

Fig 6.10. The decline in signal output from Amersham ECL Select was monitored at different time points in a two-fold dilution series of ERK 1/2 starting at 5 µg total protein. The high signal intensity of Amersham ECL Select allows for sustained detection of very low protein levels for up to 2 h.

6.1.4   Chemiluminescence hints and tips

In order to increase the signal-to-noise ratio, spend time on optimizing blocking agents and antibodies. Always start with the manufacturers' recommendations (see Appendix A).

Do not use sodium azide as a preservative for buffers as it is a potent inhibitor of HRP.

To minimize uneven staining when using ECL based detection, first mix the detection solutions and pour into a clean container. Then place the probed membrane in the detection solution and gently agitate to cover the entire surface.

👉 If the membrane is to be reused, place it in a plastic file folder to prevent drying before imaging. Be sure to remove any air bubbles.

✋ Do not allow the membrane to dry out at any time during the immunodetection procedure or between rounds of immunodetection. Any residual molecules will bind permanently to the membrane if it is allowed to dry, making it impossible to strip the membrane. This is particularly important when using PVDF membranes.

👉 For reproducible performance, allow all reagents to equilibrate to room temperature before use.

👉 Although the active, newly mixed solutions for Amersham ECL, Amersham ECL Prime and Amersham ECL Select are stable, it is recommended that reagents are mixed immediately before use. If mixed reagents must be stored before use, protect from light by wrapping the container in foil or by storing in the dark.

👉 The intensity of signals emitted by many chemiluminescent substrates can fade. If you obtain weak signals, even when you analyze your blots immediately after ECL detection:

- Ensure that the detection reagents have not expired and have been stored properly; the solutions should not be stored at room temperature or be exposed to direct light

- Ensure that the caps on the detection solution flasks have not been mixed, contaminating the solutions

- Do not cross-contaminate the solutions; use fresh, clean pipette tips

✋ If you want to strip and reprobe the membrane, do not allow it to dry out.

✋ If your detection involves a streptavidin-biotin-HRP complex, do not use low-fat milk as a blocking or dilution agent as the interaction between streptavidin and biotin can be inhibited by endogenous biotin in the milk, leading to a decreased signal. Additionally, milk can contain phosphoepitopes, which can interfere with phosphospecific antibodies and lead to an increased background.

👉 To check if the ECL reagent is working properly, you can perform the "blue light test". In a darkroom, simply prepare 1 ml of ECL reagent in a tube, add 1 to 3 µl of HRP-conjugated secondary antibody and mix well. A functional reagent will rapidly flash with an easily visible bluish-white light. Alternatively, spot 2 to 5 µl of a 1:100 dilution of HRP-conjugated secondary antibody on a membrane. Add ECL reagent and expose the membrane to a CCD camera based imager or X-ray film.

6.2 Fluorescence

The light phenomenon of fluorescence occurs when molecules called fluorophores absorb light. In their ground state, fluorophores do not emit light, but when subjected to light (excitation) their energy levels are raised to a brief but unstable excited state. As fluorophores return to their ground state, they release light at a lower energy, higher wavelength (emission) than that of the excitation light.

Fluorescence detection is a direct method where the secondary antibody is conjugated to a fluorophore, thus avoiding the need for ancillary detection reagents. Different fluorophores are available, either for detection of signals at wavelengths of visible light or at near infrared wavelengths. The fluorescence detection approach is highly sensitive, delivers a broad linear dynamic range, and is well adapted to quantitative Western blotting. In addition, more than one protein can be detected at the same time (multiplexed detection) and the signal is stable for up to 3 mo.

Fluorescence-based detection is recommended for:

- Simultaneous detection of more than one protein (multiplexing)
- Detection of proteins of identical molecular weights
- Quantitative Western blotting applications
- Applications where high sensitivity is needed
- Changes in protein abundance
- Protein:protein interactions
- Detection of isoforms
- Evaluation of protein degradation
- PTMs
- When studying low- and high-abundance proteins at the same time

6.2.1   Amersham ECL Plex

In the fluorescence-based Amersham ECL Plex system, discrete primary antibodies can be recognized by species-specific secondary antibodies conjugated to fluorescent Amersham CyDye fluorescent dyes. All CyDye fluorescent dyes have their own specific excitation and emission wavelengths in the visible light spectra and are spectrally differentiated from each other, resulting in minimal cross-talk (Fig 6.11).
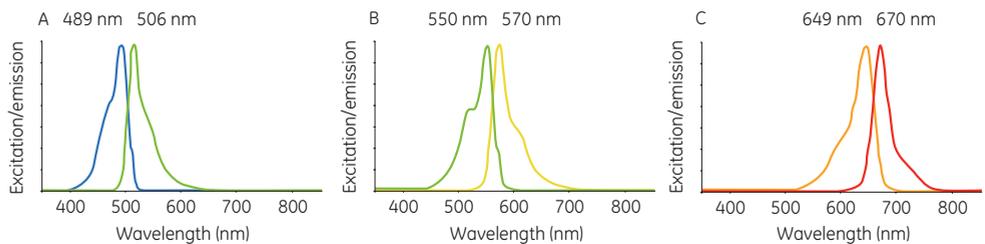

Fig 6.11. Excitation and emission spectra for fluorescent CyDye (A) Cy2, (B) Cy3, and (C) Cy5. In each example, the effect of the light wavelength generating the maximum efficiency of excitation is shown.

After excitation, the resulting fluorescent emission signals are captured using a multichannel fluorescent scanner such as Typhoon, or a CCD camera-based imager, such as Amersham Imager 600 series equipped with appropriate light sources and emission filters (see Chapter 7).

The Amersham ECL Plex system and the principle of multiplexed detection is illustrated in Figure 6.12.
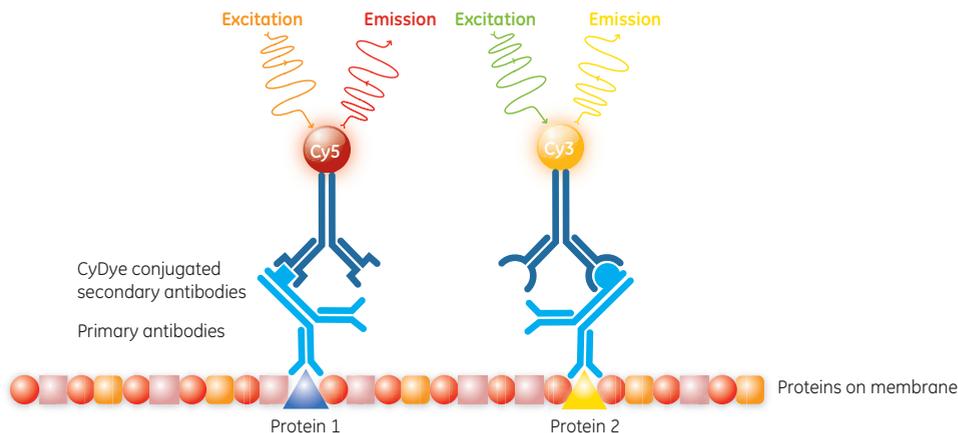
Fig 6.12. The principle of Amersham ECL Plex. Primary antibodies against two proteins are recognized by species-specific secondary antibodies conjugated to fluorescent CyDye, Cy3 and Cy5. Detection by direct fluorescence reduces the number of processing and imaging steps, which saves time and reduces errors in quantitation.

Multiplexed detection requires that the primary antibodies are raised in different species. The technique is convenient for the study of proteins of similar size or for detecting PTMs, as these investigations can be performed without the need to strip and reprobe membranes (as discussed earlier, stripping and reprobing carry a risk of uneven loss of proteins from membranes). Multiplexed detection thus improves the quantitative potential of Western blotting. It is also useful for all types of quantitation, as stripping is not required to monitor levels of housekeeping protein. In addition, fluorescence detection is highly sensitive, covers a broad linear dynamic range and emits a stable signal - factors which all contribute to the quantitative strengths of the technique.

The performance of Amersham ECL Plex is illustrated in Figure 6.13, for the detection of a dilution series of transferrin.
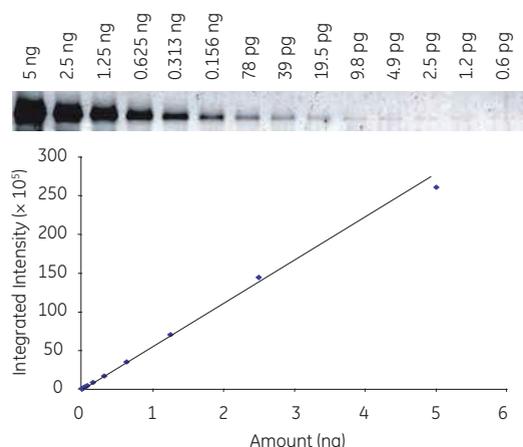

Fig 6.13. Fluorescence detection with Amersham ECL Plex gives a broad linear dynamic range, making it possible to detect and compare weak and strong bands on the same blot. This is a dilution series of transferrin detected with Cy3-labeled secondary antibodies. The dynamic range is 3.6 orders of magnitude and very linear with an LOD of 1.2 pg.

Each CyDye has a specific excitation and emission wavelength and are spectrally differentiated from each other, resulting in minimal cross-talk. After excitation, the resulting fluorescent emission signals are captured using a multichannel fluorescent scanner such as Typhoon, or a cooled CCD camera system such as Amersham Imager 600 (see Chapter 7).

Amersham ECL Plex secondary antibodies labeled with Cy2, Cy3, or Cy5 CyDye fluorescent dyes are available, directed against both rabbit and mouse primary antibodies. In addition, it is possible to label a specific antibody of interest. One advantage of CyDye is their high photostability compared to other fluorophores, such as fluorescein. In addition, the wide pH tolerance (3 to 10) of CyDye makes them compatible with most Western blotting buffers. Further benefits include the need for only a few handling steps and signal longevity (> 3 mo), enabling comparison of data across many experiments.

6.2.1.1 The multiplexing potential of Amersham ECL Plex

Studies on very small changes in protein expression, for example, in the analysis of the expression of intracellular signaling molecules after exposure to a growth factor, are only reliable if the protein load is carefully controlled. Although care should be taken to apply equal loads to lanes in a gel by measuring total protein levels, it is difficult to achieve accurate data due to pipetting errors or quantitation errors, due to variations in total protein between samples. The fastest way to simultaneously measure changes in specific proteins, while monitoring total protein levels, is to first treat the membrane with a stain prior to application of the antibodies to the membrane. This stain can thus be used to quantitate the amount of loaded protein per lane and also to confirm even transfer from gel to membrane for the whole blot.

For the highest possible precision, however, protein levels should be quantitated by comparison with a stable internal standard, or housekeeping protein. Housekeeping proteins, such as GAPDH or β-actin, are considered to be expressed at a constant level regardless of external stimulation, and are thus good indicators of the total amount of protein loaded into each well (Fig 6.14). The multiplexed detection possibilities of Amersham ECL Plex enable you to normalize each measurement to compensate for loading variations, as well as eliminating the risk of error compounded by membrane stripping and reprobing.
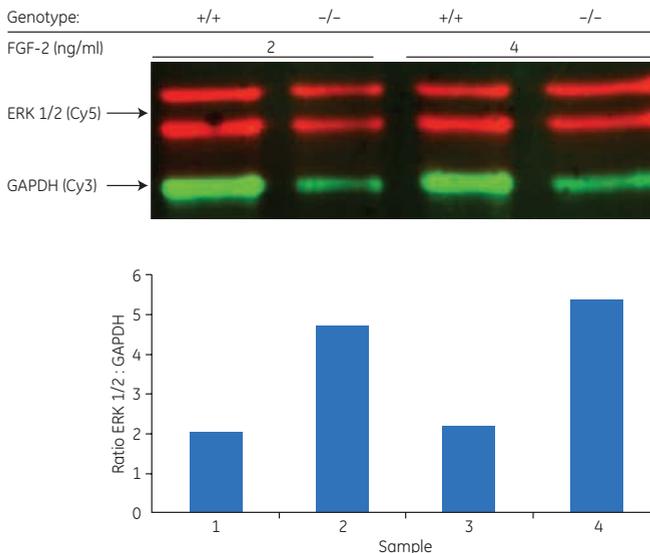

Fig 6.14. By detecting two proteins on the same blot, protein expression can be quantitated relative to a housekeeping protein such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This image shows Amersham ECL Plex detection of ERK 1/2 in wildtype (+/+) and enzyme knockout (-/-) mouse embryonic fibroblasts in response to treatment with fibroblast growth factor-2 (FGF-2). ERK1/2 and GAPDH were targeted using specific primary antibodies followed by secondary antibodies labeled with Cy5 (red) or Cy3 (green). The ratio of ERK1/2: GAPDH intensities reveals the expression of ERK 1/2 and is independent of variations in sample load. Data courtesy of Dr. Jin-Ping Li and Dr. Juan Jia, Department of Medical Biochemistry and Microbiology, Uppsala, Sweden.

PTMs that do not change the molecular weight of a protein can be very difficult to study by traditional Western blotting detection techniques. They often require the blot to be stripped and reprobed but this can lead to loss of protein and is therefore a risk when performing quantitative analysis. In this type of scenario, the potential of multiplexed detection provided by Amersham ECL Plex may be used to advantage as both the nonmodified and modified forms of the protein can be detected on the same membrane by using primary antibodies, raised in two different species and directed to the alternative forms of the protein (for example, phosphorylated and nonphosphorylated forms). The primary antibodies are then detected using species-specific secondary antibodies conjugated to different fluorophores such as Cy3 and Cy5 (Fig 6.15).
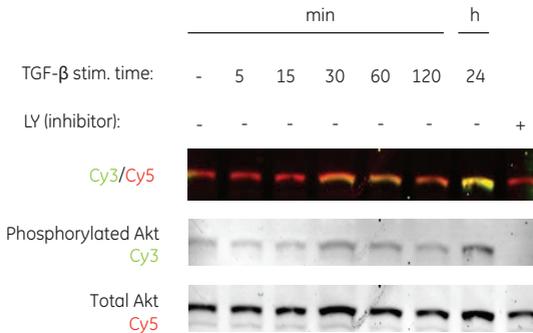

Fig 6.15. Detection, using Amersham ECL Plex, of low-abundance phosphorylated Akt protein and total Akt protein in human prostate cancer cells after stimulation with transforming growth factor-β (TGF-β). Despite the minimal change in molecular weight as a result of phosphorylation, the duplex capability of Amersham ECL Plex enables a clear distinction between the two forms of the protein. Note the complete absence of signal in the Cy3 channel for the sample treated with the kinase inhibitor, LY. Data courtesy of Marene Landström, Ludwig Institute for Cancer Research, Uppsala, Sweden.

Optimal performance of Amersham ECL Plex is achieved using low-fluorescence PVDF Amersham Hybond LFP 0.2 or nitrocellulose Amersham Protran Premium 0.2 membranes. For blocking, Amersham ECL Prime Blocking Reagent or BSA (bovine serum albumin) is recommended to minimize background noise. Antibodies should always be diluted to the concentrations recommended by the manufacturer, but as a guideline for Amersham ECL Plex, primary and secondary antibodies should be diluted from 1:100 to 1:5000 and from 1:1250 to 1:4000, respectively. It is well worth spending time to optimize antibody concentrations (see Appendix A).

6.2.3   Fluorescence hints and tips

In order to obtain high quality Western blotting results, you should become familiar with the general concerns related to fluorescence detection raised in 6.1.2. In addition, you should find the following points useful to help solve problems related to low signal-to-noise ratios.

☞   Use Amersham ECL Plex Cy5-conjugated secondary antibodies for the protein at the suspected lowest concentration in your sample. The signals emitted by Cy5 are slightly more intense than those emitted by Cy3 and especially Cy2.

☞   Anti-rabbit secondary antibodies tend to enable higher assay sensitivity than anti-mouse antibodies, although they might be less specific.

☞   To increase weak signals, use phosphate-buffered saline (PBS) supplemented with 0.1% Triton X-100 instead of 0.1% Tween-20 throughout the protocol. Note that Triton X-100 is NOT compatible with the Cy3 channel and its use might lead to high background.

☞ If you experience very strong signals from the markers, which overwhelm signals from low-abundance proteins, it is better to use a smaller amount of Amersham ECL Plex Rainbow Markers (1.5 µl). If possible, load sample loading buffer in one lane between the markers and the sample.

☞ Typhoon variable mode imagers are highly sensitive to fluorescent signals and enable detection of a wide linear dynamic range of protein quantities. As both sensitivity and dynamic range are also functions of characteristics of the antibody:protein pair, it is critical to optimize the concentrations of both the primary and secondary antibodies (Appendix A).

☞ Working with fluorescence detection carries certain risks of uneven background signal due to fluorescent contamination. Ensure that all your material is perfectly clean.

- Remove any residual pieces of gel from your membranes.

- The presence of bromophenol blue (BPB) on the membrane can generate unwanted fluorescent signals. Remove all traces of BPB from the bottom of sodium dodecyl sulfate (SDS)-PAGE gels before transfer to a membrane.

- Ensure that your trays, forceps and containers are clean and free of Coomassie Blue stain, as this can also cause background problems.

- Avoid labeling the membrane with a ballpoint pen as this can generate contaminating signals. Cut one corner of your membrane and note which corner you have cut!.

- Do not handle the membrane with your fingers; use clean forceps.

- Wear powder-free gloves when handling membranes. The powder used in laboratory gloves can fluoresce and might also scatter light, complicating the interpretation of images.

☞ If you have problems with the presence of many apparently nonspecific protein bands, try using an Amersham Hybond LFP 0.2 membrane. Amersham ECL Prime Blocking Reagent can further reduce nonspecific binding. This problem is often related to the choice of primary antibody. In addition, the dilution of primary antibody can affect specificity - optimization is crucial (see Appendix A)!

☞ Incubation of the primary antibody in blocking solution helps reduce reduce nonspecific binding and increase signal intensity. Some primary antibodies diluted in blocking solution result in a much stronger signal compared to dilution in washing buffer, even if used at a much lower concentration.

☞ A concentration of Tween-20 greater than 0.1% can result in a significantly increased background on PVDF membranes.

☞ If probing with fluorophore-conjugated antibodies:
- Aliquot the antibodies in light-protected tubes.
- Dilute the antibodies in light-protected containers.
- Perform secondary antibody probing protected from light.

☞ Rinse the membrane in PBS to remove Tween-20 prior to imaging.

Chapter 7
Imaging

| Sample preparation | Gel electrophoresis | Transfer | Antibody probing | Detection | Imaging | Analysis |

The last step in the Western blotting workflow before data analysis is image capture.

Enhanced chemiluminescence (ECL) is based on the reaction between an added luminol substrate and horseradish peroxidase (HRP)-labeled antibodies (see Chapter 6). In the presence of HRP, hydrogen peroxide catalyses the oxidation of luminol, a reaction that results in the emission of light. The light signal can then be detected on X-ray film or by digital imaging with a charge-coupled device (CCD) camera-based imager.

When using fluorescence detection, a fluorophore is conjugated to the primary or secondary antibody. Light is emitted by the fluorophore after excitation via a specific wavelength of light. A photomultiplier tube (PMT) or a CCD can be used to collect and convert the emitted light to an electrical signal. The electrical signal is then digitized for image display and analysis.

7.1   Digital imaging

In Western blotting applications, digital imaging is usually performed with a CCD camera-based imager or a scanner, depending on the characteristics of the light emitted from the detection system. In the following section, we will briefly describe these imaging systems and their suitability for capturing different kinds of emitted signals.

7.1.1   Charge-coupled device (CCD) camera-based imagers

Chemiluminescence is currently the most commonly used detection method in Western blotting. Traditionally, signals have been detected using X-ray film, which provide high sensitivity but a limited linear dynamic range. CCD camera-based imagers, on the other hand, provide detection with high sensitivity and also a broad linear dynamic range, enabling more precise quantitation. In addition, digital images allow for easier handling, archiving and analysis. There is no chemical waste and no need for a dark room as no film is involved.

CCD camera-based imagers can also be used to document membranes, stained gels, or ultraviolet (UV) light/fluorescence applications if the imager is equipped with the appropriate filter and excitation system. CCD cameras operate by collecting photons on a chip, and the collected charge is translated into a digital signal that correlates with signal strength. The signal from the imaging field is collected by a lens assembly that focuses the image (Fig 7.1).
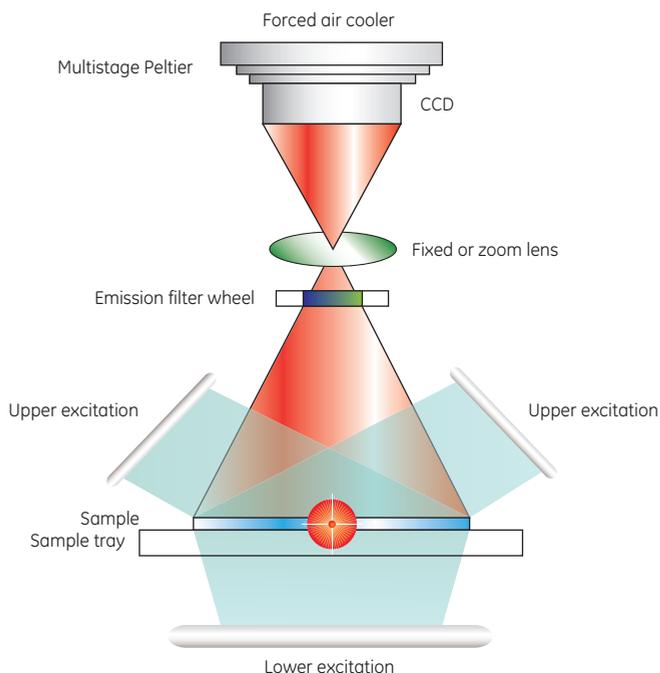
Fig 7.1. Components of a typical CCD camera-based imaging device. When the camera is used for chemiluminescence detection, the sample itself is the source of emitted light as the enzyme acts on the substrate. For fluorescence, on the other hand, light emission requires a source of excitation; the sample can be illuminated in a variety of ways depending on the nature of the label. Emission filters can be inserted in the light path to select specific wavelengths and reduce background. The camera includes focusing optics to maintain a clear image.

7.1.1.1 Excitation sources and light delivery

For visible light and fluorescence applications, illumination or excitation is provided by UV or white light gas discharge tubes, broad spectrum xenon arc lamps, or high-power, narrow-bandwidth diodes.

7.1.1.2 Light collection

Lenses are used to collect emissions from the imaging field. The lens system is either fixed, when used with an adjustable sample tray, or alternatively, has a zoom capacity, allowing capture of different sample sizes in a single view. Zoom objectives, however, decrease sensitivity and for this reason, height adjustable sample trays are preferable. Optimal systems are those in which the geometry of illumination can be adapted during image acquisition in order to accommodate the precise height of the sample tray.

In fluorescence imaging, filters are used to remove unwanted emitted light of specific wavelengths. Although chemiluminescence detection requires no filters, good lenses will optimize the image quality. As the light source is very weak, a wide aperture will collect more light in less time. The diameter of the aperture is thus particularly important; the weaker the light source, the wider the aperture.

7.1.1.3 System performance

The performance of any CCD camera-based imager is dependent on factors such as system resolution, sensitivity, and linear dynamic range. The resolution of a captured image is linked to the geometry of the CCD, with the size of each pixel varying from 6 to 30 µm. Currently, CCDs with formats from 512 × 512 to 3326 × 2504 elements are available.

CCD arrays are sensitive to light, temperature, and high energy radiation. Dark current from thermal energy, cosmic rays, and the preamplifier system cause noise that can affect instrument performance. Active cooling of the CCD significantly reduces noise levels and improves both sensitivity and linearity. This is only really an issue for capture of the typically very low light levels emitted in detection systems such as chemiluminescence.

The dynamic range of a CCD camera—the signal range over which the instrument yields a linear response in relation to sample quantity—is dependent on the light collector and the detector.

7.1.1.4   Amersham CCD camera-based imagers

Amersham imagers from GE Healthcare are digital systems for sensitive, quantitative imaging of gels and blots. A flexible combination of light sources and filters can be incorporated into the imagers, for detection by chemiluminescence, colorimetric image capture, UV, and red, green, or blue (RGB) fluorescence. All Amersham imagers from GE Healthcare have active cooling to reduce noise levels and to improve sensitivity and linearity. Amersham imagers are available with light sources for UV and RGB imaging.

7.1.2   Scanner systems

7.1.2.1   Excitation source

Scanner devices used for detecting fluorescence most commonly employ laser light for excitation. A laser source produces a narrow beam of highly monochromatic, coherent, and collimated light. A combination of focused energy and narrow beam width contributes to the excellent sensitivity and resolution of laser scanners. Alternative light sources include light-emitting diodes (LED), which are more compact and less expensive than lasers, but produce a wide band, low power output.

7.1.2.2   Light collection

The light collection optics in a scanner system must be designed to efficiently collect as much of the emitted fluorescent light as possible. In single-channel or single-label experiments, emission filters are designed to allow only a well-defined spectrum of emitted light to reach the detector. Any remaining stray excitation or scattered light (as well as autofluorescence) should be rejected.

In multiple experiments or multiplexed detection, the emitted light from each label has to be detected separately. The filter setup, in combination with the selection of labels, should be chosen to ensure that the different labels can be spectrally resolved, thus avoiding any cross-talk issues. After the fluorescent emission has been filtered, and only the desired wavelengths remain, the light is detected and quantitated. As the intensity of light at this stage is very small, a PMT must be used to amplify and detect it.

7.1.2.3   System performance

The performance of a laser scanner system can be measured in terms of system resolution, linear dynamic range, uniformity, and sensitivity.

Linear dynamic range is the signal range over which the instrument yields a linear response to sample quantity and is therefore an important parameter for accurate quantitation. A scanner with the ability to cover a wide linear dynamic range can detect and precisely quantitate signals from both very low and very high intensity targets in the same scan without reaching saturation. The linear dynamic range of most laser scanners is between 10^4 and 10^5 orders of magnitude.

Uniformity across the entire scan area is critical for reliable quantitation. A given fluorescent signal should yield the same measurement at any position within the imaging field.

Moving head scanners, in particular, deliver flat-field illumination and uniform collection of fluorescent emissions across the entire scan area and can also reduce the likelihood of photobleaching, as any single part of the sample is exposed to the excitation source for the minimal time.

The limit of detection (LOD) is the minimum amount of sample that can be detected by an instrument at a known confidence level. Instruments with better LOD require less fluorescent sample for analysis and allow more precise detection of small variations between different samples.

7.1.3   Fluorophores and filters

To generate a fluorescent signal, the excitation light directed at a sample must be within the absorption spectrum of the fluorophore. Generally, the closer the excitation wavelength is to the peak absorption wavelength of the fluorophore, the greater the excitation efficiency. It is not essential that the major absorption peak of the fluorophore exactly matches the available excitation wavelength for efficient excitation (Fig 7.2).
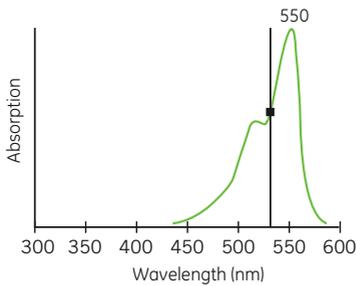

Fig 7.2. Excitation of Cy3 using 532 nm laser light. The absorption spectrum of Cy3 is overlaid with the 532 nm wavelength line of the laser.

Similarly, selecting a filter that transmits a band at or near the emission peak of the fluorochrome generally improves the sensitivity and linear dynamic range of the measurement (Fig 7.3).
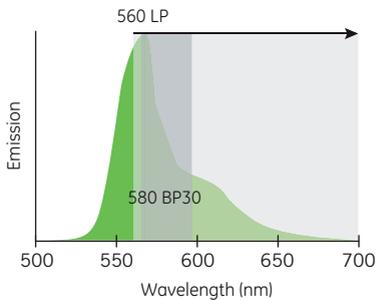

Fig 7.3. Emission filtering of Cy3 fluorescence using either a 580 BP30 filter (dark-gray area) or a 560 LP filter (light- and dark-grey areas). BP = band pass. LP = long pass.

Multicolor imaging allows the detection and resolution of multiple targets using fluorescent labels with different spectral properties. The process for multicolor image acquisition varies depending on the imaging system. An imager with a single detector acquires consecutive images using different emission filters and, in some cases, different excitation light. When two detectors are available, the combined or mixed fluorescence from two different labels is collected at the same time and is then resolved by filtering before the signal reaches the detectors.

Implementation of dual detection requires a beamsplitter filter to spectrally split the mixed fluorescent signal, directing the resulting two emission beams to separate emission filters (optimal for each fluorophore), and finally to the detectors. However, some spectral overlap between emission profiles is almost unavoidable. To minimize cross-contamination, fluorophores with well-separated emission peaks should be chosen along with emission filters that allow reasonable spectral discrimination between the emission profiles of the fluorophores. For optimal results, fluorophores with emission peaks at least 30 nm apart should be chosen.

Three types of optical emission filters are in common use:

Long pass (LP) filters pass light longer than a specified wavelength and reject all shorter wavelengths.

Short pass (SP) filters reject wavelengths longer than a specified value and pass shorter wavelengths.

Band pass (BP) filters allow a band of selected wavelengths to pass, while rejecting all shorter and longer wavelengths.

7.2 Chemiluminescence detection using film

When chemiluminescence is used as the detection system in Western blotting, X-ray films may be used to record emitted signals. X-ray films provide high sensitivity and flexible exposure times which might be needed for detection of very weak signals. However, X-ray film has limitations for quantitative analysis, as high-intensity signals tend to saturate if both weak and strong signals are to be detected on the same film. This results in a significantly narrower linear dynamic range of measurable protein quantities compared to analysis with a CCD camera-based imager. Other disadvantages to consider when using X-ray film for detection include the need to handle the chemical waste generated as well as the requirement for a dark room.

After X-ray film development, a digital image must be created for further computer analysis if required or if the image is to be published. This can be achieved by scanning the film with a densitometer or by taking a digital photo.

A variety of commercial products are available for signal detection in Western blotting, such as Amersham Hyperfilm ECL (Table 7.1).

Table 7.1. Recommended films from GE Healthcare for imaging in Western blotting analysis

Film	Detection
Amersham Hyperfilm ECL	Chemiluminescence
Amersham Hyperfilm MP	Radioisotopic

Amersham Hyperfilm ECL offers excellent sensitivity for low-concentration protein detection, excellent band resolution, and the detection of low-intensity bands. The film is also artefact-free due to an anti-static layer and produces publication-quality images due to a clear background. Amersham Hyperfilm ECL is suitable for use with Amersham ECL, Amersham ECL Prime, and Amersham ECL Select and it can be processed in automatic processors or manually by using most common X-ray film developers and fixers.

7.3 Autoradiography

The use of autoradiography as a detection method in Western blotting applications has greatly diminished due to cost and safety issues, (working in a radioactive environment, extensive waste handling etc). Despite these issues, autoradiography is very sensitive and as it is compatible with most of the imagers available from GE Healthcare, it is briefly discussed here. Autoradiographic detection is either performed using an X-ray film or a storage phosphor screen.

7.3.1   X-ray film autoradiography

Ever since radioactive isotopes were first used to tag biomolecules, autoradiography on photographic film has remained a detection option. Most radioisotopes have limited sensitivity, but this can be overcome by converting the emitted radiation to light. The resultant gain in sensitivity, however, is partly offset by decreased resolution together with a nonlinear response on film, which might result in misinterpretation of data, particularly in quantitative analysis.

7.3.2   Storage phosphor screen autoradiography

If very high sensitivity is required for Western blotting, radioisotopes, in combination with storage phosphor screens, are an excellent choice. Storage phosphor screens are reusable and are not degraded by repeated exposure to laboratory levels of radioactivity - unless you are using tritium with tritium screens. The sample is placed in contact with a storage phosphor screen (protected from light by using an exposure cassette). The location of the radioisotope is captured on the screen as the crystals of the screen are excited to a high energy state. This screen is then placed in an imager and exposed to a red light source, such as a laser, which destabilizes the crystals so they return to their ground state, releasing a lower wavelength light that can be captured with the appropriate filter and detection system.

7.4 Detection system and imager compatibility

Table 7.2 lists detection reagents available from GE Healthcare and recommended system for image capture.

Table 7.2. Summary of detection systems from GE Healthcare and compatibility with various imaging methods

	Detection method	Imager
Chemiluminescence	Amersham ECL	X-ray film/CCD camera
	Amersham ECL Prime	CCD camera/X-ray film
	Amersham ECL Select	CCD camera/X-ray film
Fluorescence	Amersham ECL Plex	Laser scanner/CCD camera[1]
	CyDye	Laser scanner/CCD camera[1]
Chromogenic	Coomassie Blue stain	CCD camera
	Silver stain	CCD camera
Radioisotopic	Autoradiography	X-ray film

[1] *Equipped with appropriate light sources and emission filters.*

Chapter 8
Analysis

| Sample preparation | Gel electrophoresis | Transfer | Antibody probing | Detection | Imaging | Analysis |

Detection of signals, using either X-ray film, scanners, or a charge-coupled device (CCD) camera-based imager, results in one or more visible protein bands on the membrane image. The molecular weight of the protein can be estimated by comparison with marker proteins and the amount of protein can be determined as this is related to band intensity (within the limits of the detection system). In most applications it is enough to confirm protein presence and roughly estimate the amount. However, other applications demand a quantitative analysis that defines protein levels in either relative or absolute terms. The aim of this chapter is to provide a guide to Western blotting analysis and how you can achieve precise quantitation in Western blotting experiments.

Qualitative protein analysis is performed in order to verify the presence or absence of a specific protein of interest (Fig 8.1). In typical Western blotting applications, the presence of a protein of interest is confirmed, the amount is estimated by visual analysis, and the size can be determined by comparison to a known molecular weight marker.
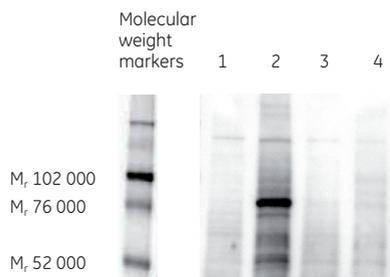

Fig 8.1. A typical Western blotting image in which a specific protein is detected in different samples. The image shows that the target protein (approx. M_r 76 000) is present only in the sample in lane 2. The size of the protein can be determined by comparing the location of the band to those in the lane containing molecular weight markers.

Quantitative protein analysis, on the other hand, implies a definition of the amount of protein on a blot either in relative or absolute terms. Both types of analyses can be performed with 1-D or 2-D gel electrophoresis followed by Western blotting. The aim of this chapter is to provide a guide to help you achieve precise quantitation of your proteins in Western blotting experiments.

8.1 Quantitative Western blotting

Western blotting has long been used for qualitative protein analysis to confirm protein presence and to approximately estimate protein amount. The development of highly sensitive detection reagents, however, together with advanced imaging techniques has made Western blotting a potential tool for quantitative protein analysis. When performing quantitative Western blotting, some important factors should be considered:

- Sensitivity
- Linear dynamic range
- Signal stability
- In-lane normalization
- Signal-to-noise ratio

8.1.1 Sensitivity

Sensitivity, in the context of Western blotting, is defined as the minimum amount of protein that can be detected using available detection systems (see Chapter 6). Sensitivity can be affected by many factors, such as antibody quality, antibody concentration, and exposure times.

Two terms are commonly used to define assay sensitivity; the limit of detection (LOD) is the minimum amount of protein that can be "seen" in a given assay. Usually, this is below the lower limit of signal intensity that can be reliably used for precise quantitation, known as the limit of quantitation (LOQ). LOQ is determined by the signal-to-noise ratio (see 8.1.5).

8.1.2 Linear dynamic range

The linear dynamic range is that over which signal intensity is proportional to the protein quantity on a blot and which thus allows precise quantitation throughout that range. Excessive amounts of protein or high concentrations of antibodies, but also excessive exposure times, can lead to saturated signals that are no longer proportional to protein concentration and must therefore be excluded from the analysis (Fig 8.2). The dynamic range is also affected by LOD: a detection system with low LOD (high sensitivity), in combination with precise quantitation of strong as well as weak signals, provides an assay with a broad linear dynamic range.
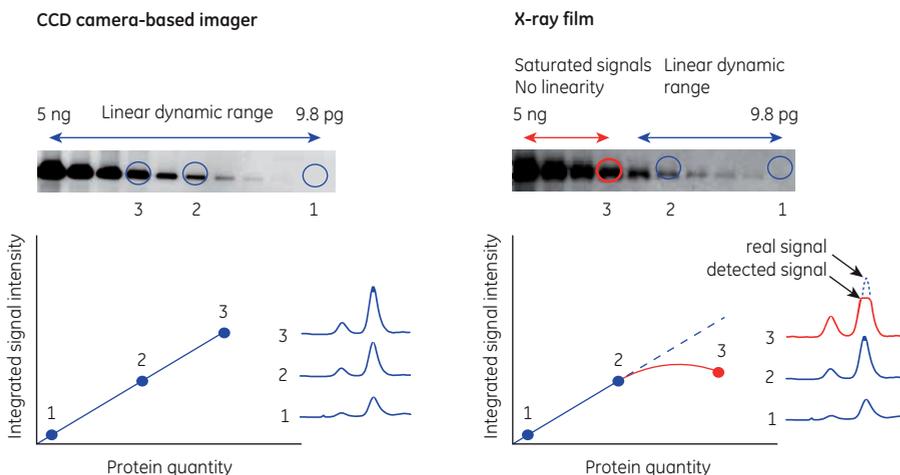

Fig 8.2. The advantage of using a CCD camera-based imager compared to X-ray film for signal detection. For the CCD camera-based system, all bands fall within the linear dynamic range of the system, whereas for film, the most intense bands (indicating more protein) are saturated, rendering quantitation impossible at this portion of the curve as the volume under the curve can no longer be determined.

For chemiluminescence detection, film provides high sensitivity, but has only a limited dynamic range. To obtain optimal sensitivity using film, increased exposure times are required, but this leads to saturated signals from high-abundance proteins and thus results in a narrower linear dynamic range.

CCD camera-based imagers (see Chapter 7), in addition to constantly increasing sensitivity, allow quantitation over a broader linear dynamic range than film. A narrow linear dynamic range is acceptable, however, if only small variations in signal intensity are expected, or where qualitative information (whether the protein is present or absent in the sample) is sufficient for your needs.

8.1.3   Signal stability

When performing quantitative Western blotting, it is an advantage to use a detection system with high signal stability, as this will impact on the linear dynamic range. With a stable signal detection reagent, the time window for reaching high sensitivity is longer. This allows multiple exposures and the possibility to detect weak bands that might be missed in a single, brief exposure.

It is important to distinguish between the terms signal "stability" and "duration". Chemiluminescence detection systems, for example, are based on an enzymatic reaction and the signal intensity thus decreases over time. This means that the signal stability achieved with a fluorescence detection system such as Amersham ECL Plex (where the signal is stable over time and is highly reproducible) cannot be matched by chemiluminescence. However, the signal duration of chemiluminescent reagents, such as Amersham ECL Prime (Fig 8.3), allows multiple exposures without the risk of a significant reduction in signal, a prerequisite for many quantitative Western blotting applications.
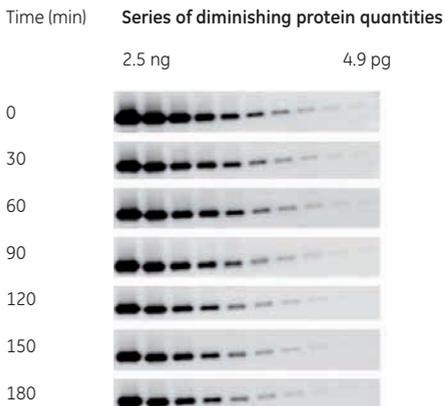

Fig 8.3. Amersham ECL Prime signal duration is monitored for 3 h after the addition of reagent by capturing images every 30 min with the same exposure time of 3 min. The majority of the bands are still detectable, and allow precise quantitation even after 3 h.

8.1.4   In-lane normalization

In order to reliably quantitate protein levels by Western blotting analysis, levels of the protein of interest should be normalized to an internal reference. This allows for variations in the amount of total protein from lane-to-lane due to errors such as inconsistent sample loading or, alternatively, different protein concentrations in the samples. If the sample is a cell lysate, an endogenous and unregulated "housekeeping" protein (a protein which is expressed at a relatively constant rate and is required for the maintenance of basic cellular functions) is commonly used as an internal standard (Fig 8.4). Another alternative is to spike the cell lysate with a known quantity of protein or to relate the protein of interest to the amount of total protein detected per lane on the blot.
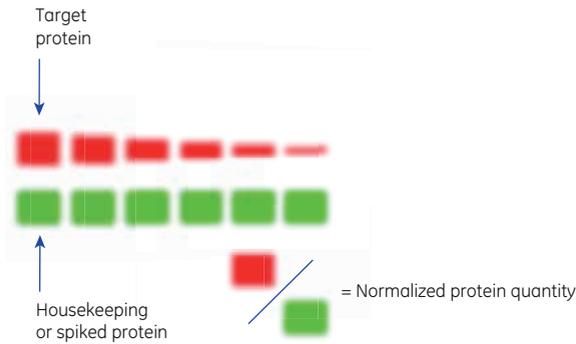
Fig 8.4. Illustration of how to normalize a target protein to a housekeeping or spiked protein. Initially, the intensity of each band is calculated using analysis software. The measured value for each target protein is then related to the corresponding value of the housekeeping or spiked protein.

Enhanced chemiluminescence (ECL) detection allows you to detect two proteins on the same blot in a single experiment, as long as their molecular weights are sufficiently different to be discretely resolved by electrophoresis (Fig 8.5). This approach requires preliminary screening of the primary antibodies to rule out any cross-reactivity issues. In the event of cross-reactivity, stripping and reprobing are necessary to detect both the housekeeping protein and the target protein. Stripping, however, carries a risk of the loss of an unknown amount of target protein from the membrane, leading to erroneous results (see Chapter 5 for more information).
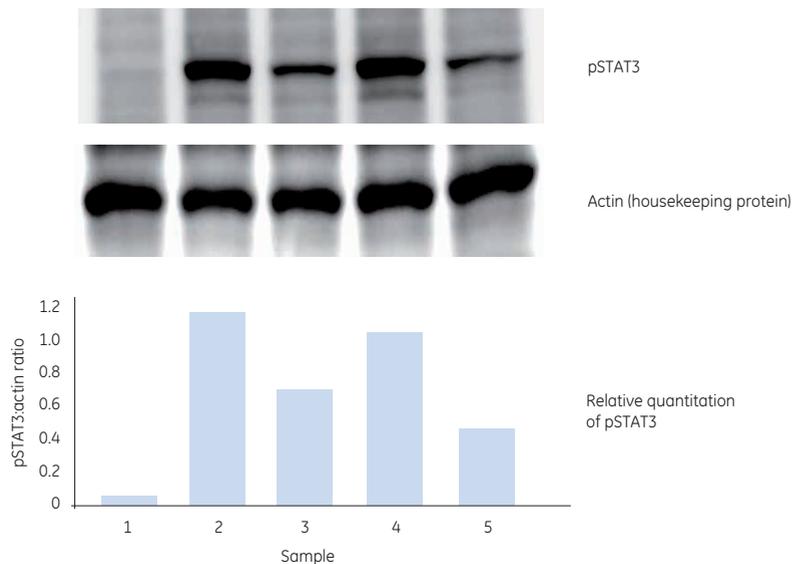

Fig 8.5. Western blotting detection of Tyr 705-phosphorylated STAT3 (pSTAT3) in five different HeLa cell lysates. In each case, pSTAT3 levels were quantitated after normalization with actin as a housekeeping protein. Detection was performed using Amersham ECL Prime and ImageQuant LAS 4000 mini and analysis was carried out using ImageQuant TL software.

By performing multiplexed, fluorescent Western blotting using Amersham ECL Plex as the detection system, it is possible to simultaneously detect two target proteins on the same blot. Proteins can thus be easily quantitated, after normalization to a housekeeping protein (Fig 8.6). This system enables the detection of proteins with very similar or even identical molecular weights. In addition, Amersham ECL Plex is optimized for minimal cross-talk between different CyDye conjugated secondary antibodies.
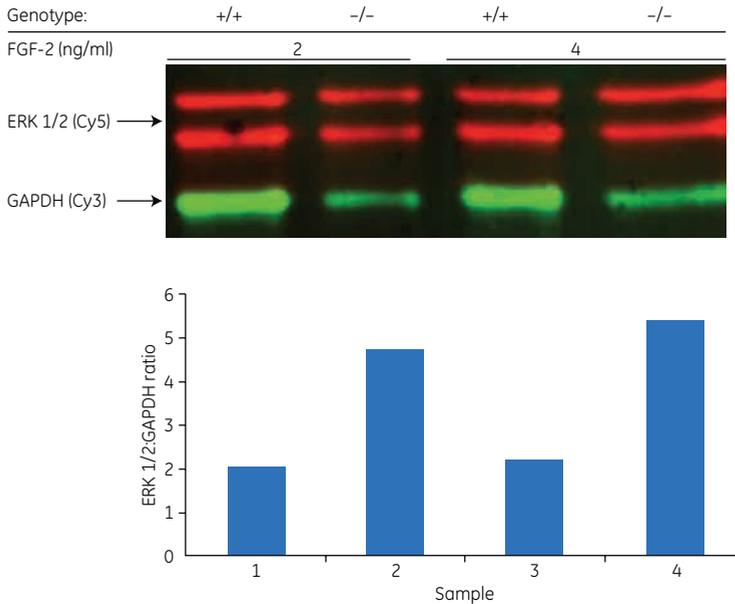
Fig 8.6. In this multiplexed Western blot, primary antibodies to ERK1/2 and the housekeeping protein, GAPDH, were simultaneously used to probe a membrane. Relating the signals to GAPDH signals indicates the amount of protein applied to each lane of the gel, allowing the true effect of FGF-2 treatment on ERK1/2 to be quantitated. +/+ indicates wildtype cells, -/- indicates knockout cells deficient in a key enzyme involved in the regulation of ERK1/2. Data courtesy of Dr. Jin-Ping Li and Dr. Juan Jia, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden.

8.1.5   Signal-to-noise ratio

During the imaging process (Chapter 7), the presence of a protein on a blot or gel gives rise to a signal of a certain intensity that is recorded and analyzed. To properly quantitate the protein, however, it is essential to consider the specific signal, as well as background (signals due to unwanted interactions). The background comprises primary antibodies binding to other proteins or the choice of membrane and blocking agent, as well as noise (system-generated signals). A signal peak corresponds generally to a protein band and the volume under that peak, but above the background level, is directly proportional to the quantity of protein (Fig 8.7).

Densitometric analysis of film is a sensitive way to quantitate ECL mediated detection. The high quantitative precision at very low levels, however, is offset by signal saturation at levels far below those at which CCD camera-based imagers continue to yield signals in proportion to protein quantity. The linear dynamic range offered by film, in other words, is significantly narrower than that of CCD camera-based imagers. In addition, the exposure time necessary to amplify weak signals to detectable levels tends to lead to high background signals that can mask the signals of the proteins of interest.
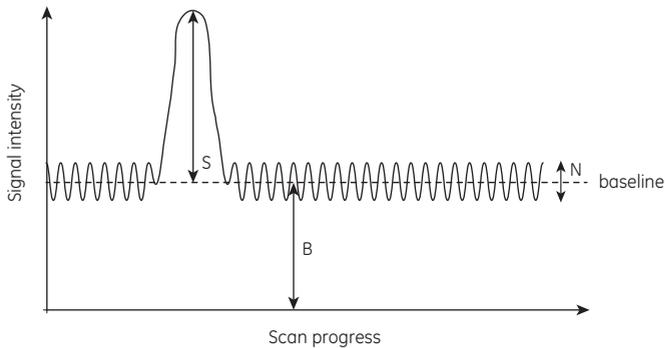
Fig 8.7. The basis of signal detection. B = background, N = noise, S = signal.

Background and noise reduce the signal-to-noise ratio and consequently decrease the sensitivity and the LOD of Western blots. High background will decrease the signal-to-noise ratio (Fig 8.8) and adversely alter linearity, leading to less precise quantitation.

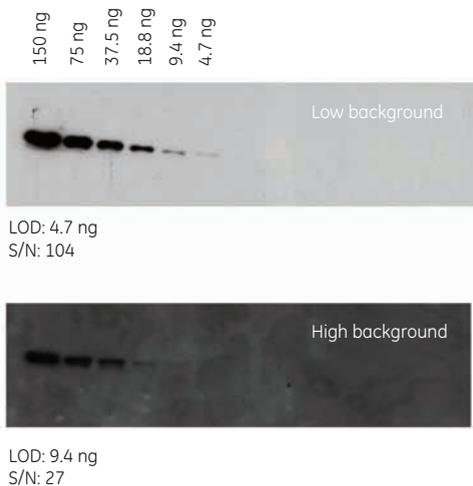**Fig 8.8.** A two-fold serial dilution of actin detected on blots using Amersham ECL: Some important consequences of elevated background levels on signal quantitation. The signals shown in the upper panel were obtained using optimized antibody concentrations. No optimization was performed for the signals shown in the lower panel. The consequence is a higher background, which affects LOD and the signal-to-noise ratio.

8.2 Analysis software

Image acquisition using imaging devices creates one or more data files for each sample analyzed. Several varieties of software are available that display the image, adjust contrast, annotate and print the image. In addition, image analysis software allows fragment sizing, quantitation, matching, pattern analysis, and generation of analysis reports.

ImageQuant TL is a high-performance, easy-to-use image-analysis software for the quantitative analysis of images from a wide range of biological samples. The program consists of four different modules: One for 1-D gel electrophoresis analysis; one for array analysis; one for colony counting/2-D spot analysis; and one general image analysis toolbox. The software provides tools for quantitation through user-defined signal integration of regions (volume) or using lane profiles and peak analysis (area). Analysis reports are dynamically linked to Microsoft Excel®.

8.2.1   Main features of ImageQuant TL software

ImageQuant TL software can be used to manually quantitate Western blotting although it is also possible to detect bands automatically using one of several types of background calculations. One of the analysis modules is shown in Figure 8.9.
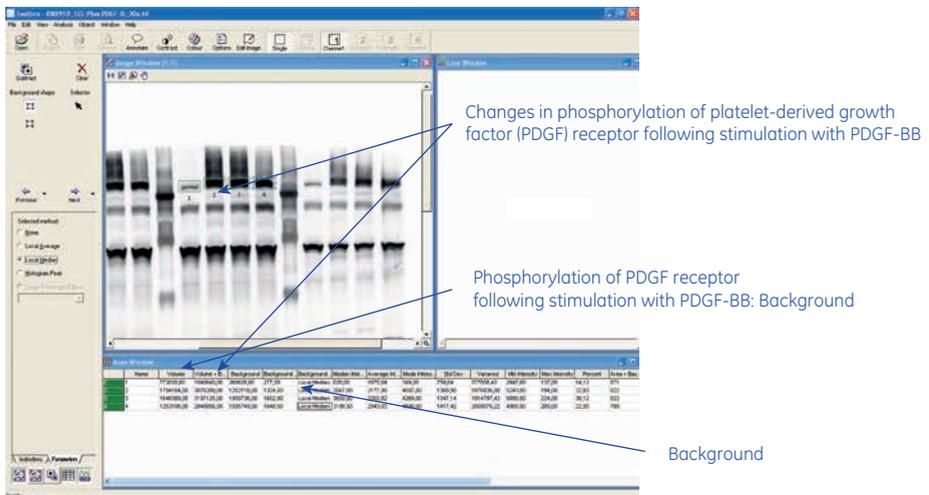

Fig 8.9. The relative increase in phosphorylation of PDGF receptor over time after stimulation with PDGF-BB as calculated using ImageQuant TL software. This screenshot shows how you can calculate the total intensity of a band of interest in the toolbox module. You can select the band for which you want to determine intensity and automatically subtract the background. In this particular example, the total band intensity for the phosphorylation of the PDGF receptor at different time points after stimulation with PDGF was determined. The background is subtracted from all values and the data can easily be exported to Microsoft Excel for further calculations.

Automatic analysis of 1-D gel electrophoresis images

For the analysis of 1-D gels, including multitiers. If the analysis requires subsequent refinement, it is possible to view each stage and make adjustments.

Accurate molecular weight determination

For the creation of a molecular weight curve and propagation by Rf to account for distorted gels.

Quantitation of bands

For the quantitation of data from 1-D gels, comprising band detection, background subtraction, calibration, and normalization.

Image handling tools

For cropping, rotating, flipping, and filtering images across all channels.

1-D analysis

1-D analysis is performed using the tools provided in ImageQuant TL including:

- Multichannel analysis capability
- Fully automatic, single-click image analysis
- Instant access to refinement of any analysis step
- Alternative stepwise image analysis for each step
- User-defined preferences that can be saved and loaded for similar experiments

Lane creation is fully flexible and can be performed manually or automatically. Lane editing tools include options for:

- Multitier detection
- Selection of the area of interest
- Multibox adjustment
- Compensation for band distortions via "Grimace" tool
- Moving, resizing, and bending individual lanes
- Deleting and adding lanes
- Importing and exporting the lane template

Background subtraction

Background can be subtracted by three automatic methods (rubber band, minimum profile, or rolling ball) or by two manual methods (image rectangle or manual baseline).

Band detection

Bands can be automatically detected using three parameters (minimum slope, noise reduction, percentage maximum peak), with band edges automatically determined or set to a fixed width. Band editing and analysis tools include:

• Manual editing of peak and edge detection

• Option to view multiple lane profiles, stacked or overlaid

• Editing in Image Window and Lane Profile Window

• Band measurements automatically displayed in Measurement Tables

• Three types of Measurement Tables (Selected lane, All lanes, and Comparisons)

• Automatic update of tables

• Export of lane profile information

Molecular size/isoelectric point (pI) calibration

Molecular size/pI calibration allows users to select from (and edit) a library of 13 standards, or to create a new standard. Editing options include:

• Fully automatic or manual assignment of standard bands

• Propagation by Rf

• Six Curve Fitting Methods

• Molecular weights automatically displayed in Measurements Table

Quantity calibration

To account for nonlinear staining effects, one of five curve fitting methods is used for quantity calibration. A choice of 10 measurement units is available.

Normalization

The normalization protocol allows the use of known values, or the bands can be expressed as a percentage or a proportion of one or more selected bands. A choice of 10 measurement units is available.

Chapter 9
Application examples

In this chapter, we present a selection of applications in which Western blotting (and also in some instances, 1-D gel electrophoresis analysis) has played a central role—from confirmation of the presence of target proteins in a chromatography fraction to quantitative multiplexed Western blotting.

Electrophoresis on mini-gels has been used, and standard transfer, blocking, and probing procedures have been applied unless otherwise stated. "Standard procedures" for various steps refer to the recommendations in this Handbook (see Chapter 11). Wet transfer has been used in all cases. Optimal antibody concentrations were established according to our recommendations, as well as information provided by vendors. A set of Western blotting applications are described and information about sample types and products used are provided for each set of results. We hope that these examples will give ideas and show how Western blotting can contribute to solve the many challenges in research, production, and quality control.

9.1 Chemiluminescent Western blotting

9.1.1   Purification of recombinant proteins

The processes employed in the production of recombinant proteins in bacteria must deliver high purity and yield. In evaluating alternative production methods, it is usually more important to compare yields or confirm the presence of the protein of interest in chromatography fractions, rather than to know precise quantities. It is also important to be able to detect impurities in recombinant proteins.

Before scale-up of a protein production process, for example, it is important to know how variations in certain parameters affect final yields. In the following example, researchers at GE Healthcare studied the effect of different induction temperatures on the yield of recombinant histidine-tagged green fluorescent protein (GFP) produced in *E. coli*. Following purification, the protein yields were determined with Western blotting using Amersham ECL detection to assess the effects of temperature variation on protein yield and batch consistency (Fig 9.1).

Materials

|	
Sample:	Purified recombinant histidine-tagged GFP
Marker:	Full-Range Rainbow Molecular Weight Markers
Membrane:	Amersham Hybond ECL
	(now replaced by Amersham Protran Premium)
Blocking solution:	Amersham ECL Blocking Agent
Primary antibody:	Mouse anti-histidine
Secondary antibody:	ECL Mouse IgG, HRP-Linked Whole Ab
Detection:	Amersham ECL
Imaging:	Amersham Hyperfilm ECL
Analysis:	ImageQuant TL 7.0
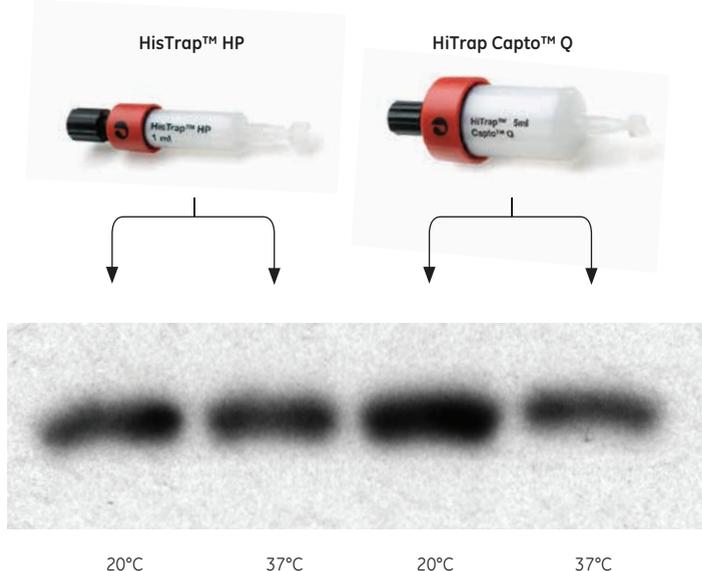
Fig 9.1. Detection of recombinant histidine-tagged GFP with Amersham ECL and Amersham Hyperfilm ECL, for comparison of protein yields after different induction temperatures (20°C or 37°C) and purification media (HisTrap HP, 1 ml or HiTrap Capto Q, 5 ml on ÄKTAexplorer). A visual analysis of the results from this purification shows that the highest yield of histidine-tagged GFP was achieved using an induction temperature of 20°C and purification on HiTrap Capto Q.

9.1.2 Analysis of IgG fractions purified from human plasma

Plasma contains a large variety of proteins including immunoglobulins, which have important therapeutic applications, such as passive prophylaxis. The efficacy of the chromatography and ultrafiltration procedures used to purify IgG from the total immunoglobulin pool in plasma can be tested by Western blotting. In this example, samples of interest for the further purification of the plasma proteins, transferrin and fibrinogen, were also identified by Western blotting.

Samples from the different purification steps were separated on a 4% to 20% gradient Amersham ECL Gel and stained using Deep Purple Total Protein Stain (Fig 9.2). Western blotting was also performed to verify the identity of the purified protein (Fig 9.3).

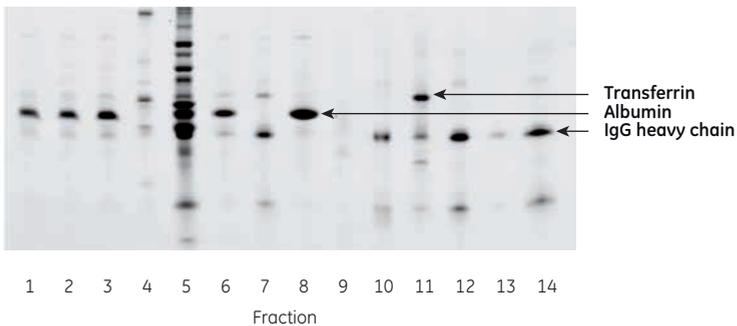|	
Gel type:	Amersham ECL Gel 4-20%, 15 wells
Sample:	Plasma fractions from an IgG purification process, 500 ng total protein
Detection:	Deep Purple Total Protein Stain
Imaging:	Typhoon laser scanner
Analysis:	ImageQuant TL 7.0

Fig 9.2. Plasma fractions in an IgG purification process, stained using Deep Purple Total Protein Stain. The enrichment and purification of IgG can be followed through different steps from the original plasma pool (1) to purified IgG heavy chain (14). Fractions run in lanes 8 and 11 show where albumin and transferrin, respectively, are separated from IgG.

Sample:	Plasma fractions from an IgG purification process, 250 ng (IgG) and 500 ng (transferrin, fibrinogen) total protein
Membrane:	Amersham Hybond-P (now replaced by Amersham Hybond 0.45 PVDF)
Blocking solution:	Amersham ECL Prime Blocking Reagent
Primary antibody:	Mouse anti-human IgG-HRP
	Mouse anti-human transferrin
	Rabbit anti-human fibrinogen
Secondary antibody:	ECL Mouse IgG, HRP-Linked Whole Ab
	ECL Rabbit IgG, HRP-Linked Whole Ab
Detection:	Amersham ECL Prime
Imaging:	ImageQuant LAS 4000
Analysis:	ImageQuant TL 7.0

Fig 9.3. Western blot detection of IgG in plasma fractionated using chromatography and ultrafiltration. Lanes 1 to 14 contain samples in the same order as indicated in Figure 9.2. With Western blotting, it was possible to monitor the different purification steps for IgG, as well as transferrin and fibrinogen content. Relative quantitation of IgG, transferrin and fibrinogen content indicated how much protein was lost (or remained) during the different purification steps. These results show the enrichment of IgG during the purification process. In addition, the fractions of most interest for the further purification of fibrinogen and transferrin were identified.

9.1.3 Detection of low-abundance proteins: Monitoring signaling pathway activation

In preclinical research, cultured cells are sometimes treated with potential drugs and biotherapeutics to study the activation of specific signaling pathways. The incidence of post-translational modifications (PTMs), such as protein phosphorylation, indicate the activation of such pathways. Phosphorylated forms of proteins, however, are often present at very low levels compared to the parent protein. For this reason, a very sensitive detection that is nevertheless able to measure a wide range of protein quantities is needed. Western blotting followed by detection using chemiluminescent Amersham ECL Prime provides a practical means to work with applications where both high sensitivity and a broad linear dynamic range are important.

STAT3 is a transcription factor involved in host immunity. The activity of the protein is regulated by cytokines, such as interferon-α (IFN-α). Stimulation of cells with IFN-α leads to the phosphorylation of two specific tyrosine residues on STAT3, resulting in the formation of homodimers or heterodimers that translocate to the nucleus and initiate transcription.

In the following application example, phosphorylation of STAT3 is evaluated in HeLa cells after treatment with IFN-α. Samples of lysates from HeLa cells (IFN-α-treated and untreated controls), were applied to SDS-PAGE followed by Western blotting using a phosphorylated STAT3-specific antibody. Phosphorylated STAT3 (pSTAT3) was detected using Amersham ECL Prime (Fig 9.4) and imaged using ImageQuant LAS 4000 mini. The membrane was then stripped and reprobed for actin, a housekeeping protein. By monitoring the expression levels of actin, it was possible to compensate for uneven sample loads on the gel. Levels of pSTAT3 were normalized against corresponding actin levels.
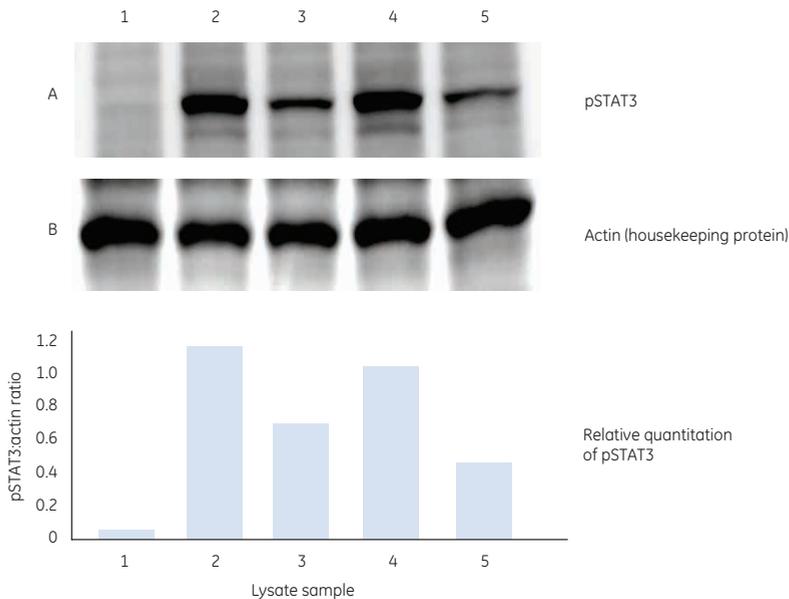
Fig 9.4. Amersham ECL Prime Western blotting detection of pSTAT3 and actin in cell lysates from untreated- (1) and IFN-α-treated HeLa cells (2 to 5). The result shows IFN-α-induced phosphorylation of STAT3 (A). The levels of pSTAT3 (A) are normalized to the levels of a housekeeping protein, actin (B), to correct for variation in total sample amount loaded. The biological variation of pSTAT3 levels in the samples is relatively quantitated to monitor the changes in STAT3 phosphorylation as a response to IFN-α treatment.

9.1.4 Detection of protein interactions by co-immunoprecipitation and Western blotting

Immunoprecipitation of intact protein complexes is known as co-immunoprecipitation and is a powerful technique for the analysis of interactions between proteins. Co-immunoprecipitation works by selecting an antibody against the protein of interest. Subsequently, the antibody:protein complex is conjugated to a solid support, such as Protein G Mag Sepharose beads from GE Healthcare. The bead complex is captured and the protein of interest is eluted together with any interacting partners.

A typical application for co-immunoprecipitation is the analysis of cell-signaling proteins following growth factor receptor activation. TAK1, for example, is a serine/threonine protein kinase involved in signaling induced by the activation of transforming growth factor-β (TGF-β) receptors. When thus stimulated, TAK1 binds and forms a functional kinase complex with a second protein known as TAB1, further propagating signal transduction.

Co-immunoprecipitation was performed on cell lysates from untreated- and TGF-β-treated human prostate cancer cells (PCU3). TAK1 antibody was added to the cell lysate followed by Protein G Mag Sepharose. The beads bound the TAK1 antibody in the cell lysate, and allowed extraction of TAK1/TAB1 complexes. TAK 1/TAB 1 complex eluted from the beads, as well as total cell lysate, was applied to a gel. TAK1 and the interacting protein, TAB1, were detected in the samples eluted from the beads and in the total cell lysate (Fig 9.5).

Materials
Sample: Cell lysates from untreated and TGF-β treated PCU3 cells
Marker: Full-Range Rainbow Molecular Weight Markers
Membrane: Amersham Hybond-P (now replaced by Amersham Hybond PVDF 0.45)
Blocking solution: Amersham ECL Prime Blocking Reagent

Primary antibodies: (A) Mouse anti-TAK1
                          (B) Rabbit anti-TAB1
Secondary antibodies: (A) ECL Mouse IgG, HRP-Linked Whole Ab
                          (B) ECL Rabbit IgG, HRP-Linked Whole Ab

Primary antibodies: (C) Mouse anti-TAK1
                          (D) Mouse anti-GAPDH
Secondary antibodies: (C, D) ECL Mouse IgG, HRP-Linked Whole Ab

Detection: Amersham ECL Prime
Imaging: ImageQuant LAS 4000
Analysis: ImageQuant TL 7.0
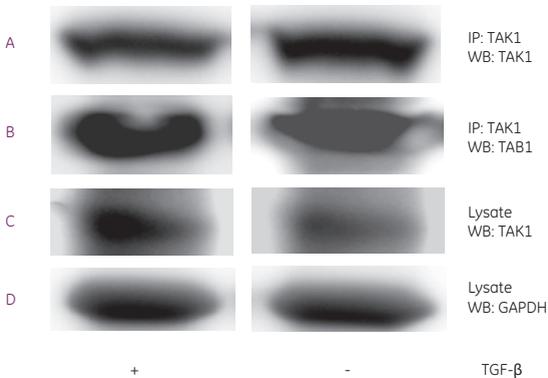
Fig 9.5. TAB1 was co-immunoprecipitated with TAK1 in cell lysates from untreated (-) and TGF-β-treated (+) PCU3 cells. Although an endogenous interaction between TAK1 and TAB1 was observed in the PCU3 cells (panels A and B), no TGF-β-dependency was observed in this particular case. Levels of TAK1 expression and GAPDH, a housekeeping protein, were confirmed in the total cell lysate (panels C and D). Data courtesy of Professor Marene Landström, Umeå University, Sweden.

9.2 Fluorescent Western blotting

9.2.1 Multiplexed detection for normalizing against a housekeeping protein

When working with chemiluminescent Western blotting, it is often necessary to strip and reprobe the membrane if you want to normalize detected protein levels against a housekeeping protein. The stripping procedure, however, carries a risk of uneven loss of proteins. With Amersham ECL Plex, stripping and reprobing are no longer required, due to the possibility of multiplexed detection – the detection of the target protein using one CyDye and the housekeeping protein with a different CyDye.

In the following example, the activation of ERK 1/2 was studied in the lysates of wildtype and knockout fibroblasts treated with fibroblast growth factor-2 (FGF-2). The lysates were run on a gel, and after blotting, the membrane was simultaneously probed with mouse anti-ERK1/2 and rabbit anti-GAPDH, followed by Amersham ECL Plex anti-mouse Cy5 and Amersham ECL Plex anti-rabbit Cy3. Although protein quantitation analysis indicated that a similar amount of total protein was loaded in each lane, the intensity of the signals emitted due to detection of the housekeeping protein, GAPDH, clearly showed that this was not the case (Fig 9.6). Without relating to the GAPDH levels, no significant pattern of ERK1/2 activation related to wildtype and knockout cells was seen. However, when normalized against GAPDH signals, ERK1/2 levels were shown to be increased in knockout cells after stimulation with 2 and 4 ng/ml of FGF-2.

Materials
Sample:	Cell lysates from FGF-2-treated wildtype (+/+) and knockout (-/-) mouse embryonic fibroblasts
Marker:	ECL Plex Fluorescent Rainbow Markers
Membrane:	Amersham Hybond-LFP (now replaced by Amersham Hybond LFP 0.2)
Blocking solution:	Amersham ECL Prime Blocking Reagent
Primary antibodies:	Rabbit anti-MAP kinase ERK1/2
	Mouse anti-GAPDH
Secondary antibodies:	ECL Plex goat anti-rabbit Cy5
	ECL Plex goat anti-mouse Cy3
Detection:	Amersham ECL Plex
Imaging:	Typhoon laser scanner
Analysis:	ImageQuant TL 7.0
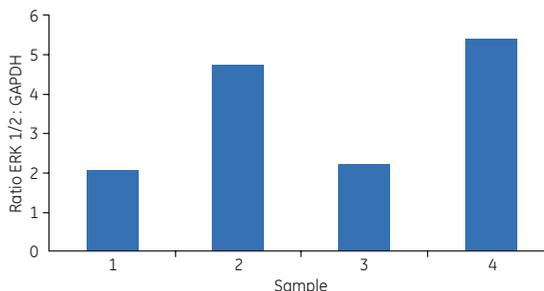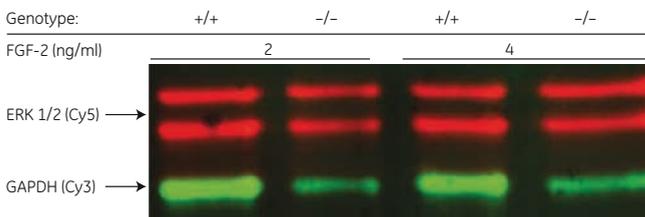
Fig 9.6. By detecting two proteins on the same blot, protein expression can be quantitated relative to a housekeeping protein such as GAPDH. This image shows Amersham ECL Plex detection of ERK 1/2 in wildtype (+/+) and knockout (-/-) mouse embryonic fibroblasts in response to treatment with FGF-2. ERK1/2 (Cy5) and GAPDH (Cy3) were targeted using specific primary antibodies followed by secondary ECL Plex Cy5 and Cy3 antibodies. The expression levels of ERK1/2 after FGF-2 stimulation is revealed after normalization to GAPDH, as illustrated in the diagram. Data courtesy of Dr. Jin-Ping Li and Dr. Juan Jia, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden.

9.2.2   Multiplexed detection of proteins with similar molecular weights

The multiplexing potential of Amersham ECL Plex is particularly powerful when it comes to visualizing proteins of similar size and PTMs, while simultaneously monitoring expression of the unmodified form of the protein. In addition, if the protein of interest is predicted to be expressed at very low levels, it is important to avoid the sample loss and reduction of protein activity inherent in the aggressive process of membrane stripping.

In the following example, the phosphorylation of tyrosine residues on Akt protein was analyzed after stimulation of cancer cells with TGF-β. PCU3 cell lysates were first separated on a Tris-glycine gel. Proteins were then transferred to an Amersham Hybond-LFP membrane (now replaced by Amersham Hybond LFP 0.2). After blocking, the membrane was probed with mouse anti-Akt and rabbit anti-phosphoAkt primary antibodies, followed by secondary Amersham ECL Plex anti-mouse Cy5 and Amersham ECL Plex anti-rabbit Cy3.

An increase in tyrosine phosphorylation of Akt was seen after stimulation with TGF-β. The results demonstrate how multiplexed detection with Amersham ECL Plex enables two epitopes on a single protein to be identified and simultaneously quantitated on a single Western blot (Fig 9.7).

Materials

Sample:	PCU3 cell lysate
Marker:	ECL Plex Fluorescent Rainbow Markers
Membrane:	Amersham Hybond-LFP (now replaced by Amersham Hybond LFP 0.2)
Blocking solution:	Amersham ECL Prime Blocking Reagent
Primary antibodies:	(1) Mouse anti-Akt
	(2) Rabbit anti-phosphoAkt
Secondary antibodies:	(1) ECL Plex goat anti-mouse Cy5
	(2) ECL Plex goat anti-rabbit Cy3
Detection:	Amersham ECL Plex
Imaging:	Typhoon laser scanner
Analysis:	ImageQuant TL 7.0
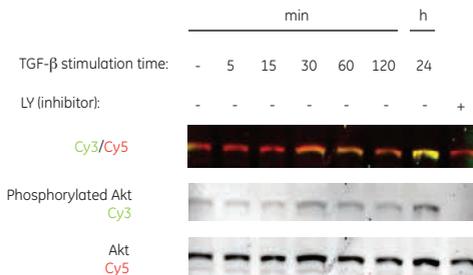
Fig 9.7. Detection, using Amersham ECL Plex, of low-abundance phosphorylated Akt protein and total Akt protein in PCU3 cells after stimulation with TGF-β. Despite the minimal change in molecular weight as a result of phosphorylation, the duplex capability of Amersham ECL Plex enables a clear distinction between the two forms of the protein. Note the complete absence of signal in the Cy3 channel for the sample treated with kinase inhibitor, LY. Data courtesy of Marene Landström, Ludwig Institute for Cancer Research, Uppsala, Sweden.

9.2.3   Triplexed detection: The simultaneous detection of three proteins

Imagine you have to study the expression of two proteins of interest, for example, a protein with and without a PTM such as phosphorylation, normalized to a housekeeping protein on the same blot. This application demands the use of three fluorophores, for example Cy2, Cy3, and Cy5. On excitation with light of the appropriate wavelength, each of these fluorophores will emit light at its own discrete, characteristic wavelength. In addition, it is necessary that the primary antibodies used to detect each of the three targets are raised in different species and that the secondary antibodies do not significantly cross-react between these species. Analysis using Amersham ECL Plex enables the simultaneous detection of two targets in a sample using different CyDye labeled goat anti-mouse and goat anti-rabbit secondary antibodies. The antibody directed against the third target, however, must be directly labeled with a third fluorophore by the user.

Triplexed detection is particularly suited for the detection of minute changes in PTMs. For example, as part of a study on the effect of irradiation on cells, three proteins, ERK, phosphorylated ERK, and the housekeeping protein, GAPDH, were simultaneously detected using primary antibodies raised in three different species. Two of the conjugated secondary antibodies (ECL Plex anti-mouse Cy3 and ECL Plex anti-rabbit Cy2) are commercially available from GE Healthcare. Cy5, on the other hand, was directly conjugated to anti-GAPDH primary antibody (Fig 9.8). For information on how to perform this labeling step, see Chapter 11.

Materials

|	
Sample:	Cell lysates from non-irradiated (C) and irradiated (T) HeLa cells
Marker:	ECL Plex Fluorescent Rainbow Markers
Membrane:	Amersham Hybond-LFP (now replaced by Hybond LFP 0.2)
Blocking solution:	Amersham ECL Prime Blocking Reagent
Primary antibodies:	(1) Rabbit anti-MAP kinase (ERK1-ERK2)
	(2) Mouse anti-phospho-ERK
	(3) Cy5-conjugated goat anti-GAPDH
Secondary antibodies:	(1) ECL Plex goat anti-rabbit Cy2
	(2) ECL Plex goat anti-mouse Cy3
Detection:	Amersham ECL Plex
Imaging:	Typhoon laser scanner
Analysis:	ImageQuant TL 7.0
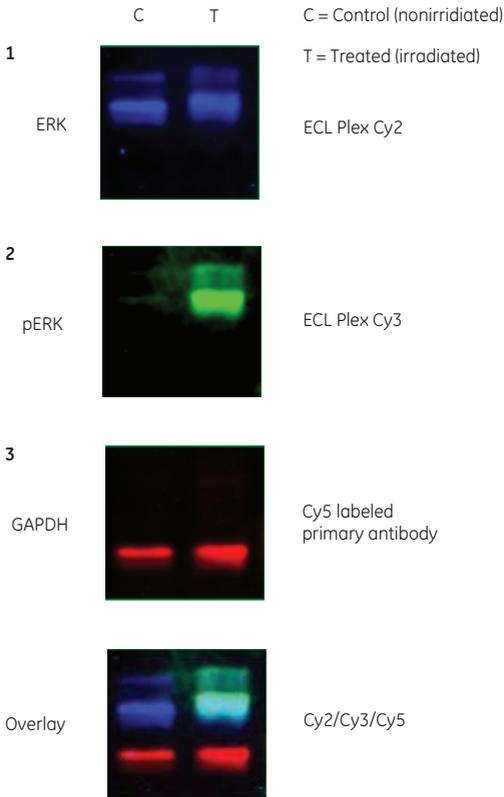
Fig 9.8. Quantitative triplex analysis of a cell lysate of HeLa cells labeled with antibodies to ERK, pERK and GAPDH. Ultraviolet irradiation is shown to induce only a small increase in native ERK, but a significant increase in phosphorylated ERK. In the same experiment, levels of the housekeeping protein, GAPDH, were monitored without the need to strip and reprobe the membrane. In a single experiment, three different parameters were thus simultaneously detected.

9.2.4 Three-layer fluorescent Western blotting for signal amplification

For the detection of extremely low-abundant proteins and other weak signals, three-layer fluorescent Western blotting is a good option.

In this technique, primary antibodies are used to probe proteins on a membrane. Biotin-labeled, species-specific secondary antibodies are then applied, followed by a third layer, usually streptavidin conjugated with HRP or a fluorophore (Fig 9.9).
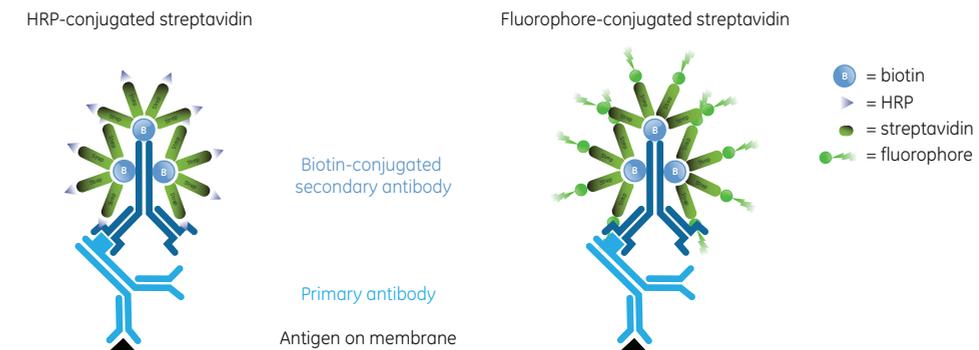
HRP-conjugated streptavidin   Fluorophore-conjugated streptavidin

B = biotin
► = HRP
● = streptavidin
●—‹ = fluorophore

Biotin-conjugated secondary antibody

Primary antibody

Antigen on membrane

Fig 9.9. The principle of three-layer fluorescent Western blotting, where the secondary antibody is labeled with biotin. A third layer of streptavidin conjugated with an enzyme, such as HRP (left) or a fluorophore, such as a CyDye (right) as the detection reagent leads to a significant increase in sensitivity compared with two-layer detection.

This approach is a particularly effective way to increase sensitivity over two-layer detection and is illustrated in the following example on the expression of ERK protein in cancer cells. ERKs are members of the mammalian MAP kinase family. They are known to activate many transcription factors, as well as other protein kinases. Disruption of the ERK signaling pathway is a common feature of many cancers. As they are expressed transiently and at very low concentrations, their detection thus requires a highly sensitive system. A Western blotting analysis of a dilution series of 3T3 mouse fibroblast cell lysates was performed targeting ERK and the housekeeping protein, GAPDH (Fig 9.10).

Materials

Sample:	3T3 cell lysates
Marker:	Full-Range Rainbow Molecular Weight Markers
Membrane:	Amersham Hybond-LFP (now replaced by Amersham Hybond LFP 0.2)
Blocking solution:	5% BSA
Primary antibody:	Rabbit anti-ERK 1/2
	Mouse anti-GAPDH
Secondary antibodies:	ECL Plex goat anti-rabbit IgG Cy5,
	ECL Plex goat anti-mouse IgG Cy3
	Biotin-conjugated donkey anti-rabbit IgG
Third layer reagents:	Cy5-conjugated streptavidin
Detection:	Amersham ECL Plex
Imaging:	Typhoon laser scanner
Analysis:	ImageQuant TL 7.0

	Two-layer standard approach	Three-layer approach
ERK 1/2 (Cy5) GAPDH (Cy3)		
Layer 1	Rabbit anti-ERK 1/2	Rabbit anti-ERK 1/2
Layer 2	ECL Plex goat anti-rabbit IgG Cy5	Biotin conjugated donkey anti-rabbit IgG
Layer 3		Cy5-conjugated streptavidin

Fig 9.10. Western blotting analysis of a two-fold dilution series of 3T3 mouse fibroblast cell lysates. ERK 1/2 (red) and GAPDH (green) were targeted by multiplexed fluorescent Western blotting. ERK 1/2 was measured by using standard two-layer Amersham ECL Plex (left) and three-layer detection with Cy5-labeled streptavidin (right). A comparison of the differences in integrated signal intensity showed an approximately 15-fold increase in signal intensity when using three-layer detection with Cy5-labeled streptavidin compared with two-layer detection. Note that the GAPDH Cy3 signal (green) is almost identical using both methods. The effect of introducing a third layer of detection was shown to improve sensitivity and is thus an option for the detection very low-abundance proteins.

9.2.5 Two-dimensional (2-D) Western blotting for detection of phosphorylated isoforms

2-D Western blotting analysis is particularly beneficial for complex analyses when high resolution is needed.

In this example, phosphorylation sites on glucogen synthase kinase 3β (GSK3β) were characterized by quantitative multiplexed analysis of PCU3 cells using Amersham ECL Plex. Cell lysates were separated by isoelectric focusing (first-dimension separation according to charge) and then by SDS-PAGE (second-dimension separation according to size). Following blotting, the membranes were probed with mouse anti-GSK3β and a rabbit antibody to its phosphorylated counterpart, pGSK3β. The membranes were then probed using ECL Plex goat anti-mouse IgG-Cy3 and ECL Plex goat anti-rabbit IgG-Cy5.

Each additional phosphate group on GSK3β induces a measurable change in migration. The 2-D Western blotting experiment gave additional information on phosphorylation states compared to 1-D Western blotting (Figs 9.11 and 9.12). The band corresponding to phosphorylated GSK3β (M_r 48 000) in the 1-D experiments was resolved into at least five distinct protein isoforms, two of which were phosphorylated on serine 9. For more detailed information on methods, see reference 1.

Materials

|	
Sample:	PCU3 cell lysate
Immobilized pH gradient (IPG) strip:	Immobiline Dry Strip pH 7–11 NL, 7 cm
Membrane:	Hybond-P (now replaced by Amersham Hybond PVDF 0.45)
Blocking solution:	Amersham ECL Prime Blocking Reagent
Primary antibodies:	Mouse anti-GSK3β
	Rabbit anti-pGSK3β
Secondary antibodies:	ECL Plex anti mouse IgG-Cy3
	ECL Plex anti rabbit IgG-Cy5
Detection:	Amersham ECL Plex
Imaging:	Typhoon laser scanner
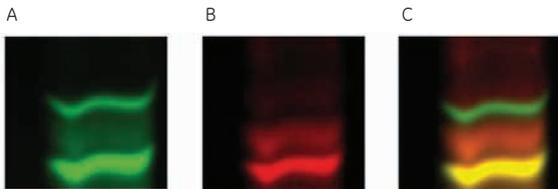
Fig 9.11. 1-D Western blotting scans of (A) ECL Plex Cy3-conjugated secondary antibodies against primary antibodies to GSK3β. (B) ECL Plex Cy5-conjugated secondary antibodies against primary antibodies to phosphorylated GSK3β, (C) Overlay of A and B.
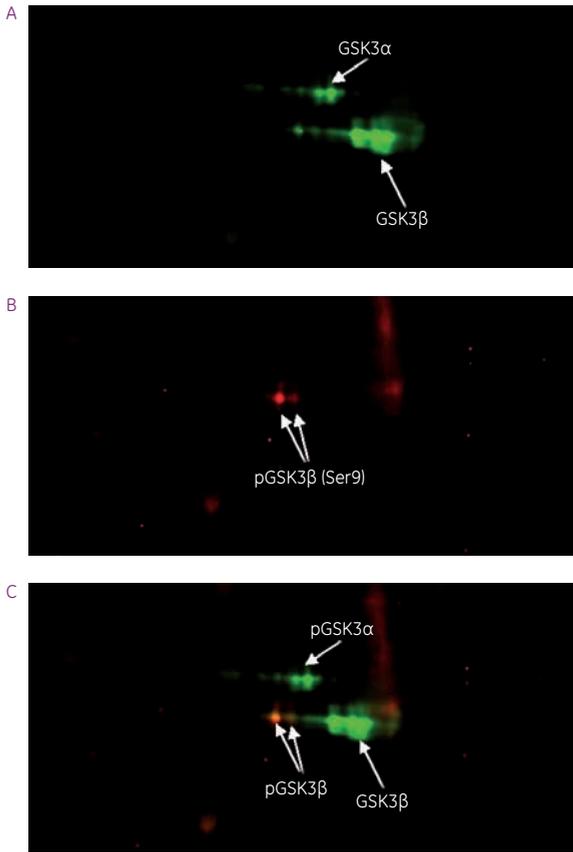
Fig 9.12. 2-D Western blotting analysis of native and phosphorylated GSK3β detected using Amersham ECL Plex. (A) Cy3 image, (B) Cy5 image, (C) Overlay of Cy3 and Cy5 images. Green image = ECL Plex Cy3-conjugated secondary antibody to primary antibody targeted against GSK3β protein (green). Red image = ECL Plex Cy 5-conjugated secondary antibody to primary antibody targeted against phosphorylated GSK3β protein (pGSK3β).

9.3 Reference

1.   Application note: Multiplex protein detection in 2-D gel electrophoresis using the Amersham ECL Plex fluorescent Western blotting system, GE Healthcare, 28-9042-34, Edition AA (2006).

Chapter 10
Troubleshooting

Symptom	Possible cause	Remedy
Problems associated with transfer		
Very little protein on membrane after transfer	Insufficient contact between gel and membrane	• Make sure the blotting unit is properly assembled, closed and sealed prior to transfer • After transfer, stain the gel with total protein stain to determine transfer efficiency or work with prestained molecular weight markers to directly monitor successful transfer • After transfer, stain a strip of membrane with a total protein dye (see Chapter 4), alternatively use a reversible stain on the whole membrane • Replace fiber pads in the blotting assembly as they can become compressed with time • Use thicker filter papers in the blotting sandwich cassette
	Wrong orientation of gel and membrane with respect to the anode (+)	• Place a membrane on both sides of the gel • Check that electrical connections are properly connected and in correct sequence • Be aware of the orientation when assembling the sandwich: the membrane should lie on the anode (+) side of the gel
	Target masked by another protein, such as IgG or albumin	• Deplete these proteins
Protein band smeared across the membrane	Excessive heat generated during transfer	*For wet transfer:* • Make sure the tank contains sufficient buffer to cover the blotting cassette. This prevents temperature gradients across the gel • Prechill the transfer buffer and carry out the transfer in a cold room • Use a cooled recirculating water bath, if possible. Alternatively, you can reduce the current or voltage and perform transfer for a longer time *For semidry transfer:* • Either shorten the run time, increase the number of filter papers in the stack, or reduce the current - never exceed the golden rule of 0.8 mA/cm^2
	Poor sample preparation leaving unwanted components, such as lipids, DNA, or excessive quantities of proteins, such as IgG or albumin that can mask the target protein	• Optimize the sample preparation procedure - there are different types of sample preparation products for cleanup, depletion, and removal of DNA (see Chapter 2 or refer to the Protein Sample Preparation Handbook from GE Healthcare) (1)
	Gel/membrane distortion or poor electrophoretic separation of proteins	• Increase equilibration time of the resolving gel in transfer buffer • Avoid unnecessary movement of component parts during assembly and handling of the stack (filter paper, gel, membrane, sponges)

Symptom	Possible cause	Remedy
Poor transfer of small proteins	Insufficient protein retention	• Optimize transfer times using relevant molecular weight markers • Use a polyvinylidene fluoride (PVDF) membrane, which has a higher protein binding capacity than nitrocellulose membranes • Use a higher percentage or gradient polyacrylamide gel to help retain proteins during equilibration and transfer • Use a membrane with pore size of 0.2 μm • Use two membranes, one on top of the other, to avoid the risk that proteins pass all the way through without binding
	Traces of sodium dodecyl sulfate (SDS) in the gel interfere with the binding of small molecular weight proteins to membranes	• Equilibrate the gel in transfer buffer for at least 15 min • Do not use SDS in transfer buffer
	Concentration of methanol in transfer buffer is too low to remove SDS	• Use a higher percentage of methanol (15% to 20%)
	Insufficient protein binding time	• A lower voltage might improve binding of small proteins to the membrane
Poor transfer of large proteins	Methanol concentration is too high	• Reducing the methanol concentration to 10% (V/V) or less should help in the transfer of high molecular weight proteins
	Insufficient protein binding time	• Increase transfer time to increase binding • Use wet transfer as this is more efficient than semidry transfer for large proteins
	Proteins are trapped in the gel as a result of high acrylamide concentration	• Reduce percentage of acrylamide in the gel to improve the resolution of large proteins • Add 0.1% SDS to transfer buffer, although this will reduce the efficiency of binding to nitrocellulose membranes *Note: Although SDS can improve transfer of large proteins, this sometimes results in deactivation of antigen binding sites*
Poor transfer of positively charged proteins	The net charge of the proteins in the transfer buffer is positive; proteins migrate to the cathode (-)	• Reverse the transfer stack so that the membrane is on the cathode (-) side of the gel

Symptom	Possible cause	Remedy
Poor semidry transfer	Current bypasses the gel	• Make sure the membrane and the blotting paper are cut exactly to the gel size and that there are no overlaps
Incomplete hydration of membrane	Poor transfer, uneven background, uneven transfer	• Ensure the entire membrane is thoroughly prewetted and equilibrated in transfer buffer before assembly in the blotting apparatus • PVDF membranes should be prewetted in methanol before use with aqueous solutions (see 10.2) - the entire membrane should change uniformly from opaque to semitransparent *Note: PVDF membranes must be kept wet at all times. If the membrane is allowed to dry out, repeat the entire wetting procedure*
Uneven transfer results	Air bubbles under the membrane or between layers in the stack	• To avoid air bubbles trapped in the interior of the membrane, prewet the membrane by carefully laying it on the surface of the methanol (see 10.1) - immersing the membrane can entrap air • Use a clean pipette to roll out any air bubbles between the gel and the membrane to ensure close contact
	Finger contamination	• Avoid touching the membrane. Always wear gloves and use blunt-ended forceps (see 10.3)
	Areas of the membrane might have dried	• Avoid drying of the membrane

Symptom	Possible cause	Remedy
Problems associated with labeling and detection		
Weak signal	Wrong blocking agent, which might have strong affinity for the protein of interest	• Try a different blocking solution with lower blocking agent concentration • Do not use a blocking agent with potential phosphatase activity, such as crude protein, if analyzing phosphorylated protein • Check the compatibility between the blocking agent and detection reagents by using the dot-blotting method (see Chapter 5 and Chapter 11) • Do not use a blocking agent that can interfere with the target protein or the antibodies used - be aware that milk contains many different proteins and the amounts can vary between batches • Do not use milk as blocking agent when working with biotin/streptavidin • Reduce the detergent concentration - usually, 0.05% to 0.1% Tween-20 or SDS are sufficient to reduce a high background without adversely affecting the specific signal (try Triton X-100 instead of Tween-20) • For monoclonal or highly purified antibodies, detergent-free buffer is preferable • Determine experimentally the number of washes required to optimize signal-to-noise ratio • Do not use a phosphate-containing buffer, such as phosphate-buffered saline (PBS), if analyzing phosphorylated proteins
	Antibody/antigen are masked by over-blocking	• Do not block for more than 1 h at room temperature. If longer blocking time is required, keep the membrane at 4°C
	Short exposure time	• Extend the exposure time if using a CCD camera-based imager or X-ray film. Some CCD camera-based imagers have auto exposure and/or increment exposure functions that can be useful if the optimal exposure time is unknown
	Insufficient antibody reaction time and incorrect temperature	• Incubation at room temperature increases specific- as well as nonspecific signals, resulting in high background (see 10.1) if the reaction time is too long • If incubation is carried out at 4°C, leave enough time (12 to 16 h) for the antibody to react with the blotted protein
	Excessive or robust washing	• Use a milder washing procedure
	Inactive antibody	• Ensure that your primary antibody is of sufficient specificity for your protein; perform a dot-blot test before the full Western blotting analysis (see Chapter 11)
	Low antibody concentration	• Optimize the antibody concentration (see Appendix A)
	Primary antibody not suitable for Western blotting	• Check manufacturers' instructions to ensure that the antibody has been approved for Western blotting • Perform a dot-blot for testing the antibody • If necessary, change the antibody

Symptom	Possible cause	Remedy
Weak signal	Secondary antibody inhibited by sodium azide	• If using a horseradish peroxidase (HRP)-based detection system, make sure the antibody solution does not contain sodium azide, a common preservative
	Outdated or incorrectly stored detection reagents	• Pay attention to manufacturers' indications of expiry date for antibodies, other detection reagents, and storage conditions
	Insufficient protein loaded or low amount of target protein in the sample	• Titrate your protein sample to make sure that you load enough protein onto the gel • Before Western blotting analysis, perform a dot-blot with your protein sample to make sure that you see a signal (see Appendix A) • Check that the protein assay is compatible with the buffer to ensure that the correct amount of protein is measured • Load more sample or enrich the target protein
No signal	Low antibody concentration	• Optimize antibody concentrations (see Appendix A)
	Wrong species of secondary antibody	• Ensure the secondary antibody is directed against the species in which the primary antibody was raised
	Inhibition of HRP	• HRP can be inhibited by sodium azide in antibody solutions or water. If so, strip and reprobe using antibodies diluted in azide-free buffer
	Antibody was raised against native protein	• Ensure that the antibody you use is validated for Western blotting applications and recognizes the form of the protein present in your samples. For example, an antibody raised against a native protein might not bind the denatured form • If necessary, strip the membrane (see Chapter 5) and reprobe using a suitable antibody (check using dot-blot or Western blotting, see Chapter 11
	Primary and secondary antibodies not compatible	• Make sure the secondary antibody is directed against the species in which the primary antibody was raised
	Dysfunctional detection reagent	• Detection reagents can be inactivated by cross-contamination - in the dark room, check the reagents by adding 1 µl of HRP-labeled antibody or similar reagent to a small quantity of prepared ECL detection reagents (blue light should be visible if functioning correctly, see Chapter 11)
	Target protein not present at detectable levels	• Use a positive control to test the blotting procedure

Symptom	Possible cause	Remedy
White bands (ECL detection)	Excessive signal generated	• Reduce antibody or protein concentration as excessive quantities can cause extremely high levels of localized signal (usually at a single band), leading to rapid, complete consumption of substrate at this point. As no light can be generated after the completion of this reaction, white bands appear when exposed
High background (see 10.1)	Nonspecific protein binding to the membrane	• Increase the concentration of the blocking reagent and/or blocking time • Ensure that equipment and components are clean and free of residues from previous transfers • Use only high quality water for your buffers and reagents
	Insufficient washes	• Increase number of washing steps and volume of the washing buffer • Add 0.1% (V/V) Tween-20 to washing buffer *Note: Increasing concentration of Tween-20 might improve the background but can also affect the binding of the antibody* • Increase the times of the wash steps • Ensure adequate movement of the washing buffer over membrane – use a tilting table or a suitably designed blot processor
	Wrong detergent concentrations in buffers	• 0.05% to 0.1% Tween-20 is commonly used in washing buffer - the addition of up to 0.5 M NaCl and up to 0.2% SDS to the washing buffer, as well as extending the times of the wash steps to 2 h, can help reduce persistent background
	Insufficient blocking	• Ensure appropriate blocking conditions are being used for the application and detection system in use • Use another blocking agent • Use freshly prepared blocking agent that is fully dissolved • Increase the concentration of blocking agent in the working antibody solution • Increase the blocking incubation time and/or temperature • Between 1% and 10% of blocking agent is usually used for blocking but you can slightly increase the concentration to improve the signal-to-noise ratio • Soaking the membrane for 1 h at 37°C can solve some persistent background issues - as a rule, a blocking time of 2 h should not be exceeded

Symptom	Possible cause	Remedy
High background (see 10.1)	High concentration of secondary antibody	• It is important to follow manufacturers' instructions for antibody dilutions, ranging usually from 1:100 to 1:500 000 • For improved results, optimize the antibody concentrations (Appendix A)
	Protein:protein interactions	• Before starting Western blotting analysis, test for the occurrence of interactions between the target protein and proteins used for blocking on a dot-blot - store all reagents properly
	Poor quality reagents	• Follow manufacturers' instructions, and take heed of the expiry date of antibodies - old antibodies can lead to high background
	Cross-reactivity between blocking agent and antibodies	• Test cross-reactivity by performing a dot-blot on a blocked membrane free of blotted proteins - a signal indicates that you should change blocking agent
	Poor quality antibodies	• Use high quality, affinity purified antibodies (see Chapter 11)
Bad resolution	Poor gel quality	• Use fresh gels - do not used expired gels, gels that have dried out, or incorrectly stored gels

Symptom	Possible cause	Remedy
High background (see 10.1)	Overexposure of detected blots	• Expose the film for the minimum period of time. • Use the autodetection feature if using a charge-coupled device (CCD) camera-based imager. Alternatively, expose initially for a short period of time and then select the optimal exposure time based on the signal intensity after the short exposure • When using light-based detection, leave the blots for several minutes before re-exposing film to allow the signal to decay - an extended period might be required with alkaline phosphatase (AP)-based chemiluminescence systems • Titrate the antibodies and/or reduce the protein load applied to the gel
	Film expiry date exceeded	• Ensure that films are stored according to the manufacturers' recommendations and that the expiry date has not been exceeded - change the film if required
	Darkroom not lightproof	• In cases of persistent background, ensure that the darkroom is fully light-proof and use a suitable safe light
Non-specific binding	High primary antibody concentration	• Optimize antibody concentrations (see Appendix A)
	High secondary antibody concentration	• Optimize antibody concentrations (see Appendix A)
	High antigen concentration	• Decrease amount of protein loaded on the gel
	Unexpected low bands	• In order to avoid protein degradation or proteolysis, minimize time between sample preparation and electrophoresis • Use only fresh samples kept on ice to avoid degradation • Use protease inhibitors to avoid the activation of proteases • Use an alternative antibody to avoid cross-reactivity with other proteins sharing similar epitopes • There are several splice variants of your protein • Under SDS reducing conditions, protein subunits can be detected – if possible, check the structure of the protein from the literature – the unexpected bands might not be artifacts!
	Unexpected high molecular weight bands	• Use agents that remove post-translational modifications (PTMs) and cause a molecular weight shift of your target protein
	Primary antibody is binding nonspecifically to other proteins	• Use more stringent washing, alter high salt and low salt concentration in wash buffer

| Symptom	Possible cause	Remedy
Nonspecific binding	Unexpected bands at very high molecular weights	• To avoid dimer interactions of proteins, use only fresh dithiothreitol (DTT) or β-mercaptoethanol • Denature your protein sample by heating before loading on the gel
	Multiple bands with different molecular weights	• Use high quality antibodies prepared for Western blotting, optimize antibody concentration (Appendix A) • Use a negative control to check for crossreactions
Distorted results	Fingerprints, fold marks, or forcep imprints on the blot	• Avoid touching the membrane (always wear gloves and use blunt ended forceps) - this form of contamination can be a particular problem when using Amersham ECL Plex • Use containers free of residual protein stains such as Coomassie Brilliant Blue
	Air bubbles trapped between sample and film (see 10.1)	• Ensure the film is in close contact with sample - if necessary, expel air bubbles by rolling a pipette over the membrane wrapped in plastic wrap • Firmly close the cassette for film exposure
Speckled background	Aggregates in the blocking agent	• Ensure the blocking agent is completely dissolved in the buffer - if necessary, warm the solution slightly and mix well • Ensure the membrane is fully immersed throughout the incubations
	Aggregates in HRP-conjugated secondary antibody	• Filter the conjugate through a 0.2 μm filter • Use a new, high quality conjugate
	Exposure equipment not clean or adequate	• If local blackening occurs, ensure that the cassette is clean • Avoid adhesive tape • Keep the sample as dry as possible, avoid condensation • If using autoradiography or fluorescence detection, allow the cassette to reach room temperature before removing the film after exposure at -70°C

Symptom	Possible cause	Remedy
Problems associated with fluorescence detection		
High background	High background fluorescence from the blotting membrane	• Use low fluorescence membranes such as Amersham Hybond LFP 0.2 (PVDF) or Amersham Protran Premium (nitrocellulose) for fluorescence-based detection techniques • Make sure that the bromophenol blue front is cut from the gel before transfer as this dye is fluorescent • Make sure to use clean incubation trays that have not previously contained stains, such as Coomassie Blue • If Triton X-100 is used as deionized, switch to Tween-20 as Triton X-100 is not compatible with the Cy3 channel • The final washes should be in PBS or TBS without detergent
Multiplexing problems	Experimental design	• Ensure that the secondary antibodies can differentiate the species-specific primary antibodies
Speckled background	Dust or powder particles on the surface of the blot	• Ensure that all material is perfectly clean and free of fluorescent contaminants • Wipe the surface of the scanner with 70% ethanol followed by deionized water before imaging or, alternatively, follow manufacturers' recommendations for cleaning the surface before and after use • Do not handle the membranes with your fingers - use clean forceps and wear powder-free gloves
	Polyacrylamide gel left on membrane	• Carefully remove gel fragments
	Ballpoint pen used on membrane (see 10.3)	• Label your membrane by cutting the corners
Low signal	Wet blot	• Use a low-fluorescence membrane • Skip the blocking step and instead dry the membrane directly after transfer for at least 2 h at ambient temperature - rewet the membrane in methanol, followed by washing in buffer
	Blot photobleached	• Some dyes are sensitive to photobleaching - protect the membrane from light during and after secondary antibody incubation
	Wrong excitation wavelength or emission filter	• Always follow manufacturers' instructions on excitation/emission wavelengths for fluorophores
	Incorrect imager settings	• Choose the correct exposure time if using a CCD camera-based imager. Choose correct voltage for the photomultiplier tube (PMT) if using a scanner

10.1   Less than perfect results: A rogue's gallery

The following pictures illustrate suboptimal results of the type referred to throughout this handbook. If you are experiencing the same sort of problems as seen in the images below, refer to the Troubleshooting table in this chapter.

No signal
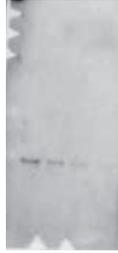
Weak signal

Faint bands after a long exposure
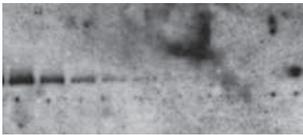
Uneven spotted background

High background
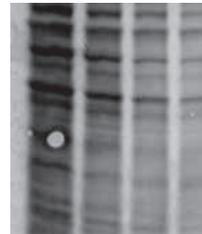
Air bubbles trapped between the blotting paper, gel, and membrane hinder protein transfer and cause "bald spots" (areas of nontransfer) to appear

White bands on film
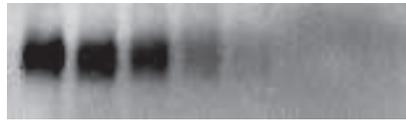
Excessive, diffuse signal

10.2   Problems associated with running buffer and membrane handling

We recommend that you scan properly dried membranes (not partially dried or wet). Scanning dried membranes usually results in a more even background and a higher signal-to-noise ratio. The membrane shown here was not fully dried and the bands to the right are very weak.

If SDS is absent from the running buffer during PAGE (right), sensitivity is reduced and the protein bands become more diffuse.
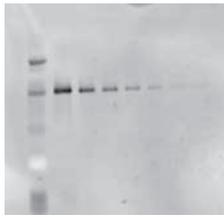
Amersham Hybond LFP 0.2 membranes must be prewetted in methanol (right) for efficient protein transfer from the gel to the membrane. The membrane on the left has not been prewetted.

10.3 Don't forget the simple things!
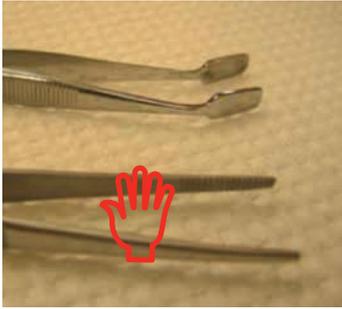
Use flat-ended, clean forceps with a smooth surface.

Do not touch the membrane without gloves - use forceps!

When using Amersham ECL Plex, avoid marking the membrane with a ballpoint pen - mark your membrane by cutting a corner or by using a pencil.

Use clean containers free of traces of protein stain from earlier work.

Use powder-free gloves when working with fluorescence detection.

When using fluorescence detection, avoid:

- All autofluorescent material
- Bromophenol blue
- The presence of unwanted, small fragments of polyacrylamide gel
- Triton X-100
- All possible sources of fluorescent contamination

10.4   Reference

1.   *Protein Sample Preparation Handbook*, GE Healthcare, 28-9887-41 Edition AA (2010).

Chapter 11
Protocols and recipes

👉 Throughout this chapter, where water is included in the composition of buffers and solutions, distilled or deionized water should be used.

11.1 Western blotting standard procedure

1. Prepare your samples by adding sample loading buffer and heating at 95°C for 5 min.
2. Centrifuge all samples in a microcentrifuge tube at 12 000 × g for 2 to 5 min prior to loading to remove any aggregates.
3. Place the gel in the electrophoresis equipment and add the appropriate running buffer.
4. Remove the comb and rinse out the wells with running buffer.
5. Load your samples and molecular weight markers in the wells.
6. Place the safety lid on the unit and plug the color-coded leads into the jacks in the power supply (red to red, black to black).
7. Run the gel under appropriate conditions.
8. When the tracking dye reaches the bottom of the gel, turn off the power supply, disconnect the leads, and remove the safety lid.
9. Release the gel cassette from the electrophoresis apparatus.
10. Cut away the stacking gel and cut one corner from the lower part of the resolving gel. Note which corner you have cut. This will enable you to correctly orientate the gel if it "flips over" during equilibration.

Note: that the steps described from step 11 refer specifically to wet transfer.

11. Equilibrate the gel in transfer buffer for 10 to 15 min.
12. Prewet and equilibrate the membrane in transfer buffer for 10 to 15 min.

👉 PVDF membranes need to be prewet in methanol and water before equilibration in transfer buffer.

13. Place the electrotransfer cassette in a tray filled to a depth of 3 cm with chilled transfer buffer. Assemble the transfer stack so that proteins migrate toward the membrane. For negatively charged proteins, build the stack on the half of the cassette that will face the anode (+).
14. Prewet a sponge and place it on the submerged part of the cassette. Press gently to remove any air bubbles.
15. Place two prewetted blotting papers on to the sponge.
16. Place the membrane on top of the blotting papers.
17. Place the gel on top of the membrane.
18. Place two additional prewetted blotting papers on the gel.
19. Finally place a prewetted sponge on top of the stack and close the cassette, after gently pressing to remove air bubbles.
20. Add prechilled transfer buffer to the transfer tank.

👉 Optional! Add a stirring magnet to circulate the buffer during transfer.

21. Place the cassette in the transfer tank.

22. Connect the transfer tank to the power supply and run the transfer according to manufacturers' recommendations.

23. After transfer, block the membrane in appropriate blocking solution for 1 h at room temperature.

24. Incubate with primary antibody, 1 h at room temperature or 4°C overnight.

25. Wash the membrane three to six times for 5 min per wash in phosphate-buffered saline (PBS) containing Tween-20 in (PBS-Tween) or Tris-buffered saline (TBS) containing Tween-20 (TBS-Tween).

26. Incubate with secondary antibody for 1 h at room temperature or 4°C overnight.

27. Wash the membrane three to six times for 5 min per wash in PBS-Tween or TBS-Tween.

28. Continue with protein detection according to instructions for the selected detection system.

11.2   Sample preparation

The choice of sample preparation method depends on the nature of the sample. In general, gentle methods should be used if the sample consists of easily lysed cells, whereas more vigorous methods will be needed to disrupt more robust bacterial or plant cells, or mammalian cells embedded in connective tissue. The measures adopted to cater for the analysis of only partially soluble connective tissue proteins demand yet another approach. Regardless of the source and protein of interest, however, any extraction procedure must be aggressive enough to extract proteins from their *in situ* environment but mild enough not to disrupt important structural features. At the same time, the extraction procedure must result in sufficient yield of material at an acceptable level of purity. Table 2.1 in Chapter 2 lists some of the most popular extraction methods and indicates their applicability to the treatment of specific cell types or tissue sources.

11.2.1   Extraction of proteins with Mammalian Protein Extraction Buffer

Here we describe how to use Mammalian Protein Extraction Buffer from GE Healthcare, a solution specifically suited to extracting proteins from mammalian cells, both adherent as well as in suspension.

☞   Depending on the application, dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) may be added to the buffer.

☞   Add a cocktail of protease inhibitors during the extraction procedure. Use prechilled buffers and work on ice.

11.2.1.1 Protein extraction from cells in suspension

1. Pellet the cells by centrifugation at 3000 × g for 5 min. Remove and discard the supernatant.

2. Wash the cell pellet once with 5 to 10 ml of prechilled PBS. Pellet the cells again by centrifugation. Remove and discard the PBS wash.

3. Add Mammalian Protein Extraction Buffer and suspend the cell pellet. For each 10 ml of suspension culture, add 1 ml of Mammalian Protein Extraction Buffer. Alternatively, add 1 ml of Mammalian Protein Extraction Buffer for each 0.05 g of wet cell pellet.

   ☞ For a more concentrated cell extract, the volume of Mammalian Protein Extraction Buffer added to the pellet may be reduced. In such cases, one freeze and thaw cycle will ensure complete lysis of the cells.

4. Use a pipette to suspend the cells until you have a homogeneous suspension. Incubate the lysate on ice for 15 to 30 min. Periodically shake or briefly vortex the suspension.

5. Centrifuge the lysate at 20 000 × g for 30 min in a refrigerated centrifuge. Collect the lysate for downstream processing and analysis.

11.2.1.2 Protein extraction from adherent cells

1. Remove the culture medium from the adherent cells.

2. Wash the cells once with PBS. Remove the PBS wash.

3. Add an appropriate volume of Mammalian Protein Extraction Buffer to cover the culture surface area (see Table 11.1). Alternatively, cells can be scraped from the surface and treated as cells in suspension in subsequent steps.

Table 11.1. Volume of Mammalian Protein Extraction Buffer to add to adherent cells

Volume of buffer/well (µl)	Type of culture plate
50 to 100	96-well plate
100 to 200	24-well plate
200 to 400	6-well plate
250 to 500	60 mm diameter culture plate
500 to 1000	100 mm diameter culture plate

4. Shake the culture plate gently for 10 min. The cells should be gently detached from the plate using a plastic scalpel or "rubber policeman".

   ☞ For a more concentrated cell lysate, the volume of Mammalian Protein Extraction Buffer added to the culture plate may be reduced. Subject the culture plate or well to one freeze and thaw cycle.

5. Lysate, including cellular debris may be used directly from the culture wells/plates. Alternatively, transfer the lysate to a tube for centrifugation at 20 000 × g for 30 min. Collect the clear lysate for downstream processing and analysis.

11.2.2 Sample cleanup

In order for protein samples in a gel containing sodium dodecyl sulfate (SDS) to yield clear, distortion-free bands of constant width, each sample must have the same buffer and ionic composition. Laboratory samples, however, often vary in buffer, salt or detergent content, which can result in suboptimal resolution and lane distortions. Note that while alternative strategies of varying degrees of complexity may be employed to prepare samples prior to electrophoresis, simple cell lysis and centrifugation may well be sufficient for Western blotting analysis of cell-based samples.

Guidelines on how to use two cleanup products from GE Healthcare are given in the following sections.

11.2.2.1 SDS-PAGE Clean-Up Kit

SDS-PAGE Clean-Up Kit is designed to prepare samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) that are otherwise difficult to analyze due to high conductivity or low protein concentration. The procedure works by quantitatively precipitating proteins while leaving behind in solution interfering substances such as detergents, salts, lipids, phenolics, and nucleic acids. The proteins are then resuspended and mixed with SDS-PAGE sample loading buffer. The procedure can be completed in under 2 h with quantitative yield. The kit contains sufficient reagents to process 50 samples of up to 100 µl each. The procedure can be scaled up for larger volumes or more dilute samples.

☞ Always position the microcentrifuge tubes in the centrifuge rotor with the cap hinge facing outward. This way the pellet will always be on the same side of the tube so it can be left undisturbed, minimizing loss.

Materials required

- 1.5 ml microcentrifuge tubes
- Microcentrifuge capable of spinning tubes at 12 000 × g or more at 4°C
- Vortex mixer
- β-mercaptoethanol or DTT
- Boiling water bath or heat block set at 95°C

Proteases are generally inactive in the solutions employed in this procedure, but protease inhibitors can be added to the sample solution if desired.

Preliminary preparations

Stand the washing buffer at -20°C for at least 1 h before starting the procedure. The washing buffer may be stored in a -20°C freezer.

Add reducing agent to the sample loading buffer. The reducing agent can be either DTT or β-mercaptoethanol. If using DTT, add 3.1 mg per 100 µl of SDS-PAGE sample loading buffer. Make sure it is fully dissolved. If using β-mercaptoethanol, add 5 µl per 100 µl of sample loading buffer. Once the reducing agent has been added, the sample loading buffer should be used immediately, so reducing agent should only be added to the amount of sample loading buffer needed for a single experiment. Alternatively, reducing agent in sample loading buffer may be aliquoted and stored at -20°C.

The protein sample should be substantially free of particulate material. Clarify by centrifugation if necessary.

Process the protein samples in 1.5 ml microcentrifuge tubes. All steps should be carried out with the tubes on ice unless otherwise specified. Always position the microcentrifuge tubes in the centrifuge rotor with the cap hinge facing outward. This way the pellet will always be on the same side of the tube so it can be left undisturbed, minimizing loss.

1. Transfer 1 to 100 µl of protein sample (containing 1 µg to 1 mg of protein) into a 1.5 ml microcentrifuge tube.

2. Add 300 µl of precipitant (labeled "1") and mix well by vortexing or inversion. Incubate on ice for 15 min.

3. Add 300 µl of coprecipitant (labeled "2") to the mixture of protein and precipitant. Mix by vortexing briefly.

4. Centrifuge the tubes in a microcentrifuge set at maximum speed (at least 12 000 × g) for 5 min. Remove the tubes from the centrifuge as soon as centrifugation is complete. A small pellet should be visible. Proceed rapidly to the next step to avoid resuspension or dispersion of the pellet.

5. Remove as much of the supernatant as possible by decanting or careful pipetting. Do not disturb the pellet.

6. Carefully reposition the tubes in the microcentrifuge as before, with the cap hinge and pellet facing outward. Centrifuge the tubes again to bring any remaining liquid to the bottom of the tube. A brief pulse is sufficient. Use a micropipette tip to remove the remaining supernatant. There should be no visible liquid remaining in the tubes.

7. Pipette 25 µl of water on top of each pellet. Vortex each tube for 5 to 10 s. The pellets should disperse, but not dissolve in the water.

8. Add 1 ml of washing buffer (labeled "3"), prechilled for at least 1 h at -20°C, and 5 µl of wash additive (labeled "4"). Vortex until the pellet is fully dispersed.

   ☞ The protein pellet will not dissolve in the washing buffer.

9. Incubate the tubes at -20°C for at least 30 min. Vortex for 20 to 30 s once every 10 min.

   ☞ The tubes can be left at this stage at -20°C for up to 1 w with minimal protein degradation or modification.

10. Centrifuge the tubes in a microcentrifuge set at maximum speed (at least 12 000 × g) for 5 min.

11. Carefully remove and discard the supernatant. A white pellet should be visible. Allow the pellet to air dry briefly (no more than 5 min).

    ✋ Do not dry the pellet for longer than 5 min. If it becomes too dry, it will be difficult to resuspend.

12. Resuspend the pellet in 5 to 40 µl of buffer I (labeled "5"). Vortex briefly and incubate on ice for 5 min.

    ☞ The appropriate resuspension volume for the sample depends on a number of factors, including the protein concentration of the original sample, the capacity of the gel system used for SDS-PAGE and the sensitivity of the detection method used to visualize the proteins in the gel. If the pellet is large or too dry, it might take time to resuspend fully. Sonication or treatment with the Sample Grinding Kit from GE Healthcare can speed resuspension.

13. Add 1 µl of buffer II (labeled "6") for each 5 µl of buffer I used in Step 12. Vortex briefly and incubate on ice for 5 to 10 min.

14. Add an equal volume (6 to 48 µl) of SDS-PAGE sample buffer (labeled "7") to which reducing agent (DTT or β-mercaptoethanol) has been added (see preliminary preparations). If the solution turns yellowish, add buffer I in increments of 0.5 µl until the solution turns blue.

15. Vortex the sample for 5 to 10 s and incubate at room temperature for 5 to 10 min. Place the sample tube in a boiling water bath or 95°C heat block for 3 min.

16. Centrifuge the tube briefly to bring the contents to the bottom of the tube. A brief pulse is sufficient. Gently tap the tube to ensure that the contents are mixed. The sample is now ready for loading. The protein concentration of the sample is determined using 2-D Quant Kit from GE Healthcare, which can accurately quantitate protein in sample loading buffer.

11.2.2.2 2-D Clean-Up Kit

The 2-D Clean-Up Kit is designed to prepare samples for 2-D gel electrophoresis that otherwise produce poor results due to high conductivity, high levels of interfering substances, or low concentration. The procedure works by quantitatively precipitating proteins while leaving behind in solution interfering substances such as detergents, salts, lipids, phenolics, and nucleic acids. The proteins are then resuspended in a solution compatible with first-dimension isoelectric focusing (IEF). The procedure can be completed in 1 h and does not result in spot gain or loss. The kit contains sufficient reagents to process 50 samples of up to 100 µl each. The procedure can be scaled up for larger volumes or more dilute samples.

Materials required

- 1.5 ml microcentrifuge tubes
- Microcentrifuge capable of spinning tubes at 12 000 × g or more at 4°C
- Rehydration or sample solution for resuspension
- Vortex mixer

Preliminary preparations

Place the washing buffer at -20°C for at least 1 h before starting the procedure. The washing buffer may be stored in a -20°C freezer. The protein sample should be substantially free of particulate material. Clarify by centrifugation if necessary.

Process the protein samples in 1.5 ml microcentrifuge tubes. All steps should be carried out with the tubes in an ice bucket unless otherwise specified.

1. Transfer 1 to 100 µl of protein sample (containing 1 to 100 µg of protein) into a 1.5 ml microcentrifuge tube.

2. Add 300 µl of precipitant and mix well by vortexing or inversion. Incubate on ice for 15 min.

3. Add 300 µl of coprecipitant to the mixture of protein and precipitant. Mix by vortexing briefly.

4. Centrifuge the tubes in a microcentrifuge set at maximum speed (at least 12 000 × g) for 5 min. Remove the tubes from the centrifuge as soon as centrifugation is complete. A small pellet should be visible. Proceed rapidly to the next step to avoid resuspension or dispersion of the pellet.

5. Remove as much of the supernatant as possible by decanting or careful pipetting. Do not disturb the pellet.

6. Carefully reposition the tubes in the microcentrifuge as before, with the cap hinge and pellet facing outward. Centrifuge the tubes again to bring any remaining liquid to the bottom of the tube. A brief pulse is sufficient. Use a pipette tip to remove the remaining supernatant. There should be no visible liquid remaining in the tubes.

7. Carefully add 40 µl of coprecipitant on top of the pellet. Let the tube sit on ice for 5 min.

8. Carefully reposition the tube in the centrifuge as before, with the cap hinge facing outward. Centrifuge the tube again for 5 min. Use a pipette tip to remove and discard the wash.

9. Pipette 25 µl of water on top of each pellet. Vortex each tube for 5 to 10 s. The pellet should disperse, but not dissolve in the water.

10. Add 1 ml of washing buffer (prechilled for at least 1 h at -20°C) and 5 µl of wash additive. Vortex until the pellet is fully dispersed.

    ☞ The protein pellet will not dissolve in the washing buffer.

11. Incubate the tubes at -20°C for at least 30 min. Vortex for 20 to 30 s once every 10 min.

    ☞ The tubes can be left at this stage at -20°C for up to 1 w with minimal protein degradation or modification.

12. Centrifuge the tubes in a microcentrifuge set at maximum speed (at least 12 000 × g) for 5 min.

13. Carefully remove and discard the supernatant. A white pellet should be visible. Allow the pellet to air dry briefly (for no more than 5 min).

    ✋ Do not dry the pellet for longer than 5 min. If it becomes too dry, it will be difficult to resuspend.

14. Resuspend each pellet in an appropriate volume of rehydration or IEF sample loading solution for first dimension IEF. Vortex the tube for at least 30 s. Incubate at room temperature and either vortex or work up and down in a pipette to fully dissolve.

    ☞ If the pellet is large or too dry, it might take time to resuspend fully. Sonication or treatment with the Sample Grinding Kit from GE Healthcare can speed resuspension.

15. Centrifuge the tubes in a microcentrifuge set at maximum speed (at least 12 000 × g) for 5 min to remove any insoluble material and to reduce any foam. The supernatant may be loaded directly onto first-dimension IEF or transferred to another tube and stored at -80°C for later analysis.

11.2.3 Concentration measurement using 2-D Quant Kit

The 2-D Quant Kit from GE Healthcare is specially designed for the accurate determination of protein concentration in samples prior to electrophoresis. The assay has a linear response for up to 50 µg of solubilized protein in a volume of between 1 and 50 µl. The procedure is compatible with common sample preparation reagents such as 2% SDS, 1% DTT, 8 M urea, 2 M thiourea, and 4% of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS).

Materials required

Precipitant, coprecipitant, copper solution, color reagents A and B, and bovine serum albumin (BSA) standard solution are provided with the kit.

2 ml microcentrifuge tubes

Vortex mixer

Microcentrifuge

Visible light spectrophotometer

1. Prepare an appropriate volume of working color reagent by mixing 100 parts of color reagent A with 1 part of color reagent B.

   ☞ Each individual assay requires 1 ml of working color reagent. Working color reagent can be stored at 4°C to 8°C for up to 1 w or for as long as the optical density (OD) of the solution remains below 0.025 at 480 nm.

2. Prepare a standard curve with BSA standard solution (2 mg/ml) added to six tubes to the following final quantities: 0 (blank), 10, 20, 30, 40, and 50 µg.

   ☞ The accuracy of the assay is unaffected by the volume of the sample as long as the sample volume is 50 µl or less. It is therefore unnecessary to dilute standard or sample solutions to a constant volume.

3. Prepare samples by adding 1 to 50 µl of the sample to be assayed in separate tubes. Adjust this volume to make sure that you add a quantity of protein within the working range of the assay (0.5 to 50 µg).

4. Add 500 µl of precipitant to each tube (including the standard curve tubes). Vortex briefly and incubate the tubes for 2 to 3 min at room temperature.

5. Add 500 µl of coprecipitant to each tube and mix briefly by vortexing or inversion.

6. Centrifuge the tubes at a minimum of 10 000 × g for 5 min. Discard the supernatants by using a pipette. It is recommended to apply a brief additional pulse and then to remove any remaining supernatant using a micropipette.

7. Add 100 µl of copper solution and 400 µl of water to each tube. Vortex briefly to dissolve the precipitated protein.

8. Add 100 µl of working color reagent to each tube. Ensure instantaneous mixing by introducing the reagent as rapidly as possible. Mix by inversion and incubate at room temperature for 15 to 20 min.

9. Read the absorbance of each sample and standard at 480 nm using water as the reference. The absorbance should be read within 40 min of the addition of working color reagent.

10. Generate a standard curve by plotting the absorbance of the standards against the quantity of protein. Use this standard curve to determine the protein concentration of the samples.

☞ As this assay is extremely sensitive to time, all readings should be made as close as possible to each other. An automated cell changer is the optimal solution but if this is not available, the addition of reagent should be staggered every 30 s and readings should be taken after the same time interval has elapsed. Although this must be tested empirically, 20 min elapsed time is a good starting point.

☞ Unlike most protein assays, the absorbance of the assay solution decreases with increasing protein concentration. Do not subtract the blank reading from the sample reading or use the assay blank as the reference.

11.2.4 2× sample loading buffer

Before loading samples on a gel, an equal volume of 2× sample loading buffer (Table 11.2) is added to each sample. Sample loading buffer increases the density of the sample to ease loading, gives the protein the right properties for optimal separation, and provides a color that functions as a leading, visible front during the run. Note that in order to minimize the dilution effect of adding sample to sample loading buffer, it is possible to use 5× sample loading buffer for the analysis of low-abundance target protein.

Table 11.2. 2× sample loading buffer

	Final concentration	Volume or mass
0.5 M Tris-HCl (pH 6.8)	0.125 M	2.5 ml
10% SDS	4%	4 ml
Glycerol	20%	2 ml
Bromophenol blue	0.02%	2 mg
DTT[1]	200 mM	0.31 g
Water		Added to make the final volume 10 ml

[1] DTT should be freshly prepared and added to the sample loading buffer just before adding the sample loading buffer to the samples. β-mercaptoethanol (500 µl per 10 ml) can be used as an alternative to DTT.

1. Add 1 volume of 2× sample loading buffer to microtubes.

2. Add 1 volume of protein sample containing the desired quantity of protein.

3. Heat the microtubes at 95°C for 5 min.

4. Spin samples in a centrifuge.

☞ If the samples are not going to be used at once they can be stored in a freezer. After storage in a freezer, the samples need to be heated before loading to dissolve precipitated SDS.

11.3   Electrophoresis

A variety of precast gels are commercially available from different vendors. The attraction of using these gels includes the fact that you do not have to handle acrylamide, and they are simple and convenient to use. GE Healthcare offers Amersham ECL Gel and Amersham ECL Gel Box, a horizontal mini-gel system for high quality protein electrophoresis. The gel enables high electrotransfer efficiency of proteins, and convenient integration into the Amersham ECL Western blotting workflow.

11.3.1   Preparation of polyacrylamide gels containing SDS

🤚 Acrylamide and bisacrylamide are potent neurotoxins and should be handled with care. Follow local safety rules!

👉 Always wear gloves and a lab coat. Use a fume hood!

Polyacrylamide gels for use in PAGE are cast in two steps. First, the resolving gel should be cast and after this gel has polymerized, the stacking gel is cast on top. The resolving gel is usually prepared in a Tris-based buffer at a higher concentration and with a higher pH than the stacking gel, as well as a higher concentration of acrylamide.

Acrylamide monomer stock solutions should be prepared by adding 30 g of acrylamide and 0.8 g of bisacrylamide to water and making the final volume to 100 ml with water.

Buffers used in gel casting

4× resolving gel buffer (1.5 M Tris-Cl, pH 8.8): Add 36.3 g of Tris (relative molecular mass, M_r 121.1) to 150 ml of water. Adjust the pH to 8.8 using HCl and then make the final volume to 200 ml with water.

4× stacking gel buffer (0.5 M Tris-HCl, pH 6.8): Add 3 g of Tris (M_r 121.1) to 40 ml of water. Adjust the pH to 6.8 using HCl and then make the final volume to 50 ml with water.

10% SDS: Add 10 g of SDS to 50 ml of water and then make the final volume to 100 ml with water.

10% ammonium persulfate (APS): Add 100 mg of APS to 1 ml of water – this should be freshly prepared before use.

Preparation of resolving and stacking gels containing SDS: Mini-gel systems

1. Assemble the vertical slab gel unit in the gel casting stand. Use an appropriate thickness of spacer to create the "sandwich".

2. In a 125 ml side-arm vacuum flask, mix the resolving gel solution (see Table 11.3), omitting APS and N, N, N', N'-tetramethylethylenediamine (TEMED).

3. Stopper the flask and apply a water vacuum for several minutes while swirling to degas the solution.

4. Add the TEMED and APS and gently swirl the flask to mix, being careful not to generate bubbles.

5. Pipette the solution between the spacers into each sandwich to a level about 4 cm from the top.

6. Gently add a top layer of approximately 0.6 ml of water-saturated n-butanol or isopropanol (or water) so that the entire surface of the resolving gel is covered with n-butanol. A very sharp liquid-gel interface will be visible when the gel has polymerized. The gel should be fully polymerized after approximately 1 h.

7. Prepare the stacking gel by mixing the reagents (see Table 11.4) in a 50 ml conical tube.

8. Degas under vacuum for 10 to 15 min.

9. Add 50 µl of 10% APS and 10 µl of TEMED. Swirl gently to mix.

10. After polymerization of the resolving gel, tilt the casting stand to pour off the n-butanol overlay.

   ☞ Traces of n-butanol will affect polymerization of the stacking gel. Make sure that all n-butanol is decanted before addition of the stacking gel. This can be achieved by rinsing the surface of the resolving gel with water.

11. Pipette the stacking gel solution between the spacers.

12. Carefully place a comb into the unpolymerized gel, taking care not to trap any air bubbles. Allow the gel to polymerize for about 1 h.

   ☞ The gels can be stored with the combs in place, tightly wrapped in plastic wrap inside a sealed bag at 4°C for 1 w. Keep the gels moist. Do not store gels in the caster.

Table 11.3. Resolving gel solutions, 40 ml

Final gel concentration	5%	7.5%	10%	12.5%	15%
Acrylamide monomer stock solution	6.7 ml	10 ml	13.3 ml	16.7 ml	20 ml
4× resolving gel buffer	10 ml	10 ml	10 ml	10 ml	10 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
Water	22.7 ml	19.4 ml	16.1 ml	12.8 ml	9.5 ml
10% APS[1]	200 µl	200 µl	200 µl	200 µl	200 µl
TEMED[1]	13.3 µl	13.3 µl	13.3 µl	13.3 µl	13.3 µl

[1] Added after degasing (step 3 above).

Table 11.4. Stacking gel solution, 10 ml

Final gel concentration	4%
Acrylamide solution	1.33 ml
4× Stacking gel buffer	2.5 ml
10% SDS	0.1 ml
Water	6 ml
10% APS[1]	50 µl
TEMED[1]	5 µl

[1] Added after degasing (step 8 above).

11.3.2 Electrophoresis running buffer for SDS-PAGE

Most Western blotting applications are performed following the separation of proteins by SDS-PAGE. A recipe for the frequently used Laemmli buffer, based on Tris-glycine, is therefore presented here. By excluding SDS, the buffer can also be used for native PAGE applications.

Materials required

5× electrophoresis running buffer containing SDS

0.125 M Tris

0.96 M glycine

0.5% SDS

Dissolve 15.1 g of Tris base, 72 g of glycine, and 5 g of electrophoresis-grade SDS in water and make to a total volume of 1000 ml with water. Do not adjust the pH of the solution (this should be pH 8.3 when diluted). Store at room temperature. Add 1 part buffer to 4 parts water to make a working solution (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3).

☞ Make a stock solution of 5× or 10× Tris-glycine buffer without SDS (according to recipe above). This can then be used as a stock solution for transfer buffer.

11.4 Western blotting

Here, we describe methods for the electrotransfer of proteins separated by PAGE onto a solid membrane, usually made of nitrocellulose or polyvinylidine difluoride (PVDF). Electrotransfer is by far the most frequently used technique in Western blotting. Two types of electrotransfer, wet transfer and semidry transfer, are in common use.

11.4.1 Wet transfer

The following protocol is recommended for wet transfer (Fig 11.1). Manufacturers' recommendations should always be followed due to system-specific rquirements.

Materials required

- Polyacrylamide gel, post-electrophoresis
- PVDF or nitrocellulose membrane
- Four sheets of Whatman™ 3 mm filter paper or equivalent, cut to the same dimensions as the gel
- Two foam pads
- Tank transfer system, power supply
- 100% methanol (can be replaced with ethanol)
- Water
- Tris-glycine transfer buffer: 25 mM Tris base, 192 mM glycine, ≤ 20% (V/V) methanol, pH 8.3 (see Chapter 4)

☞ Prepare 10× Tris-glycine buffer as a stock solution (this can also be used as running buffer to which SDS is added once the working dilution is prepared). Make 1× Tris-glycine containing 20% methanol as transfer buffer. Prechill prior use.

☞ Prechill the transfer buffer before use.

Blotting protocol: Wet transfer

1. Prepare sufficient transfer buffer to fill the transfer tank, plus an additional 200 ml to equilibrate the gel, membrane, and filter papers.

2. After electrophoresis, cut one corner of the gel with a clean sharp razor blade or scalpel to allow you to orientate the gel.

3. Equilibrate the gel in transfer buffer for 10 to 15 min. Bear in mind that incomplete equilibration of the gel might cause band smearing.

4. Cut, prewet, and equilibrate a membrane according to manufacturers' recommendations.

   ☞ Prewet PVDF membranes in methanol for 15 s and then rinse in water. Equilibrate in transfer buffer for at least 10 min.

   ☞ Prewet nitrocellulose membranes in water and then equilibrate in transfer buffer for at least 10 min.

5. Soak the filter papers and foam pads in transfer buffer.

6. Place an electrotransfer cassette, anode (+) side facing down, in a tray filled to a depth of 3 cm with chilled transfer buffer.

7. Load the cassette, starting at the anode (+) side, with a foam sponge, followed by two filter papers, the prepared membrane, the gel, two more wet filter papers and, finally, a second foam sponge.

8. Place a stirring magnet in the transfer tank.

   ✋ Ensure that any air bubbles any air bubbles between the filter papers, gel, and membrane are removed.

9. Close the cassette and place the anode (+) side in the same orientation as any other additional cassettes in the electrotransfer tank filled with cold transfer buffer.

10. Connect the positive end of the cables of the lid of the electrotransfer tank to the positive labeled sockets of the power supply (see Fig 11.1 and Chapter 4).

11. Run the transfer according to manufacturers recommendations.

12. Following transfer, remove the membrane from the electrotransfer cassette, cut a corner from the membrane to allow orientation, and rinse briefly in PBS or TBS.

    ☞ Membranes can be air dried and stored between sheets of 3 mm filter paper wrapped in plastic wrap at 2°C to 8°C for up to 3 mo.

    ☞ After drying, PVDF membranes need to be activated in methanol and water prior to blocking and probing.

    ☞ After drying, nitrocellulose membranes need to be prewet in water prior to blocking and probing.

    ☞ Blotted membranes can be stored in PBS or TBS at 4°C for up to 2 w. For optimal results, however, continue with the Western blotting procedure immediately.

The setups of wet and semidry transfers are illustrated in Figures 11.1 and 11.2, respectively.
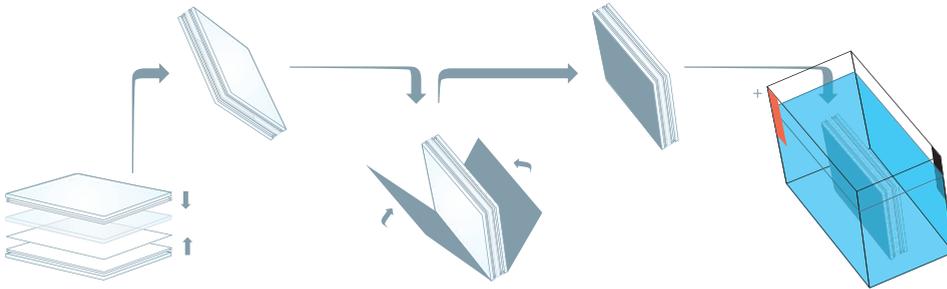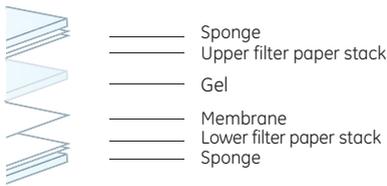
Fig 11.1. Wet transfer system. Assembly of the transfer sandwich is best performed in a tank filled with transfer buffer to a depth of at least 3 cm. The sandwich is built on the side of the transfer cassette facing the anode (+) and starts with a sponge, followed by two wetted filter papers, the membrane of choice, the gel, two additional wetted filter papers, and, finally a second sponge. Take care to avoid wrinkles, folds, or air bubbles between the different layers of the sandwich. This construct is then securely fixed in the transfer cassette and submerged in an electrotransfer tank containing transfer buffer. The orientation of the construct must be so that the membrane is on the anode (+) side of the gel.

11.4.2 Semidry transfer

The following protocol is recommended for semidry transfer (Fig 11.2). Manufacturers' recommendations should always be followed due to system-specific requirements.

Depending on the system used and the total area of combined gels, multiple gels can be transferred at the same time by placing several small gels of the same thickness side by side. The following protocol is for the semidry transfer of one gel.

✋ It is not advisable to stack gels although this is possible if reproducibility is not an issue.

Materials required

- Polyacrylamide gel, post-electrophoresis
- PVDF or nitrocellulose membrane
- At least six 3 mm filter papers or equivalent, cut to the same dimensions as the gel
- Semidry blotter instrument, power supply
- 100% methanol (can be replaced with ethanol)
- Water
- Tris-glycine transfer buffer: 25 mM Tris base, 192 mM glycine, ≤ 20% (V/V) methanol, pH 8.3 (see Chapter 4)

  👉 Prepare a 10× Tris-glycine buffer as stock solution (see 11.3.2). Prepare 1× Tris-glycine buffer with up to 20% methanol as transfer buffer. Prechill prior use.

Blotting protocol: Semidry transfer

1. Prepare the electrotransfer by rinsing the anode (+) and the cathode (-) with water.
2. After electrophoresis, cut one corner of the resolving gel with a clean sharp razor blade or scalpel to allow you to orientate the gel.
3. Remove the stacking gel and dye front from the gel.
4. Equilibrate the gel in transfer buffer for 10 to 15 min. Bear in mind that incomplete equilibration of the resolving gel might cause band smearing.
5. Cut at least six pieces of filter paper to the same dimensions as the gel or slightly smaller.

    ☞ Gauge the amount of buffer required according to the thickness or number of blotting paper layers.

6. Soak at least three pieces of blotting paper with transfer buffer. One-by-one, center each sheet on the lower electrode (anode [+]) and remove all trapped air by rolling a clean pipette or roller from the center toward the edges.
7. Cut a membrane to the same dimensions as the gel or slightly smaller. Prewet and equilibrate the membrane in transfer buffer for at least 10 min.

    ☞ PVDF membranes must be prewetted in methanol and rinsed in water before equilibration in transfer buffer. Nitrocellulose membranes should be prewetted in water before equilibration. Always wear clean gloves when handling membranes to avoid fingerprints.

8. Place the prewetted membrane onto the stack of blotting paper.

    ☞ The blotting paper and membrane must be the same size as the gel or 1 to 2 mm smaller. Larger sizes will provide an electrical path for current to bypass the gel, leading to poor transfer. This can be avoided by the use of a plastic gasket with a rectangular hole cut to the same size as the gel and placed between the upper and lower stacks of filter paper.

9. Place the gel on the membrane. Proteins bind to the membrane as soon as contact occurs, so it is important to place the gel correctly on the first try.
10. Cover the gel with three layers of wetted blotting paper. Stack each layer with care, with edges parallel. As each layer is added, remove all air pockets by rolling a clean pipette from the center to the edges.

    ☞ The addition of a few drops of buffer to the blotting paper will help remove air pockets.

11. Connect the color-coded leads to the power supply. Before connecting, ensure that the power is switched off.
12. Set the current and the timer according to the manufacturer's recommendations. Start the transfer. Semidry transfer is usually performed at a constant current.

    ☞ Recommendations for most units suggest a limiting current of 0.8 mA/cm^2. Transfer time is usually about 1 h.
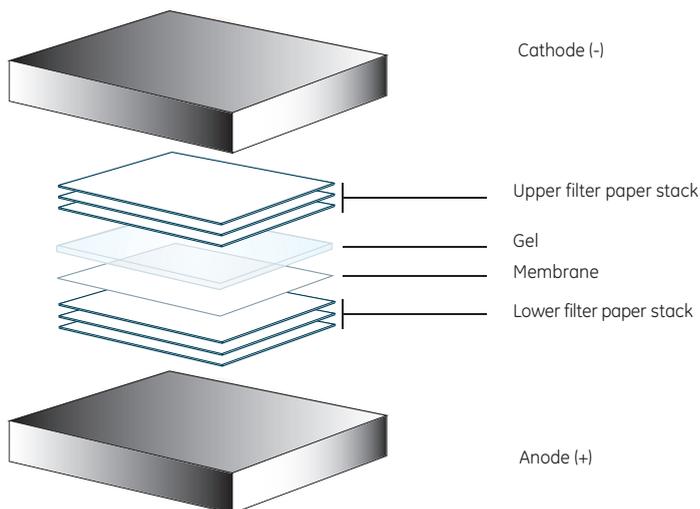
Fig 11.2. Semidry transfer system. The sandwich is built on the lower electrode (anode [+]). The first layer consists of at least three pieces of wetted filter papers, followed by the membrane, the gel and, finally, three additional wetted filter papers. As each layer is added, take care to avoid wrinkles, folds, or trapped air bubbles. This construct is placed and compressed between two flat conduction plates that also serve as cathode (-) and anode (+). The orientation of the construct must be so that the membrane is on the anode (+) side of the gel.

11.5   Western blotting buffers

After transfer, two alternative buffers may be considered for the dilution of blocking agents, dilution of antibodies, and for the different washing steps. PBS is often used, but an alternative buffer is TBS, which is particularly appropriate if the blotted proteins are phosphorylated. Descriptions of how to prepare these buffers are provided below. The performance of PBS and TBS in their multiple uses in Western blotting can be improved by the addition of Tween-20.

☞   High purity water should always be used in the preparation of buffers used in Western blotting.

TBS, pH7.6

Add 12.1 g of Tris base and 40 g of NaCl to water. Adjust to pH 7.6 with HCl and make to a final volume of 5 l with water. Store at room temperature.

TBS-Tween

Dilute the required volume of Tween-20 in TBS to give a 0.1% (V/V) solution.
Store at 2°C to 8°C.

PBS, pH7.5

Add 11.5 g of anhydrous disodium hydrogen orthophosphate, 2.96 g of sodium dihydrogen orthophosphate, and 5.84 g of NaCl to water. Adjust to pH 7.5 and make to a final volume of 1 l with water. Store at room temperature.

PBS-Tween

Dilute the required volume of Tween-20 in PBS to give a 0.1% (V/V) solution.
Store at 2°C to 8°C.

☞   Store at 2°C to 8°C. Make a 20% Tween-20 solution. This makes it easier to quickly dissolve Tween-20 in PBS.

11.6   Blocking

After transfer, the membranes can be probed by immunodetection. Prior to antibody probing, nonprotein binding sites on the membrane are blocked using a suitable blocking agent (see Chapter 5). A description of how to prepare Amersham ECL Prime Blocking Reagent and Amersham ECL Blocking Reagent is given in the following sections. Amersham ECL Prime Blocking Agent is recommended for fluorescence applications.

1. Shake the powdered block to ensure even distribution of components.
2. Weigh out the appropriate amount of blocking agent for a 2% (W/V) solution (Amersham ECL Prime Blocking Reagent), or a 5% (W/V) solution (Amersham ECL Blocking Agent).
3. Add an appropriate volume of PBS-Tween or TBS-Tween, shake vigorously and stir for 15 min until all components are fully dissolved.

   ☞ Prepared blocking solution can be stored at 2°C to 8°C but should be used within 24 h.
4. Place the membrane in blocking solution and incubate with agitation for 1 h at room temperature, or at 37°C if the background is persistently and unacceptably high. Alternatively, membranes may be left in the blocking solution overnight at 2°C to 8°C, if more convenient.
5. Briefly rinse the membrane in washing buffer.

11.7   Antibody probing and detection

Usually, membranes are first probed using an unlabeled primary antibody directed against the target protein, followed by a species-specific, labeled secondary antibody directed against the primary antibody. This technique maximizes the potential sensitivity of the assay, and is presented below.

☞ All containers used to store antibody solutions must be rigorously clean and all solutions used to dilute the antibodies must be prepared using high quality water.

☞ To achieve good quality results, optimize your antibody dilutions.

11.7.1   Chemiluminescence detection with Amersham ECL, Amersham ECL Prime, and Amersham ECL Select

1. Dilute the primary antibody in PBS-Tween/TBS-Tween.
2. Place the membrane (protein side up) in the primary antibody solution and incubate with agitation for 1 h at room temperature or overnight at 4°C. Always refer to manufacturers' recommendations.
3. Wash the membrane three to six times in PBS-Tween or TBS-Tween for 5 min per wash or according to manufacturers' recommendations.
4. Place the membrane in the secondary antibody diluted in PBS-Tween or TBS-Tween and incubate with agitation for 1 h at room temperature or overnight at 4°C.
5. Place the membrane in washing solution and wash four to six times for 5 min per wash.
6. Continue with detection as recommended for the selected detection reagent and imaging system.

11.7.1.1 Charge-coupled device (CCD) camera-based imaging

1. Allow the detection solutions to equilibrate to room temperature before opening the vials.

2. Mix an equal volume of detection solutions A and B, allowing sufficient total volume to cover the membranes. A volume of $0.1 \ ml/cm^2$ of membrane is required.

3. Drain the excess washing solution from the washed membranes and place them, protein side up, on a sheet of plastic wrap or other suitable clean surface. Pipette the mixed detection reagent onto the membrane.

4. Incubate for 1 min (Amersham ECL) or 5 min (Amersham ECL Prime and Amersham ECL Select) at room temperature.

5. Drain excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue.

6. Place the membrane, protein side up, on the CCD camera sample tray.

7. Place the sample tray in the CCD-camera and operate according to instructions. Choose an exposure time and capture the image.

☞ Choose exposure times according to expected signal intensity. A recommended starting point is to begin with 1 min and then adjust the time to find the optimal exposure. Alternatively, an increment function can be used, in which the camera captures images at predetermined time points during a given time.

11.7.1.2 X-ray film detection

1. Allow the detection solutions to equilibrate to room temperature before opening the vials.

2. Mix an equal volume of detection solutions A and B, allowing sufficient total volume to cover the membranes. A volume of $0.1 \ ml/cm^2$ of membrane is required.

3. Drain the excess washing solution from the washed membranes and place them, protein side up, on a sheet of plastic wrap or other suitable clean surface. Pipette the mixed detection reagent onto the membrane.

4. Incubate for 1 min (Amersham ECL) or 5 min (Amersham ECL Prime and Amersham ECL Select) at room temperature.

5. Drain off excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue. Place the blots, protein side down, on to a fresh piece of plastic wrap, wrap the blots and gently smooth out any air bubbles.

6. Place the wrapped blots, protein side up, in an X-ray film cassette.

7. Place a sheet of X-ray film (for example, Hyperfilm ECL) on top of the membrane. Close the cassette and expose for 15 s.

☞ Choose exposure times according to expected signal intensity. A good starting point is to begin with 1 min. Develop the film, and perform additional exposures if necessary. Adjust to optimal exposure time.

11.7.2 Fluorescence detection with Amersham ECL Plex

1. Dilute the primary antibody of mouse or rabbit origin to optimal concentration in washing solution or blocking solution.

2. Incubate a blocked membrane (protein side up) with the diluted primary antibody for 1.5 h at room temperature, or overnight at 4°C.

3. Rinse the membrane twice in washing solution, then wash the membrane twice for 5 min per wash in washing solution with shaking at room temperature.

4. Dilute the ECL Plex CyDye conjugated secondary antibody, (prepared at a concentration of 1 µg/ml) to optimal concentration.

5. Incubate the washed membrane in the secondary antibody solution with shaking for 1 h at room temperature. Ensure that the membrane is protected from light.

6. Rinse the membrane three times in washing solution, followed by four washes in washing solution for 5 min per wash with shaking at room temperature Protect the membrane from light.

   ☞ When using antibodies labeled with fluorophores, incubation should be performed in the dark.

7. Rinse the membrane three times in PBS or TBS (without Tween-20).

8. Detect the secondary antibody signal by scanning the membrane using a laser scanner. For optimal results, dry the membrane before scanning by placing it on Hybond blotting paper and incubate at 37°C to 40°C for 1 h, or at room temperature. Protect the membrane from light.

11.7.2.1 Protocol for multiplexed detection

1. Dilute the primary antibodies in PBS-Tween or TBS-Tween.

   ☞ More than one antibody may be mixed in a single solution for multiplexed detection.

   ☞ To prevent cross-talk, primary antibodies must be raised in different species. If using chemiluminescence detection, proteins of interest must be well separated following electrophoresis.

   ☞ If primary antibodies are to be reused, it is possible to incubate the primary antibodies separately when multiplexing.

2. Place the membrane (protein side up) in the primary antibody solution and incubate with agitation for 1 h at room temperature or overnight at 4°C.

3. Rinse the membrane twice in washing solution, then wash the membrane twice for 5 min per wash in washing solution with shaking at room temperature.

4. Mix the secondary antibodies in a single solution.

5. Place the membrane in the mixed secondary antibody solution and incubate for 1 h at room temperature, or alternatively at 4°C overnight. Protect the membrane from light.

   ☞ When using antibodies labeled with fluorophores, incubation should be performed in the dark.

6. Rinse the membrane three times in washing solution, followed by four washes in washing solution for 5 min per wash with shaking at room temperature. Protect the membrane from light.

7. Rinse the membrane three times in PBS or TBS (without Tween-20).

8. Detect the secondary antibody signal by scanning the membrane using a fluorescent laser scanner. For optimal results, dry the membrane before scanning by placing it on Hybond blotting paper and incubate at 37°C to 40°C for 1 h, or at room temperature. Protect the membrane from light.

11.7.2.2 Protocol for three-layer probing

1. Place the membrane in the primary antibody solution and incubate with agitation for 1 h at room temperature or overnight at 4°C.

2. Place the membrane in washing solution and wash three times for 5 min per wash.

3. Place the membrane in the biotinylated secondary antibody solution and incubate with agitation for 1 h at room temperature or 37°C.

4. Place the membrane in washing solution and wash three times for 5 min per wash.

5. Dilute the streptavidin-HRP conjugate (or streptavidin CyDye conjugate if using Amersham ECL Plex detection) in PBS-Tween or TBS-Tween.

   ☞ We highly recommend that you optimize the concentration (see Appendix A for optimization protocol).

6. Incubate the membrane in the solution for 45 to 60 min at room temperature on an orbital shaker.

7. Briefly rinse the membrane with two changes of washing solution.

8. Wash the membrane by suspending it in enough washing solution to cover the membrane and agitate for 5 min at room temperature. Repeat the procedure at least four to six times.

9. Continue with detection as recommended for the selected detection reagent and imaging system.

11.7.2.3 Conjugation of CyDye to antibody

Amersham ECL Plex has been optimized for optimal signal performance and minimal cross-reactivity between secondary antibodies. Labeling your own primary antibodies will enable triplexed detection but these antibodies will have a lower limit of detection (LOD) and can give rise to cross-reactivity issues. The performance of such reagents should therefore be tested before use.

1. Add the protein solution (1 ml) to the vial of coupling buffer and mix thoroughly by gentle vortexing or by manually inverting the capped tube 10 times.

2. Transfer the entire volume of protein and coupling buffer to the vial of reactive dye, cap the vial, and mix thoroughly.

   ✋ Care should be taken to prevent foaming of the protein solution.

3. Incubate at room temperature in the dark for 30 min, with additional mixing approximately every 10 min.

4. While the labeling reaction is incubating, decant the buffer from the top of the column. Mount the column on a ring stand.

5. Add 13 ml of fresh elution buffer.

6. Remove the tip from the column to start the outflow of the column and allow all the buffer to run through the column into a collection tube or small beaker.

☞ Flow will automatically stop when the meniscus reaches the disk at the top of the column packing. There is no need to worry about the column drying out.

7. Carefully transfer the antibody labeling mixture to the top of the column and allow the solution to enter the packing.

8. Add 2 ml of elution buffer. As this volume of buffer moves through the column, a faster moving colored band of labeled protein will separate from any unconjugated dye. These bands might be difficult to distinguish by color, but they can be readily visualized by fluorescence using a longwave ultraviolet (UV) lamp.

9. When the elution buffer has completely run into the column packing, the leading edge of the faster moving colored band should be near the bottom of the packing.

10. Add an additional 2.5 ml of elution buffer to the top of the column and collect the faster moving colored band in a clean tube as it elutes from the column. The labeled protein should be entirely eluted by the 2.5 ml of buffer and collected in a single tube.

11.7.3   Affinity purification of antibodies

Antisera contain a mixture of antibodies (polyclonal antibodies) that bind to target epitopes with different specificities and affinities. There is also a risk for cross-reaction with irrelevant target molecules (nonspecific interactions). To enrich for antibodies with the highest specificity and affinity for the antigen of interest, polyclonal antibodies (and also monoclonal antibodies) can be purified by affinity methods. There are several affinity methods available for the purification of antibodies such as chromatography, magnetic beads or, less commonly, by binding to a membrane. Descriptions of how to purify polyclonal antibodies by affinity methods using products from GE Healthcare are provided in the following sections.

11.7.3.1   Antibody purification with prepacked HiTrap columns

HiTrap Protein G HP and HiTrap Protein A HP are prepacked, ready-to-use columns for the convenient purification of antibodies. Purification can be performed using a syringe, pump, or chromatography system such as ÄKTA design systems from GE Healthcare or fast protein liquid chromatography (FPLC). Furthermore, purification capacity can be greatly increased by connecting columns in series. For more information and selection guides, see Antibody Purification Handbook from GE Healthcare (1).

Materials required

Binding buffer: 20 mM sodium phosphate, pH 7.0
Elution buffer: 100 mM glycine-HCl, pH 2.7
Neutralizing buffer: 1 M Tris-HCl, pH 9.0

1. Prepare collection tubes by adding 60 to 200 µl of 1 M Tris-HCl, pH 9.0 per milliliter of fraction to be collected.

2. Fill the syringe or pump tubing with water. Remove the stopper and connect the column to the syringe (with the provided adapter) or pump tubing. Add the water drop-by-drop to avoid introducing air into the column.

3. Remove the snap-off end at the column outlet.

4. Remove the ethanol storage solution with 3 to 5 column volumes (CV) of water.

5. Equilibrate the column with at least 5 CV of binding buffer. Recommended flow rates are 1 ml/min (1 ml column) and 5 ml/min (5 ml column)[1].

6. Apply the sample using a syringe fitted to the adapter or by pumping it onto the column. For optimal results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application[1].

7. Wash with binding buffer (generally at least 5 to 10 CV) until the absorbance reaches a steady baseline or no material remains in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing[1].

8. Elute with elution buffer using a one-step or linear gradient. For step elution, 5 CV are usually sufficient. For linear gradient elution, 10 to 20 CV are usually sufficient. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for elution[1].

   [1] 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a 1 ml HiTrap column; 5 ml/min corresponds to approximately 120 drops/min when using a 5 ml HiTrap column.

9. After elution, regenerate the column by washing it with 3 to 5 CV of binding buffer. The column is now ready for a new purification.

   ☞ The purified fractions can be buffer exchanged using a HiTrap Desalting Column, HiPrep™ 26/10 Desalting Column or PD-10 Desalting Column if necessary.

   ☞ The reuse of HiTrap Protein G HP depends on the nature of the sample and should only be performed to purify identical monoclonal antibodies to prevent cross-contamination.

   ☞ If a P1 pump is used, a maximum flow rate of 1 to 3 ml/min can be run on a HiTrap 1 ml column packed with Sepharose High Performance media.

11.7.3.2 Antibody affinity purification with Protein A Mag Sepharose Xtra or Protein G Mag Sepharose Xtra

Protein A Mag Sepharose Xtra and Protein G Mag Sepharose Xtra magnetic beads are designed for rapid, small-scale purification and screening of monoclonal and polyclonal antibodies from sera and cell supernatants. The beads are intended to be used together with microcentrifuge tubes and a magnetic rack, such as MagRack 6 from GE Healthcare. They are easily separated from the liquid phase during the different steps of the purification protocol.

1. Prepare the magnetic beads by thoroughly vortexing the medium slurry.

2. Dispense 100 µl of homogenous medium slurry into a microcentrifuge tube.

3. Place the tube in a magnetic rack, such as MagRack 6 and decant the storage solution.

4. Equilibrate the beads by adding 500 µl of binding buffer.

5. Resuspend the medium.

6. Remove the liquid by placing the tube in the magnetic rack.

7. Immediately after equilibration, add 300 µl of sample.

   ☞ If the sample volume is less than 300 µl, dilute to 300 µl with binding buffer.

8. Resuspend the medium and incubate for 30 min with slow end-over-end mixing or by using a benchtop shaker.

9. Remove the liquid by placing the tube in the magnetic rack.

10. Wash the beads with 500 µl of binding buffer.

11. Resuspend the medium.

12. Remove the liquid.

13. Repeat the washing step.

14. Add 100 µl of elution buffer to the beads and resuspend the medium.

15. Remove and collect the elution fraction; this fraction contains most of the purified antibody. If desired, repeat the elution.

☞ As a safety measure to preserve the activity of acid-labile antibodies, we recommend the addition of 1 M Tris-HCl (pH 9.0) to tubes used for collecting antibody-containing fractions.

11.7.3.3 Antibody purification with Protein A HP SpinTrap columns

Protein A HP SpinTrap columns consist of small scale spin columns prepacked with Protein A Sepharose HP. The columns can be used for efficient, small scale purification of monoclonal and polyclonal antibodies from sera and cell culture supernatants in less than 20 min.

Before purification, prepare two collection tubes per sample for eluted fractions, each containing 30 µl of neutralizing buffer.

1. Remove storage solution from the column: Resuspend the medium, remove the bottom cap from the column and centrifuge for 30 s at 70 to 100 × g.

☞ Save the bottom cap for later.

2. After centrifugation, equilibrate by adding 600 µl of binding buffer to the medium.

3. Centrifuge for 30 s at 70 to 100 × g.

4. Bind the antibody by adding a maximum of 600 µl of the antibody solution.

5. Secure the top cap tightly and incubate for 4 min while gently mixing.

6. Centrifuge for 30 s at 70 to 100 × g.

☞ Multiple samples can be applied to the column as long as the capacity of the column is not exceeded.

7. Wash by adding 600 µl of binding buffer followed by centrifugation for 30 s at 70 to 100 × g. Add 600 µl of binding buffer once more and centrifuge for 30 s at 70 to 100 × g.

8. Repeat the washing step.

9. Elute the antibody by adding 400 µl of elution buffer and mix by inversion.

10. Place the column in a 2 ml microcentrifuge tube containing 30 µl of neutralizing buffer (see Step 1).

11. Centrifuge for 30 s at 70 × g and collect the eluted material.

12. Place the column in a new 2 ml microcentrifuge tube containing 30 µl of neutralizing buffer (see Step 1).

13. Centrifuge for 30 s at 70 × g and collect the eluted material.

☞ Most of the bound antibody is eluted after two elution steps.

11.8 Stripping and reprobing

The following protocol describes how to remove primary and secondary antibodies from membranes. The membranes may be stripped and reprobed several times. For further information on stripping and reprobing, see Chapter 5. If stripping and reprobing is planned, PVDF membranes are more robust than nitrocellulose and are therefore recommended for this process.

11.8.1 Stripping using high pH and high temperature

1. Submerge the membrane in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) and incubate at 70°C for 30 min, with agitation.

    ☞ Lower temperatures (50°C to 70°C) might also work well, but this should be determined empirically with the antibodies used (see section 5.3).

2. Wash the membrane twice for 10 min per wash in PBS-Tween or TBS-Tween at room temperature using large volumes of washing solution.
3. Block the membrane in a suitable blocking solution for 1 h at room temperature.
4. Repeat the probing and detection procedure.

11.8.2 Stripping using low pH

1. Submerge the membrane in stripping buffer (100 mM β-mercaptoethanol, 1% SDS, 25 mM glycine-HCl [pH 2.0]) and incubate for 30 min, with agitation.
2. Wash the membrane twice for 10 min per wash in PBS-Tween or TBS-Tween at room temperature using large volumes of washing solution.
3. Block the membrane in a suitable blocking solution for 1 h at room temperature.
4. Repeat the probing and detection procedure.

11.8.3 Stripping using high pH

1. Submerge the membrane in 0.2 M NaOH and incubate for 5 min at room temperature, with agitation.
2. Remove and add fresh 0.2 M NaOH and incubate for an additional 5 min.
3. Wash for 5 min in water.

    ☞ If any trace of signal still remains, increase the concentration of NaOH to as high as 2 M and the incubation time to 30 min.

4. Repeat the immunodetection protocol (without blocking).

    ☞ Reblocking is normally not necessary after stripping using NaOH. However, depending on the NaOH concentration and soaking time, it might be necessary to reblock.

11.8.4 Stripping using high salt solution

1. Soak the membrane in PBS or TBS supplemented with 0.5 M NaCl and 0.2% SDS for between 30 min and 2 h.
2. Rinse the blot with water.
3. Repeat the immunodetection protocol (without blocking).

    ☞ Reblocking is normally not necessary after stripping using salt. However, depending on the soaking time, it might be necessary to reblock.

11.9   Optimization protocols for finding optimal primary and secondary antibody concentrations

It is necessary to optimize primary and secondary antibody concentrations in order to achieve excellent sensitivity and specificity. The dot-blot method is frequently used but a more reliable method is to perform Western blotting and divide the membrane into strips (Fig 11.3). Two methods for antibody optimization are given below. These methods can also be used for optimizing additional parameters, such as blocking agents, species of primary antibody, and quality of primary antibody.

1. Perform electrophoresis and blotting. Use Rainbow Molecular Weight Markers between each loading series (M in Fig 11.3).

2. Cut the membranes into strips containing different quantities of sample (c1 to c3 in Fig 11.3). Label the different strips by cutting a different number of notches for each strip, as indicated in Figure 11.3.

3. Incubate the strips with different dilutions of primary and secondary antibodies.

   ☞ It is convenient to use 15 or 50 ml tubes for the incubations to save primary antibody. Ensure that the whole strip is covered with antibody solution during incubation.

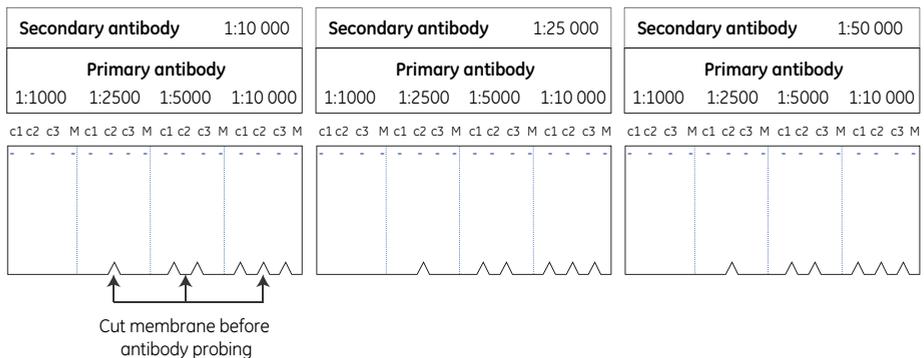4. Proceed to detection as previously described.

Fig 11.3. Membrane strips are used to determine the optimal dilutions of primary and secondary antibody dilutions. c1, c2, and c3 = three alternative quantities of sample. M = Rainbow Molecular Weight Markers. Note that this example is only illustrative. Choose the antibody dilutions recommended for the detection system. Highly sensitive chemiluminescence detection reagents usually require lower antibody concentrations, especially if X-ray film is used, while fluorescence detection requires higher antibody concentrations.

11.9.1   Optimization of primary antibody concentration by dot-blotting

1. Spot a two-fold dilution series of protein sample (five dilutions) on to a nitrocellulose membrane and allow to air dry. Prepare one blot for each primary antibody dilution to be tested.

2. Incubate in blocking solution for 1 h at room temperature, with agitation.

3. Rinse the membranes briefly with two changes of washing solution. Prepare a number of solutions within the recommended antibody dilution range. Incubate one blot in each solution for 1 h at room temperature, with agitation.

4. Briefly rinse the membrane with two changes of washing solution. Wash the membrane by suspending it in washing solution and agitate for 5 min at room temperature. Replace the washing solution three to four times.

5. Dilute the secondary antibody (using only one concentration) and incubate the membranes for 1 h at room temperature, with agitation.

6. Rinse the blots in two changes of washing solution, then wash three to six times for 5 min and then three times for 5 min per wash in fresh changes of washing solution.

7. Detect according to the protocol for the selected detection reagent. The antibody dilution that gives the strongest signal with the minimum background should be selected.

11.9.2 Optimization of secondary antibody concentration by dot-blotting

1. Spot a two-fold dilution series of protein sample (five dilutions) on to a nitrocellulose membrane and allow to air dry. Prepare one blot for each primary antibody dilution to be tested.

2. Incubate in blocking solution for 1 h at room temperature, with agitation.

3. Incubate in diluted primary antibody for 1 h at room temperature, with agitation.

4. Wash membranes three to six times for 5 min per wash or according to the protocol for the selected detection reagent.

5. Prepare a number of solutions within the recommended secondary antibody dilution range. Incubate one blot in each solution for 1 h at room temperature, with agitation.

6. Briefly rinse the membranes with two changes of washing solution.

7. Wash the membranes three to six times for 5 min per wash, or according to the protocol for the selected detection reagent.

8. Detect according to the protocol for the selected detection reagent. The antibody dilution that gives the strongest signal with the minimum background should be selected.

11.10 Reference

1. *Antibody Purification Handbook*, GE Healthcare, 18-1037-46, Edition AD (2007).

Chapter 12
Glossary

The following terms are defined according to their common usage in Western blotting applications.

Acrylamide C_3H_5NO	The monomeric unit which, when polymerized, forms the matrix of polyacrylamide gels.
Affinity purification	A chromatographic method of purification, based on a highly specific interaction, such as that between antigen and antibody, where target protein is immobilized on a solid support. Affinity purification can be used to improve the specificity of monoclonal and polyclonal primary antibodies as Western blotting reagents.
Alkaline phosphatase (AP)	A hydrolase enzyme that can be conjugated to secondary antibodies and can thus be used as part of the detection system in Western blotting.
Ammonium persulfate (APS) $(NH_4)_2S_2O_8$	The initiator of acrylamide polymerization. Used along with TEMED to catalyze the polymerization of acrylamide to polyacrylamide.
Analysis software	Tools to enable the automated analysis, storage and archiving of images collected on CCD camera-based imagers and/or scanners.
Anode	The positively charged electrode in an electrophoresis or blotting apparatus.
Antigen	Any structure that induces the generation of specific antibodies in a challenged animal.
Autoradiography	The process of capture and analysis of an image on an X-ray film/phosphoscreen produced by the decay emissions of a radioactive substance.
Background	Signals generated due to interactions of primary and/or secondary antibodies with the membrane itself, or impurities in the sample.
Biotin	A vitamin used as a protein tag in many molecular biological contexts, due to its extraordinarily strong affinity for streptavidin.
Bisacrylamide $C_7H_{10}N_2O_2$	A cross-linking agent used to polymerize acrylamide to polyacrylamide in the formation of gels.
Blocking	The process of saturating a membrane with, for example, protein after blotting to prevent nonspecific binding of antibodies to areas of the membrane not occupied by target protein.
Blotting	The process of transferring proteins from a gel to membrane, usually in an electric field. Also known as immunoblotting.
β-mercaptoethanol $HOCH_2CH_2SH$	A reducing reagent used to reduce disulfide bonds in proteins, resulting in unfolded proteins.
Bovine serum albumin (BSA)	Protein with numerous applications, including use as blocking agent and as standard in total protein concentration assays.

Bromophenol blue $C_{19}H_{10}Br_4O_5S$	A blue-colored compound that is a component in sample loading buffer. Migrates at the front in PAGE and thus serves as a marker for progress of electrophoresis.
Butanol	Used in its saturated form to temporarily overlay newly cast resolving gels before the addition of the stacking gel, preventing the formation of bubbles and forming a perfectly smooth interface between the two phases.
Cathode	The negatively charged electrode in an electrophoresis or blotting apparatus.
Charge-coupled device (CCD)	A device that converts electrical charge into a digital value. CCD-cameras are integrated with imagers, producing high-quality image data.
Chemifluorescence	Chemically and/or enzymatically induced generation of an active fluorophore that emits light after excitation with light of a specific wavelength.
Chemiluminescence	The emission of light by a molecule as a result of a chemical reaction.
Comb	A plastic manifold inserted into a stacking gel before polymerization to form the wells into which samples are injected.
Conjugate	Any chemical stably attached to a carrier molecule of interest and which can then serve as a marker. The conjugate must not interfere with the function of its carrier.
CyDye	Fluorophores from GE Healthcare that emit red (Cy5), green (Cy3), or blue (Cy2) light after excitation with light of the appropriate wavelengths.
Denaturing gel	A polyacrylamide gel containing SDS.
Densitometry	The quantitative measurement of optical density on film. Optical density is usually given a relative value in a scale.
Detection limit (LOD)	The smallest amount of protein that can be detected using given detection reagents and systems (compare with LOQ).
Detergent	A surfactant added to buffers and solutions in Western blotting applications that helps to increase the solubility of proteins.
Discontinuous buffer system	Electrophoresis using a gel comprised of a spacer gel and a larger resolving gel.
Dithiothreitol (DTT) $C_4H_{10}O_2S_2$	A reducing reagent. In the context of electrophoresis and Western blotting, it is used to reduce disulfide bonds in proteins, disrupting tertiary structure.
Dot-blot	A rapid method used to monitor the interaction of proteins with a membrane and their subsequent interactions with probes. By testing conditions using a dot-blot, there is no need to perform gel electrophoresis or gel-to-membrane blotting.
Dynamic range	The range of blotted protein quantities on a blot that can be measured using a given system. The greater the linearity of the dynamic range, the more precisely proteins can be quantitated over that range.
Emission	The release of light from a fluorophore when an electron in the molecule falls from an excited state to a lower energy state.
Enhanced chemiluminescence (ECL)	HRP-catalyzed conversion of an ECL substrate into a sensitized reagent, which on further oxidation by hydrogen peroxide, emits detectable light when it decays.
Electrophoresis (1-D)	The process of the separation of a mixture of proteins on a gel in an electric field according to size, shape, and charge.

Electrophoresis (2-D)	Separation of proteins in two dimensions, first according to isoelectric point (pI) and subsequently according to molecular weight.
Epitope	The specific molecular region of an antigen recognized by an antibody.
Excitation	Absorption of light energy by a fluorophore, during which an electron in the fluorophore molecule is boosted to a higher energy level.
Filter	A component of an imager that allows light of a certain wavelength to pass while obstructing light of other wavelengths.
Fluorescence	Light of a specific wavelength emitted by a fluorophore after excitation via a light source of shorter wavelength.
Fluorophore	Any compound, which, when transformed to a temporary high-energy state, emits light as it returns to its ground state.
Glycerol	A colorless and viscous liquid used in sample loading buffer to increase the density of the samples. Glycerol enables loading and helps anchor the sample in the sample wells until an electric field is applied to the gel.
Horseradish peroxidase (HRP)	An enzyme that catalyzes the conversion of an ECL reagent into a reactive, light-emitting compound.
Housekeeping protein	Any intracellular protein that does not significantly change in expression level in response to external stimulation.
Imaging	The process of converting the signals generated by a detection system into a format that enables visualization, analysis, and storage of data.
Immunoprecipitation	The mechanical removal of a protein, or complex of proteins from a sample by incubation with an antibody coupled to a solid matrix, such as Sepharose beads.
Isoelectric focusing (IEF)	The separation of proteins on a pH gradient according to isoelectric point (pI).
Isoelectric point (pI)	The pH at which a protein has no net charge.
Isotype	The class of an antibody, defined according to the heavy chain. The antibody isotype of most relevance to Western blotting is IgG.
Laemmli buffer	The classic electrophoresis running buffer based on Tris-glycine.
Lysis	The disruption of cells in sample preparation prior to electrophoresis. Lysis can be purely mechanical or mediated by the use of buffers containing detergents.
Membrane capacity	The maximum amount of protein that can bind per unit area of a membrane.
Methanol	An important component in transfer buffer, minimizing swelling and distortion of polyacrylamide gels at transfer. In addition, methanol counters the inhibitory effect of SDS on the contact between proteins and membranes.
Molecular weight markers	A mixture of proteins of known molecular weights. Prestained molecular weight markers can be used in Western blotting to verify successful transfer.
Monoclonal antibodies	Antibodies that only bind to one epitope and are typically highly specific, pure, and consistent in performance, and generally give rise to low background.
Multiplexing	The practice of detecting several different proteins on a single blot, in a single experiment.

Native gel	A gel that does not contain any denaturing reagents (e.g., SDS) or reducing reagents (e.g., β-mercaptoethanol).
Nitrocellulose	A membrane material.
Nonspecific binding	Interactions between antibodies used for detection and impurities on the membrane or the membrane itself.
Normalization	The process of adjusting for variations in the amount of total protein from lane-to-lane in order to reliably quantitate protein levels.
Optimization	A general term used to cover the preliminary steps that should be carried out to determine optimal conditions for a specific experimental system, for example, blocking reagent, antibody concentrations, as well as incubation times/temperatures.
Phosphatase inhibitors	Agent that prevents dephosphorylation of phosphorylated proteins by phosphatases. Should be added to lysis buffers if the aim of the experiment is to detect transient phosphorylation of proteins.
Phosphate-buffered saline (PBS)	Common buffer solution in biological research, consisting of NaCl and sodium phosphate at physiological concentrations and pH conditions.
Photomultiplier tube (PMT)	A photoelectric device that converts light into electric current and amplifies the current.
Polyclonal antibodies	Mixture of antibodies with affinities for different epitopes on a target molecule.
Polyvinylidine difluoride (PVDF)	A membrane material.
Post-translational modification (PTM)	Chemical modification of a protein after translation that regulates and changes the function of the protein. Typical PTMs include phosphorylated, glycosylated, and acetylated groups.
Primary antibody	The first antibody, specific to a target protein, used as a probe on a blotted membrane. Primary antibodies are usually unlabeled.
Protease inhibitors	A cocktail of chemicals inhibiting the activity of proteases, thereby preventing degradation of proteins.
Protein A/Protein G	Proteins derived from the cell wall of bacteria that bind immunoglobulins, most notably to the Fc region of IgG.
Quantitation limit (LOQ)	The smallest amount of protein that can be reliably quantitated using given detection reagents and systems (compare with LOD).
Radioisotope	A radioactive isotope of an element, some of which have found use as labels for secondary antibodies in Western blotting.
Resolution	The quality of separation of protein bands in a gel after electrophoresis of a sample.
Resolving gel (also known as separating gel)	The main body of a gel, in which proteins separate according to size.
Sample loading buffer	A solution added to protein sample before loading into the wells of a spacer gel.
Secondary antibody	A labeled antibody directed to the constant region of a primary antibody. Increases sensitivity of the assay by multiple binding of a labeled antibody to a primary antibody.
Semidry transfer	Electrotransfer of proteins from a gel to a membrane, by placing a stack comprised of the gel and membrane sandwiched between blotting paper, and soaked in transfer buffer.
SDS-PAGE	Electrophoresis in a polyacrylamide gel carried out in the presence of SDS (see sodium dodecyl sulfate).

Signal duration	The time interval over which a signal can be detected.
Signal stability	The quality of consistency of signal intensity over time.
Signal-to-noise ratio	A measure of how well a true signal can be resolved from the noise. "Noise" is signals generated by the detection system.
Sodium azide	A preservative added to antibody solutions and buffers to extend their shelf life. Azide inhibits HRP and AP activities.
Sodium dodecyl sulfate (SDS) $C_{12}H_{25}SO_4Na$	An anionic detergent used in SDS-PAGE to denature and coat proteins with a negative charge. Also known as sodium lauryl sulfate.
Stacking gel (also known as spacer gel)	The sample well-containing region of a gel in which proteins are concentrated before entering the resolving gel.
Streptavidin	A bacterial protein with extraordinarily high affinity for biotin. Streptavidin/biotin systems are extensively used in molecular biology.
Stripping	The physical removal of the molecular components of a detection system from a blotted membrane. After stripping the membrane is open to reprobing with a second primary antibody.
Tetramethylethylenediamine (TEMED) $(CH_3)_2NCH_2CH_2N(CH_3)_2$	Polymerization initiator. Used together with APS to catalyze the polymerization of acrylamide in the formation of polyacrylamide gels.
Tris/Tris-buffered saline (TBS)	Common buffer solution in biological research, with a buffering range of pH 7 to 9, the typical physiological pH of most living organisms.
Wet transfer	The process of transferring proteins from a gel to a membrane, by total immersion of both gel and membrane in transfer buffer and the application of an electric field. The method is highly efficient and is recommended for the transfer of large proteins.
Zwitterionic (also known as amphoteric)	The quality of possessing both acidic and basic groups (positively and negatively charged amino acids at given pH) within the same molecule. All proteins are zwitterionic.

Appendix A
Optimization

Before you run precious samples and use expensive antibodies in your Western blotting experiment, it is well worth the effort to invest some time in selecting the optimal experimental design to help you achieve the results you want. Optimization of Western blotting is desirable whether your main goal is sensitivity, precision of quantitation, or an assay of sufficient robustness and consistency for reliable day-to-day laboratory use. Once properly optimized, it should then be a simple matter to achieve results you can trust, making the time you invest in optimization pay off in terms of both quality and time saved further down the line.

The following sections provide some guidelines to help you design your experiments correctly from the start. For detailed optimization protocols, see 11.10.

A.1   Choice of membrane

The choice of membrane depends on whether your experiment involves chemiluminescence or fluorescence detection. The choice also depends on whether you plan to strip and reprobe the membrane and if you want to post-stain the blot. Polyvinylidine difluoride (PVDF) membranes are recommended for experiments involving stripping and reprobing, as these membranes are stronger and more robust than nitrocellulose membranes. Nitrocellulose membranes are compatible with total protein post-stains. PVDF membranes can also be post-stained, but destaining might be less effective. Nitrocellulose membranes are therefore recommended when low background is important for the detection of weak total protein signals.

Membranes with low autofluorescence properties are critical for fluorescence detection, especially when detecting weak signals. Amersham Hybond LFP 0.2 and Amersham Protran Premium membranes are recommended for this mode of detection, and are particularly suitable in combination with the Amersham ECL Plex fluorescent Western blotting system.

For most applications, a membrane pore size of 0.45 µm is suitable, but if you are working with small target proteins (M_r 5000 to 25 000), membranes with a smaller pore size (0.2 µm) are recommended as they have higher binding capacity for smaller proteins during transfer from the gel and reduce sample loss.

Recommended membranes for each detection system from GE Healthcare are provided in Table A.1.

Table A.1. First-choice nitrocellulose and PVDF membranes for each Amersham ECL detection system

Detection system	Nitrocellulose	PVDF
Amersham ECL	Amersham Protran Premium 0.2 or 0.45	Amersham Hybond 0.2 and 0.45 PVDF
Amersham ECL Prime	Amersham Protran Premium 0.2 or 0.45	Amersham Hybond 0.2 and 0.45 PVDF
Amersham ECL Select	Amersham Protran Premium 0.2 or 0.45	Amersham Hybond 0.2 and 0.45 PVDF
Amersham ECL Plex	Amersham Protran Premium 0.2 or 0.45	Amersham Hybond LFP 0.2

☞ These Amersham Hybond and Amersham Protran Premium membranes are sensitive to their environment and should be stored at room temperature in a clean dry atmosphere away from excessive heat, light, and noxious fumes. To prevent contamination, handle membranes using gloves or blunt forceps. Correct storage achieves consistent protein binding capacity, solvent absorbance, and minimal handling problems.

A.2 Choice of blocking agent

Selection of blocking agent is very important to achieve optimal results, especially for low-abundance proteins. Nonspecific binding and background levels, as well as signal intensity, can all be affected by the choice of blocking agent. The optimal blocking agent depends on compatibility with primary and secondary antibodies as well as the detection system used. Note that not all blocking agents are compatible with fluorescent Western blotting and background problems might arise if the blocking agent itself possesses fluorescent properties. One way to avoid these problems in applications using Amersham ECL Plex is by using Amersham ECL Prime Blocking Reagent.

Compatibility between the blocking agent and detection reagent should be tested by using the dot-blotting method described in Chapter 11. Briefly, the blocking solution is spotted onto a blank membrane. Detection reagents are then added to the blot, which is then incubated for 5 min and then imaged. The appearance of a signal indicates that the blocking agent is not compatible with the detection reagent. A more comprehensive method that mimics true experimental conditions is to prepare a Western blot and divide it into strips. This takes more time than dot-blotting, but more parameters can be monitored in a single experiment.

If you use milk-protein and have a problem with high background, this might be because the target protein is also found in milk. As milk contains a complex mixture of proteins (that can vary depending on where the cows have been feeding) blocking by milk-protein can lead to rather unpredictable results.
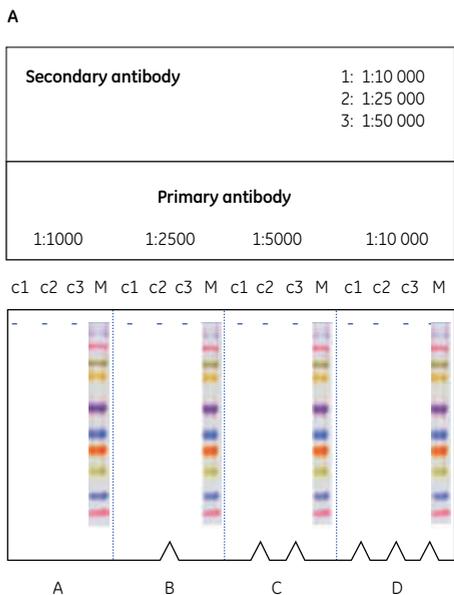
A.3 Choice of washing buffer

Phosphate-buffered saline (PBS)-Tween is a suitable washing buffer for most applications. For phosphorylated target proteins, however, we recommend using Tris-buffered saline (TBS)-Tween as washing buffer, as the phosphate in PBS-Tween can interfere with antibody binding. Under these circumstances, remember to use TBS-Tween or TBS in all washing buffer-based solutions, as well as the blocking solution.

☞ When a buffer solution is prepared, water quality is very important, as minute quantities of impurities can interfere at different levels. The enzymatic activity of horseradish peroxidase (HRP), for example, is inhibited by pyrogens commonly present in even high purity water.

A.4 Optimization of antibody concentrations using membrane strips

The optimal antibody concentration is usually determined by testing a series of antibody dilutions around those recommended by the vendor. If the recommended dilution is 1:100, for example, an appropriate titration should span two-fold dilutions either side of this mark, that is, 1:25, 1:50, 1:100, 1:200, and 1:400. To further optimize the probing conditions, the same titration may be performed for extended or reduced times, such as 1, 2, and 3 h at room temperature or overnight at 4°C. This may be done by dot-blotting, but it is more reliable to perform Western blotting and then divide the membrane into strips (Fig A.1). This takes more time than dot-blotting, but more parameters can be monitored in a single experiment. By using this method you will obtain information on specific signal intensity, background level, and levels of nonspecific detection. You will also be able to choose optimal dilutions of primary and secondary antibodies, optimal sample load, blocking agent, and species of primary antibody, as well as the highest quality of primary antibody. An alternative and convenient way to screen antibodies is to use a blotting manifold, where selected individual lanes on a membrane can be incubated with antibodies.

A

Secondary antibody	1: 1:10 000
	2: 1:25 000
	3: 1:50 000

| Primary antibody | | | |
| 1:1000 | 1:2500 | 1:5000 | 1:10 000 |

c1 c2 c3 M   c1 c2 c3 M   c1 c2 c3 M   c1 c2 c3 M

A   B   C   D

B

Incubation of strips in different dilutions of primary antibody

a   b
c
d

Incubation of strips in different dilutions of secondary antibody

a b c d   a b c d   a b c d

1:10 000   1:25 000   1:50 000

Fig A.1. In the optimization process, the steps of blocking, antibody incubation, and washing are similar to normal Western blotting procedures. (A) Suggested scheme for selecting optimal primary and secondary antibody concentrations in Western blotting. c1, c2, c3 = three concentrations of sample. M = Rainbow Molecular Weight Markers or Amersham ECL DualVue Western Blotting Markers. (B) For antibody evaluation, the membrane should first be cut into strips after transfer and incubated with different primary antibody dilutions (a = 1:1000, b = 1:2500, c = 1:5000, d = 1:10 000). The antibody dilutions here are only illustrative - you should choose the antibody dilution ranges appropriate for the detection system. It is convenient to use 15 or 50 ml tubes for this procedure. After washing, the strips that are to be incubated with the same dilution of secondary antibody can be combined and incubated in a single tray. The antibody dilutions giving the best signal with the minimum background should be selected for your Western blotting application.

The PR 150 Deca-Probe Incubation Manifold from GE Healthcare (Fig A.2) allows the user to individually screen 10 sample lanes on a single membrane (10 × 10 cm). The product minimizes the risk of cross-reactivity, facilitates multiprobing, and requires only enough reagents to wet the membrane. For larger gels, GE Healthcare also provides Amersham ECL Multiprobe (25 lanes) and ECL Multiprobe XL (45 lanes).

Fig A.2. The PR 150 Deca-Probe Incubation Manifold from GE Healthcare allows convenient multiple sample screening on a single membrane.

Ordering information

Sample preparation

Products	Quantity	Code number
Mammalian Protein Extraction Buffer	1 × 500 ml	28-9412-79
Yeast Protein Extraction Buffer Kit	1	28-9440-45
Vivaspin sample concentrators with 30 000 molecular weight cut off (MWCO)[1]		
2-D Quant Kit	500 assays	80-6483-56
Vivaspin 500	25 × 500 µl	28-9322-35
Vivaspin 2	25 × 2 ml	28-9322-48
Vivaspin 6	25 × 6 ml	28-9323-17
Vivaspin 20	12 × 20 ml	28-9323-61
SDS-PAGE Clean-Up Kit	50 samples	80-6484-70
2-D Clean-Up Kit	50 samples	80-6484-51

Gel electrophoresis, transfer, and blotting equipment

Products	Quantity	Code number
Transfer units		
TE 62 Transfer Unit	1	80-6209-58
TE 22 Mini Tank Transfer Unit	1	80-6204-26
TE 70 Semi-Dry Transfer Unit, 14 × 16 cm	1	80-6210-34
TE 70 PWR Semi-Dry Transfer Unit, 14 × 16 cm[2]	1	11-0013-41
TE 77 Semi-Dry Transfer Unit, 21 × 26 cm	1	80-6211-86
TE 77 PWR Semi-Dry Transfer Unit, 21 × 26 cm[2]	1	11-0013-42
Multiphor II NovaBlot Kit, Semi-Dry	1	18-1016-86
Blotting equipment		
ECL Multiprobe, 25 lanes	1	11-0033-95
ECL Multiprobe XL, 45 lanes	1	11-0033-96
PR 150 Deca-Probe Incubation Manifold	1	80-6087-98

[1] Other MWCO (3000, 5000, 10 000, 50 000, and 100 000) are available for all Vivaspin column volumes.

[2] Includes EPS 301 Power Supply.

Products	Quantity	Code number
Amersham ECL Gel Electrophoresis System		
Amersham ECL Gel Box	1	28-9906-08
Amersham ECL Gel 10%, 10 wells	Pack of 10	28-9898-04
Amersham ECL Gel 12%, 10 wells	Pack of 10	28-9898-05
Amersham ECL Gel 4-12%, 10 wells	Pack of 10	28-9898-06
Amersham ECL Gel 8-16%, 10 wells	Pack of 10	28-9898-07
Amersham ECL Gel 4-20%, 10 wells	Pack of 10	28-9901-54
Amersham ECL Gel Running Buffer	10 runs	28-9902-52
Vertical electrophoresis systems		
miniVE Vertical Electrophoresis System	1	80-6418-77
miniVE Blot Module	1	80-6418-96
SE 600 Ruby Standard Dual Cooled Vertical Unit	1	80-6479-57
Power supplies		
EPS 301 Power Supply	1	18-1130-01

[2] Includes EPS 301 Power Supply.

Amersham molecular weight markers

Products	Quantity	Code number
Low-Range Rainbow Molecular Weight Markers	250 µl	RPN755E
High-Range Rainbow Molecular Weight Markers	250 µl	RPN756E
Full-Range Rainbow Molecular Weight Markers	250 µl	RPN800E
ECL DualVue Western Blotting Markers	1 pack (25 loadings)	RPN810
ECL Plex Fluorescent Rainbow Markers	120 µl	RPN850E
ECL Plex Fluorescent Rainbow Markers	500 µl	RPN851E

Blotting membranes

Products	Quantity	Code number
Amersham Protran 0.45 (300 mm × 4 m)	1 roll	10-6000-02
Amersham Protran 0.2 (200 × 200 mm)	25 sheets	10-6000-43
Amersham Protran 0.1 (100 × 100 mm)	10 sheets	10-6000-77
Amersham Protran Premium 0.45 (80 × 90 mm)	25 sheet	10-6000-96
Amersham Protran Premium 0.2 (140 × 160 mm)	25 sheet	10-6000-66
Amersham Protran Supported 0.45 (200 mm × 4 m)	1 roll	10-6000-36
Amersham Protran Supported 0.2 (150 mm × 4 m)	1 roll	10-6000-54
Amersham Hybond 0.45 PVDF (300 × 600 mm)	5 sheets	10-6000-38
Amersham Hybond 0.2 PVDF (260 × 600 mm)	5 sheets	10-6000-39
Amersham Hybond LFP 0.2 (100 × 100 mm)	25 sheets	10-6000-90
Amersham Hybond SEQ 0.2 (200 × 200 mm)	10 sheets	10-6000-61
Amersham Hybond LFP Sandwich (140 × 160 mm)	10 sandwiches	10-6001-12
Amersham Protran Premium 0.2 Sandwich (80 × 90 mm)	10 sandwiches	10-6001-18
Whatman blotting papers		
Hybond Blotting Paper (20 × 20 cm)	100 sheets	RPN6101M
3 MM Chr (20 x20 cm)	100 sheets	3030-861

Blocking reagents

Products	Quantity	Code number
Amersham ECL Prime Blocking Reagent	20 g	RPN418
ECL Blocking Agent	1	RPN2125

Primary antibodies

Products	Quantity	Code number
Anti-His Antibody	170 µl	27-4710-01

CyDye antibody labeling kits

Products	Quantity	Code number
Cy2 Ab Labeling Kit	1 kit	PA32000
Cy2 mAb Labeling Kit	1 kit	PA32001
Cy3 Ab Labeling Kit	1 kit	PA33000
Cy3 mAb Labeling Kit	1 kit	PA33001
Cy5 Ab Labeling Kit	1 kit	PA35000
Cy5 mAb Labeling Kit	1 kit	PA35001

ECL HRP-linked secondary antibodies

Products	Quantity	Code number
ECL Mouse IgG, HRP-Linked Whole Ab (from sheep)	100 µl	NA931-100UL
ECL Mouse IgG, HRP-Linked Whole Ab (from sheep)	1 ml	NA931-1ML
ECL Human IgG, HRP-Linked Whole Ab (from sheep)	1 ml	NA933-1ML
ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey)	100 µl	NA934-100UL
ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey)	1 ml	NA934-1ML
ECL Mouse IgG, HRP-Linked F(ab´)$_2$ Fragment (from sheep)	1 ml	NA9310-1ML
ECL Rabbit IgG, HRP-Linked F(ab´)$_2$ Fragment (from donkey)	1 ml	NA9340-1ML

Amersham detection reagent packs and kits

Products	Quantity	Code number
Amersham ECL Western Blotting Detection Reagents	Reagents for 4000 cm^2 membrane	RPN2106
Amersham ECL Western Blotting System	1 kit	RPN2108
Amersham ECL Western Blotting Detection Reagents	Reagents for 1000 cm^2 membrane	RPN2109
Amersham ECL Western Blotting Detection Reagents	Reagents for 6000 cm^2 membrane	RPN2134
Amersham ECL Prime Western Blotting Detection Reagent	Reagents for 1000 cm^2 membrane	RPN2232
Amersham ECL Prime Western blotting Detection Reagent	Reagents for 3000 cm^2 membrane	RPN2236
Amersham ECL Select Western Blotting Reagent	Reagents for 1000 cm^2 membrane	RPN2235

Amersham ECL Plex CyDye conjugated antibodies

Products	Quantity	Code number
Amersham ECL Plex Western Blotting Combination Pack (Cy3, Cy5, Amersham Protran Premium 0.2)	1	RPN998
Amersham ECL Plex Western Blotting Combination Pack (Cy3, Cy5, Amersham Hybond LFP 0.2) for two slab gels	1	RPN999
ECL Plex goat-α-rabbit IgG-Cy3, for 1000 cm^2 membrane area	150 µg	28-9011-06
ECL Plex goat-α-rabbit IgG-Cy3, for 4000 cm^2 membrane area	600 µg	28-9011-07
ECL Plex goat-α-mouse IgG-Cy2, for 1000 cm^2 membrane area	150 µg	28-9011-08
ECL Plex goat-α-mouse IgG-Cy2, for 4000 cm^2 membrane area	600 µg	28-9011-09
ECL Plex goat-α-rabbit IgG-Cy2, for 1000 cm^2 membrane area	150 µg	28-9011-10
ECL Plex goat-α-rabbit IgG-Cy2, for 4000 cm^2 membrane area	600 µg	28-9011-11
ECL Plex goat-α-mouse IgG-Cy3, for 1000 cm^2 membrane area	150 µg	PA43009
ECL Plex goat-α-mouse IgG-Cy3, for 4000 cm^2 membrane area	600 µg	PA43010
ECL Plex goat-α-mouse IgG-Cy5, for 1000 cm^2 membrane area	150 µg	PA45009
ECL Plex goat-α-mouse IgG-Cy5, for 4000 cm^2 membrane area	600 µg	PA45010
ECL Plex goat-α-rabbit IgG-Cy5, for 1000 cm^2 membrane area	150 µg	PA45011
ECL Plex goat-α-rabbit IgG-Cy5, for 4000 cm^2 membrane area	600 µg	PA45012

Autoradiography films

Products	Quantity	Code number
Amersham Hyperfilm ECL (5 × 7 inches)	50 sheets	28-9068-35
Amersham Hyperfilm ECL (18 × 24 cm)	50 sheets	28-9068-36
Amersham Hyperfilm ECL (18 × 24 cm)	100 sheets	28-9068-37
Amersham Hyperfilm ECL (8 × 10 inches)	50 sheets	28-9068-38
Amersham Hyperfilm ECL (8 × 10 inches)	100 sheets	28-9068-39

Imaging systems

Products	Quantity	Code number
Typhoon variable mode imagers		
Typhoon FLA 9500	1	29-0040-80
Typhoon FLA 7000	1	28-9558-09
Amersham imagers		
Amersham Imager 600	1	29-0834-61
Amersham Imager 600 UV	1	29-0834-63
Amersham Imager QC	1	29-0834-64
Amersham Imager 600 RGB	1	29-0834-67
ImageQuant imager		
ImageQuant LAS 500	1	29-0050-63

Software and accessories

Products	Quantity	Code number
CD and getting started:		
ImageQuant TL 7.0 and IQTL SecurITy 8.0 Software package (with Getting Started Guide)	1 user	28-9380-94
Licenses for ImageQuant TL only:		
ImageQuant TL, single user license	1 user	28-9236-62
Licenses for ImageQuant TL v7.01 and IQTL SecurITy v8.0:		
ImageQuant TL 7.01 and ImageQuant TL SecurITy 8.0	1 user	28-9332-73

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imagination at work