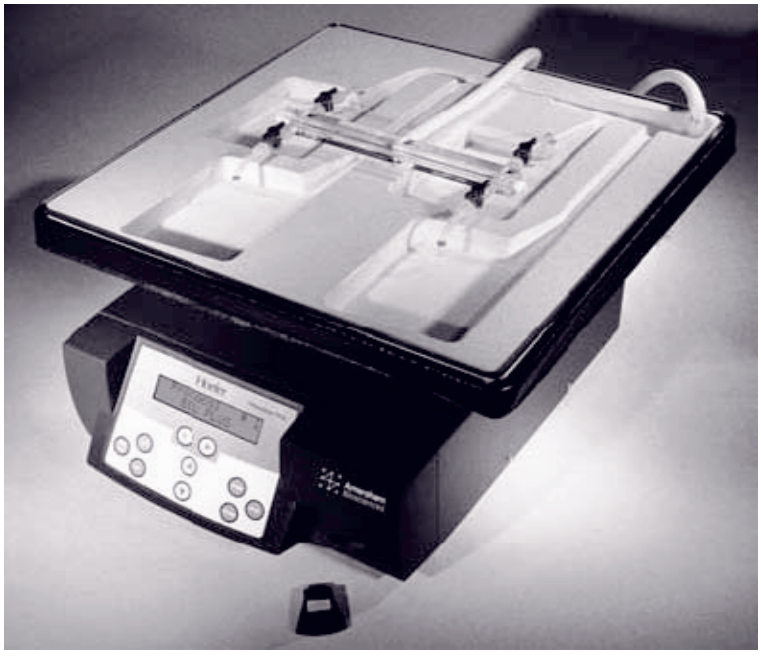
GE Healthcare

Processor Plus

Protocol Guide for Western Blotting



Important user information

All users must read this entire manual to fully understand the safe use of Processor Plus.

Important!

Processor Plus is intended for research use only, and should not be used in any clinical or in vitro procedures for diagnostic purposes.

Safety notices

This manual contains warnings and cautions concerning the safe use of the product. See definitions below.

WARNING! The WARNING symbol and notice highlight instructions that must be followed to avoid personal injury. Do not proceed until all stated conditions are clearly understood and met.

CAUTION! The CAUTION notice highlights instructions that must be followed to avoid damage to the product or other equipment. Do not proceed until all stated conditions are met and clearly understood.

Note: A Note is used to indicate information that is important for trouble-free and optimal use of the product.

Recycling

This symbol indicates that the waste of electrical and electronic equipment must not be disposed as unsorted municipal waste and must be collected separately. Please contact an authorized representative of the manufacturer for information concerning the decommissioning of equipment.

WARNING! This is a Class A product. In a domestic environment, it might cause radio interference, in which case the user might be required to take appropriate measures.

WARNING! All repairs should be done by personnel authorized by GE Healthcare. Do not open any covers or replace parts unless specifically stated in the instructions.

CE-certification

This product complies with the European directives listed below, by fulfilling corresponding standards. A copy of the Declaration of Conformity is available on request.

- 2006/95/EC, Low Voltage Directive
- 2004/108/EC, EMC Directive

The **CE** logo and corresponding declaration of conformity, is valid for the instrument when it is:

- used as a stand-alone unit, or
- connected to other CE-marked GE Healthcare instruments, or
- connected to other products recommended or described in this manual, and
- used in the same state as it was delivered from GE Healthcare except for alterations described in this manual.

Note: *The Declaration of conformity is valid only for systems that are marked with the **CE** logo:*

CE

Contents

1 Principles of Western blotting

1.1 Introduction 7
1.2 The steps of automated immunoblotting 9
Transfer of proteins from gel to membrane 9
Membranes 10
Transfer methods 10
Confirmation of transfer 11
Prestained markers 11
Blot staining 11
Gel staining 11
Blocking 12
Wash 12
Primary antibody 13
Secondary reagents for detection of the primary antibody-antigen complex 13
Detection method 14

2 Processing protocols

2.1 Automated ECL Plus western blot detection 16
Protocol 1: ECL Plus 17
2.2 Automated ECL Western blot detection 18
Protocol 2: ECL 18
2.3 Automated immunodetection with primary and secondary antibodies 19
Protocol 3: Standard 19
2.4 Automated immunodetection with tertiary complexes 19
Protocol 4: Enhanced 20
2.5 Instrument wash 21

3 Automating manual processing protocols

1 Primary antibody incubation 22
2 Washes 22
3 Minimum volume 22
4 Temperature 22

4 Troubleshooting tips

5 Useful references

6 Ordering information

1 Principles of Western blotting

1.1 Introduction

Immunoblotting is a widely-used and powerful technique for the detection and identification of proteins using antibodies. The process involves the separation of sample proteins by polyacrylamide gel electrophoresis (PAGE) followed by the transfer of the separated proteins from the gel onto a thin support membrane. The membrane binds and immobilizes the proteins in the same pattern as in the original gel. The membrane (or "blot") is then exposed to a solution containing antibodies that recognize and bind to the specific protein of interest. The antibodies bound to the membrane are detected by any of a variety of techniques, usually involving treatment with a secondary antibody (Fig 1-1). Although not difficult, the processing of the membrane is very time-consuming, the result of numerous successive incubations with a series of reagents and washes. The Processor Plus simplifies the processing of membranes by automating all of the solution changes and incubation steps once the blot has been generated. The processor delivers specified volumes of solution to the membrane surface, gently agitating the solutions evenly across the membrane surface for a set time, and then removes the solution. The precise timing and control of all of the steps results in increased reproducibility of immunoblots.

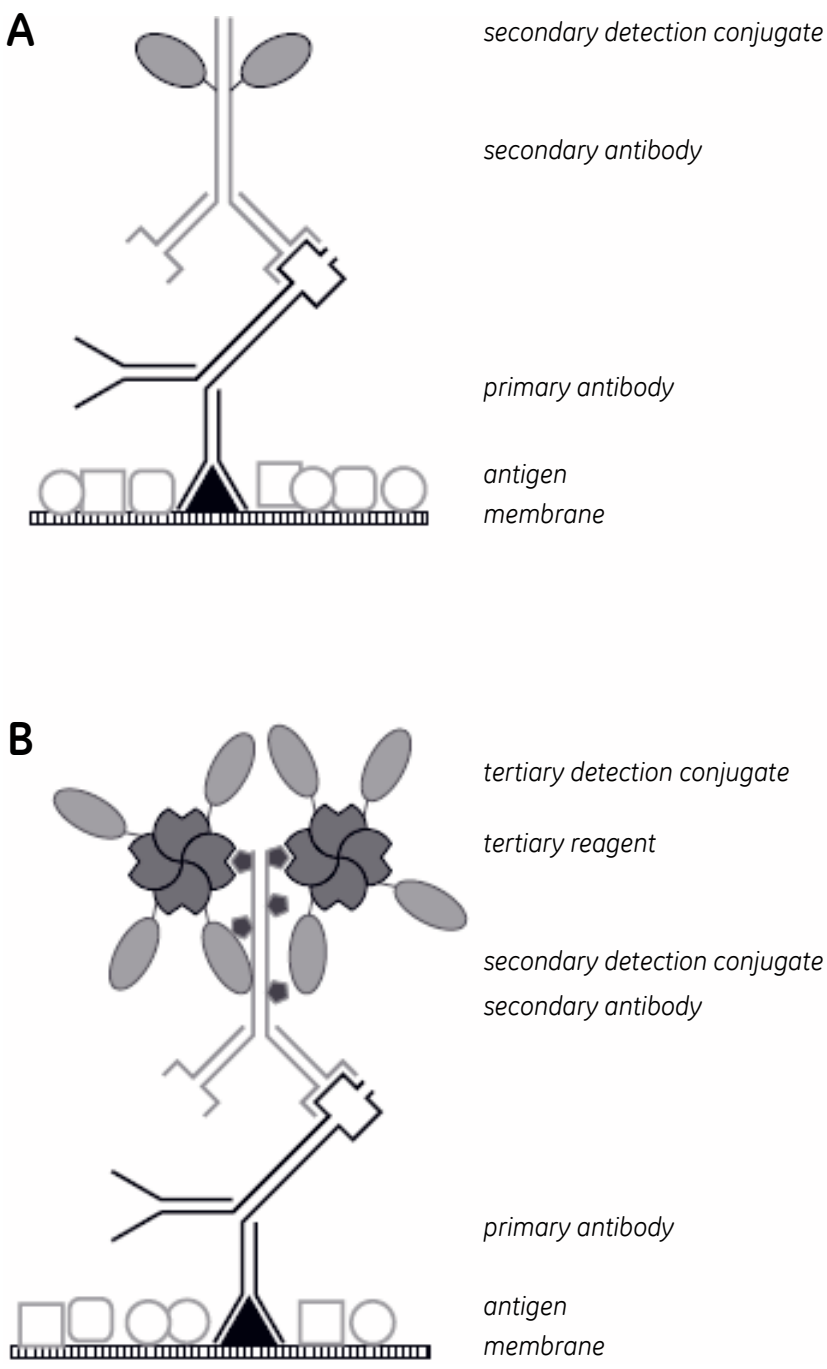


Fig 1-1. Indirect detection of blotted antigens.
A. Basic detection. A detection molecule is directly conjugated to a species-specific secondary antibody, or Protein A or G. Detection reporter systems include enzymes (horseradish peroxidase, alkaline phosphatase), fluorophores, colloidal gold, and radioisotopes.
B. Enhanced detection. A small ligand or antigen, such as biotin, is conjugated to the species-specific secondary antibody. The tertiary detection system, consisting of a binding component and a detection component, binds specifically to the ligand at multiple sites on the secondary antibody. Reporter systems are as described in A.

1.2 The steps of automated immunoblotting

The Processor Plus is designed to automate the reagent changes and incubation steps of immunoblotting techniques. Although numerous variant methods have been published, a common series of steps are involved in any immunoblotting procedure:

1 Block excess protein binding sites on the membrane after transfer

2 Wash away unbound blocking reagent (optional)

3 Treat with primary antibody

4 Wash away excess primary antibody

5 Treat with secondary reagent, often an antibody

6 Wash away excess secondary antibody

7 Detect bound secondary antibody.

All of the steps leading to detection can be carried out in the Processor Plus. The user chooses a detection method programme (see discussion below), provides the required solutions, and starts the instrument. Since the length of the last visualization step may be critical and not easily predictable, the instrument offers the choice of automated or manual control of that step.

Two detection methods most often used are chemiluminescent, producing light at the site of the target which can be detected by exposing the membrane to photographic film, and chromogenic, producing a colored precipitate on the membrane at the site of the target protein. The Processor Plus menu includes preprogrammed protocols for use with ECL™ and ECL Plus™ chemiluminescent detection systems as well as “general” programmes that can be modified for specific applications.

Converting an existing manual protocol to an automated protocol may require some adjustments to step times, number of washes, and/or reagent concentrations. See Section 3 for conversion suggestions.

Note: This instrument is not suitable for use with radioisotopes.

The basic steps of immunoblotting are summarized below:

Transfer of proteins from gel to membrane

After separation of the protein sample on a polyacrylamide gel, the proteins are transferred to a membrane in a pattern replicating the separation seen on the gel. There are several advantages to working with a membrane instead of the original gel. These include:

- Small amounts of samples are more readily detected because they are easily accessible to antibodies and concentrated on the surface of the membrane, rather than spread throughout the thickness of the gel.
- A membrane is easier to handle than a gel.
- Membrane staining and destaining is faster.
- The blot can be a convenient permanent record of the gel.

Membranes

Nitrocellulose and polyvinylidene difluoride (PVDF) membranes are the most commonly-used membranes for Western blotting. Nitrocellulose membranes give good sensitivity, resolution and low background, but they tend to be brittle and can require extra care in handling. Supported nitrocellulose improves the mechanical strength by incorporating a polyester support web. PVDF membranes offer the advantages of high protein binding capacity, physical strength and chemical stability, and are often the membrane of choice for immunoblotting. Nylon membranes can be used for protein blotting but often exhibit unacceptably high background (due to high protein binding capacity). Some processing kits may recommend a particular membrane for optimum signal. ECL Plus detection system recommends using Hybond-P PVDF membranes for the stronger signal generated, compared to nitrocellulose.

Transfer methods

Proteins can be transferred onto membranes by a variety of methods, ranging from capillary and diffusion blotting to various electrophoretic transfer techniques. Electrophoretic transfer is by far the most widely used because of its speed and precision. In “tank” electrophoretic transfer, as in the TE 62 Transphor II Cooled Transfer Electrophoresis Unit, the gel and the membrane are mounted in a cassette and suspended vertically in a buffer-filled tank between electrode panels. Commonly, a Tris-glycine buffer is used, containing up to 20% methanol to increase the binding capacity of the blotting membrane and up to 0.1% SDS to improve transfer, particularly of larger proteins. The electrodes are parallel to the plane of the gel. When power is applied, the electrodes should create a uniform voltage field over the whole surface of the gel. Proteins migrate electrophoretically from the gel to the membrane.

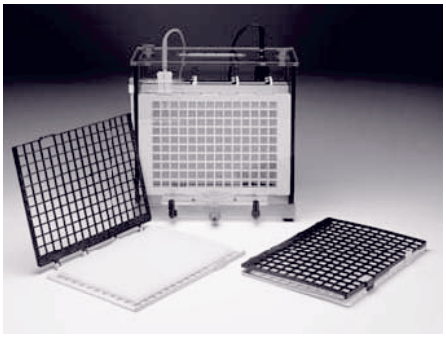


Fig 1-2. Transphor Tank with built-in heat exchanger, electrode panels and gel cassettes.

“Semi-dry” electrophoretic transfer, such as with a TE 70, requires very little transfer buffer and low voltages. The gel and membrane are saturated with transfer buffer and are stacked together horizontally between buffer-saturated blotter paper, then sandwiched between solid or perforated planar electrodes. The electrodes are separated only by the thickness of the stack, creating a uniform, high field strength for rapid transfer at relatively low voltages. When longer transfer times are required, tank transfer is preferable because of its greater buffering capacity and temperature control capability.

Slot or dot blots, a rapid variation of immunoblots, are produced by direct application of proteins to the membrane, without prior separation of the sample by electrophoresis. Typically the protein samples are applied to the membrane in a multi-slot/dot vacuum filtration apparatus. Dot or slot blots are used for quick screening of multiple samples, confirmation that a sample treatment does not alter the affinity of the antibody for the antigen, and optimization of the processing conditions (dilution of the antibodies, for example). Serial dilutions of the

protein or protein mixture to be analyzed can be loaded into various lanes of the slot blot apparatus and processed with various dilutions of both the primary and secondary antibody.

Fig 1-3. Slot Blot Manifold with 48 sample filtration slots.

Confirmation of transfer

Before continuing with blot processing, it is advisable to confirm that the transfer of proteins from the gel to the membrane was complete, especially when starting work with a new sample or procedure. Factors affecting transfer include the concentration of acrylamide in the separating gel, the type of gel (denaturing vs. non-denaturing), gel thickness, the electrical parameters used for transfer, the transfer buffer used, the length of time of transfer and the efficiency of binding of the protein to the membrane during transfer. In addition, the type of equipment used for transfer may affect the results. Follow the recommendations given by the instrument manufacturer and optimize transfer protocols before proceeding with blot processing.

Prestained markers

A rapid method of confirming transfer is to include one or more lanes of pre-stained protein molecular weight markers (such as Rainbow™ Markers) on the separating gel. The individual colored bands are visible during the separation and on the blot membrane to provide a simple visual check of the efficiency of transfer. In addition, Rainbow Markers identify the side of the membrane to which the proteins are bound (the side that should be subsequently exposed to the antibody solution).

Blot staining

To confirm that transfer of sample proteins to the membrane occurred, a total protein stain such as Ponceau S is most commonly used. It is rapid, reversible, and does not interfere with subsequent immunodetection. Membranes are stained for approximately five minutes in an acidic solution of Ponceau S, then rinsed with several changes of water or buffer to visualize the proteins. This provides a rapid transfer check, as well as a way to detect and mark unstained molecular weight standards. With further rinses, the proteins will destain entirely and the membrane will be ready for processing. One disadvantage of the Ponceau S method is its low sensitivity; only relatively high amounts of proteins (> 1 µg) can be detected. Permanent total protein stains include amido black, India ink, and colloidal gold.

Gel staining

Staining the acrylamide gel after transfer confirms the complete migration of the proteins out of the gel. Coomassie blue is the most commonly used protein stain. As long as the blot has protein (as detected above), proceed with membrane processing without waiting for the results from staining the gel.

Blocking

After transfer of the proteins from the gel to the membrane, the remaining protein-binding sites on the membrane must be blocked to avoid non-specific binding of the antibodies or detection reagents in subsequent steps. A number of different blocking reagents are available, including detergent Tween 20, non-fat dry milk, caseins, bovine serum albumin (BSA), ovalbumin, gelatins and various types of serum. The two blocking solutions compatible with nearly all immunodetection systems are non-fat dried milk and BSA. Blocking in Tween 20 is also recommended if high backgrounds are a problem. Table 1-1ists several commonly used blocking reagents used in immunoblotting. The blocking agent selected should be tested to confirm that it does not react with primary or secondary antibodies or other reagents used in processing (it should be free of endogenous phosphatases or peroxidases if those enzymes are used for detection).

Table 1-1. Common membrane blocking reagents

Reagent	Formulation	Comments
Dried milk	5% non-fat dried milk in PBS or TBS	Masks some antigens
Milk/Tween-20	5% non-fat dried milk in PBS or TBS, 0.1% Tween 20	Masks some antigens
Tween-20	0.1% Tween 20, 0.02% NaN3 in PBS or TBS	Can stain after detection
BSA	0.3–3% bovine serum albumin, 0.02% NaN3 in PBS	Lower endogenous cross-reactivity

All processing buffers should be prepared as close to the time of use as possible to reduce opportunities for microbial contamination. Phosphate-buffered saline (PBS) and Tris-buffered saline (TBS) are primarily used for blot processing. In cases where alkaline phosphatase is used in the detection process, Tris-buffered saline is preferred to minimize the phosphate concentration.

Wash

Because immunoblotting involves several successive binding steps, it is essential that the processing minimize any non-specific binding likely to increase the background signal. A thorough removal of the unbound reagents is required after each of the binding steps in the procedure. This is done by performing series of washes. The Processor Plus is normally programmed for several washes between each binding step.

Note: When implementing a particular blot processing method on the Processor Plus, alteration of the length and number of some of the steps may be required. Frequently the number and/or length of wash steps may need to be increased or decreased to obtain the best results. (See Section 3 for more detail on conversion of manual protocols.)

Primary antibody

The primary antibody recognizes and binds to the target antigen bound to the membrane. For the best results the appropriate dilution of the antibody preparation must be determined empirically for each new antibody lot used. Concentrations of antibody and other reagents used in processing must be optimized to achieve high specificity for the protein(s) of interest and a low non-specific background. With the development of biotinylated enzymes, streptavidin/avidin enzyme conjugates and chemiluminescent detection, very high dilutions of primary antibodies can be used to achieve highly sensitive detection of proteins.

Secondary reagents for detection of the primary antibody-antigen complex

To detect the bound primary antibody, numerous techniques are available. Protein A and protein G, both bacterial wall components, exhibit strong affinity for the Fc domain of antibodies. This, combined with the fact that they can be labelled with enzymes, gold or radio-iodine, has made them useful secondary reagents for immunochemical detection. However, the most common primary antibody detection system is a secondary antibody. Secondary antibodies are typically commercially prepared, specific to the species in which the original primary antibody was prepared (for instance, a goat anti-rabbit secondary for detection of a rabbit primary antibody). The secondary antibody recognizes a general class of antibodies (such as all mouse or all rabbit IgGs). Secondary antibodies bind to the blot only by binding to the primary antibody.

Five general types of labels can be linked to the secondary antibody: radiochemicals, fluorescent compounds, colloidal gold, enzymes, or biotin. Most commonly, the antibody has a reporter enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) directly conjugated to it. If the secondary antibody is biotinylated, biotin-avidin-HRP or -AP complexes can be formed. Addition of a suitable enzyme substrate results in production of a colored precipitate or fluorescent or chemiluminescent product through oxidation (HRP) or dephosphorylation (AP) at the site of the secondary antibody-enzyme complex.

Fluorescent compounds or gold particles and also be linked to the secondary antibody. Gold-labeled antibodies produce a pink coloration at the site of binding which can further be intensified by a silver enhancement process.

Detection method

Table 1-2summarizes some substrates available for chromogenic and chemiluminescent detection methods.

Table 1-2. Commonly used chromogenic and chemiluminescent detection systems

System	Substrate[1]	Product
Chromogenic		
HRP	DAB	brown precipitate, addition of Ni or Co ions increases sensitivity
	TMB	blue precipitate
	4CN	blue/black precipitate
AP	BCIP/NBT	black/purple precipitate
Chemiluminescent (ECL, ECL Plus)		
HRP	Luminol	light, glow or flash
AP	Dioxetane-phosphate	light, glow

[1] Abbreviations:
DAB: 3,3'-diaminobenzidine
4CN: 4-chloro-1-naphtol
BCIP: 5-bromo-4-chloro-3-indolyl phosphate
NBT: nitroblue tetrazolium
TMB: 3,3'5,5' tetramethylbenzidine

The choice of detection method usually depends on experimental conditions and the expected abundance of the antigen. If the target protein is relatively abundant, an enzyme-linked secondary antibody with appropriate chromogenic substrate may be sufficient. Chromogenic detection is simple to perform, however, many of the chemicals used are hazardous and the colored precipitates have a tendency to fade over time. Chemiluminescent detection (ECL system) offers fast process times and permits multiple film exposure times from the blot, which increases the sensitivity of detection and the range of reliable quantitation. Additionally, unlike chromogenic detection methods, blots detected with chemiluminescent systems can be easily stripped and re-tested for a second antigen.

If higher sensitivity is required, biotinylated secondary antibodies and an avidin or streptavidin-enzyme conjugate with a ECL Plus chemiluminescent detection system can be used. Sensitivity of such tertiary systems can approach that obtained with radiolabelled antibodies. Secondary antibody/AP- or HRP-conjugates usually have one molecule of enzyme per molecule of antibody. However, antibodies can be multiply-labelled with biotin and thus bind multiple avidin- or streptavidin-enzyme conjugates, each with multiple AP or HRP molecules. Both chromogenic and chemiluminescent detection sensitivities are enhanced by this method.

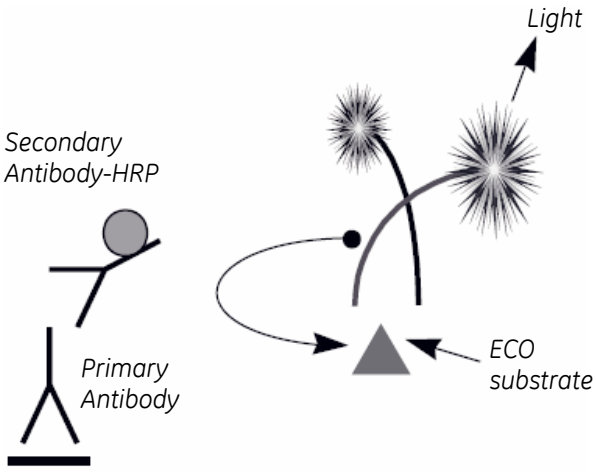


Fig 1-4. ECL detection: Chemiluminescent substrate emits visible light upon enzyme induced decomposition. ECL is based on the oxidation of the cyclic diacylhydrazide, luminol.

2 Processing protocols

The Processor Plus has 10 programmes available for automating blot processing. Two of these are pre-programmed protocols for ECL and ECL Plus Western Blotting Systems. Two additional programs are generic outlines intended to be adapted for other protocols using a secondary antibody-enzyme conjugate or tertiary detection systems. The pre-programmed protocols assume that the various reagents used are delivered through the ports listed in Table 2-1. These reagent line assignments are standardized among the pre-programmed protocols to avoid cross-contamination or cross-reactions between staining and blotting protocols run on the same Processor Plus unit.

A description of each protocol, applications, and the approximate total processing time are summarized on the next page.

Table 2-1. Default reagent line assignments

Port	Reagent	Description
0	Air vent	
1	Primary (1°) antibody	
2	Secondary (2°) antibody	
4	Enhancement reagent	Avidin or streptavidin conjugate or complex (optional)
5	Blocking solution	Protein and/or detergent solution to block open protein binding sites on membranes
6	Wash buffer	Tris-buffered saline plus Tween 20 (TBST) or phosphate-buffered saline plus Tween 20 (PBST)

2.1 Automated ECL Plus western blot detection

Blot Protocol 1 is programmed for the ECL Plus Western Blotting system detection protocol. It includes 24 steps to perform the following functions:

1 Block the membrane.

2 Wash away excess blocking agent.

3 Add and incubate with the primary antibody.

4 Wash away excess primary antibody.

5 Add and incubate with a biotinylated secondary antibody.

6 Wash away excess secondary antibody.

7 Add and incubate with a streptavidin-HRP conjugate.

8 Wash.

Because of the high sensitivity of the ECL Plus Detection System, numerous washes are programmed into this protocol to assure complete removal of antibodies and reagents between steps. Following the final wash, the membranes are treated with the ECL Plus detection reagents manually. This can be done either in the instrument while the tray rests in the level position, or outside the instrument on a sheet of plastic wrap. After wetting the membrane thoroughly and evenly with the detection reagents, proceed with exposure to film.

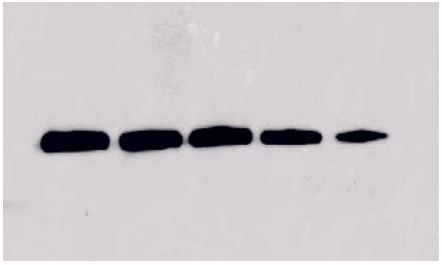


Fig 2-1. Dilutions of an E. coli extract were analysed for GroEL with ECL Plus detection. After separation on 12% SDS acrylamide gel, proteins were transferred onto Hybond™-P membrane in a TE 22 Tank Transfer Unit. Amounts of extract loaded range from 5.8 to 1.15 ìg of total protein. Processor Plus blot protocol #1 was used with rabbit anti-GroEL antibody, Biotinylated Goat Anti-Rabbit IgG (H+L), Streptavidin-Alkaline Phosphatase Conjugate (RPN1234),

Protocol 1: ECL Plus

Step	Reagent	In-Port	Multiplier	Time (min)
1	Block	5	2	60
2	Wash	6	3	2
3	Wash	6	3	2
4	1° antibody	1	1	60
5–6	Wash	6	2	1
7	Wash	6	3	15
8–10	Wash	6	3	5
11	Biotinylated 2° antibody	2	1	60
12–13	Wash	6	2	1
14	Wash	6	3	15
15–17	Wash	6	3	5
18	Streptavidin HRP	4	1	60
19 –20	Wash	6	2	1
21	Wash	6	3	15
22–24	Wash	6	3	5
	Total time (approx.)			**340**

2.2 Automated ECL Western blot detection

Protocol 2 is programmed for the ECL Protein Detection System. It includes 18 steps, to perform the following functions:

1 Block the membrane.

2 Wash away excess blocking agent.

3 Add and incubate with the primary antibody.

4 Wash away excess antibody.

5 Add and incubate with a biotinylated secondary antibody-HRP conjugate.

6 Wash away excess antibody.

Following the final wash, the membranes are treated with the ECL detection reagents manually. This can be done either in the instrument while the tray rests in the level position, or outside the instrument on a sheet of plastic wrap.

Protocol 2: ECL

Step	Reagent	In-Port	Multiplier	Time (min)
1	Block	5	2	60
2	Wash	6	3	15
3– 4	Wash	6	3	5
5	1° antibody	1	1	60
6	Wash	6	3	15
7–8	Wash	6	3	5
9	Biotinylated 2° ab or 2° ab HRP conjugate	2	1	60
10	Wash	6	3	15
11–12	Wash	6	3	5
13	Enhancement (optional)	4	1	60
14	Wash	6	3	15
15 –18	Wash	6	3	5
	Total time (approx.)			**340**

2.3 Automated immunodetection with primary and secondary antibodies

This is a generalized protocol for use with any system that requires two antibody incubation steps. This protocol is referred to as “standard” in the Processor Plus menu. It consists of 9 steps with the following functions:

1 Block the membrane.

2 Add and incubate with the primary antibody.

3 Wash away excess antibody.

4 Add and incubate with secondary antibody-enzyme conjugate.

5 Wash away excess antibody.

Following the final wash, the membrane is ready for addition of chromogenic or chemiluminescent detection reagents. Since many chromogens form insoluble precipitates that may stain the processor trays, the membranes may be transferred to other containers for development. Excessively stained processor trays should be discarded.

Protocol 3: Standard

Step	Reagent	In-Port	Multiplier	Time (min)
1	Block	5	2	60
2	1° antibody	1	1	60
3–5	Wash	6	3	5
6	2° ab conjugate	2	1	60
7–9	Wash	6	3	5
	Total time (approx.)			**210**

2.4 Automated immunodetection with tertiary complexes

Protocol 4 is programmed for a general protocol that uses a tertiary avidin or streptavidin-enzyme conjugate for enhanced sensitivity. It is referred to as “enhanced” in the Processor Plus menu. It includes 13 steps to perform the following functions:

1 Block the membrane.

2 Add and incubate with the primary antibody.

3 Wash away excess antibody.

4 Add and incubate with a biotinylated secondary antibody.

5 Wash away excess antibody.

6 Add and incubate with an avidin or streptavidin-enzyme conjugate.

7 Wash.

Following the final wash, the membrane is ready for addition of chromogenic or chemiluminescent detection reagents. Since many chromogens form insoluble precipitates that may stain the processor trays, the membranes may be transferred to other containers for development. Excessively stained processor trays should be discarded.

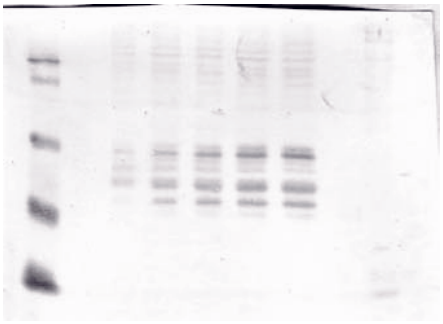


Fig 2-2. SDS acrylamide gel separations of Chinese Spring wheat seed gliadin proteins. Gel was blotted onto Hybond-C pure nitrocellulose membrane using the miniVE Electrophoresis Unit, then developed with Processor Plus blot processing protocol #4 (primary antibody: rabbit anti-gliadin; detection with biotinylated goat anti-rabbit IgG (H+L) antibody, streptavidin-alkaline phosphatase conjugate, BCIP/NBT substrate).

Protocol 4: Enhanced

Step	Reagent	In-Port	Multiplier	Time (min)
1	Block	5	2	60
2	1° antibody	1	1	60
3–5	Wash	6	3	5
6	Biotinylated 2° ab	2	1	60
7–9	Wash	6	3	5
10	A conjugate or complex of: streptavidin HRP or AP, or avidin HRP or AP	4	1	60
11–13	Wash	6	3	5
	Total time (approx.)			**285**

2.5 Instrument wash

Protocol 5 is a cleaning protocol that washes all reagent lines with water or a cleaning solution. A 5% sodium hypochlorite (bleach) or dilute detergent solution can be used to help remove residual proteins between processor runs. One or two cleaning cycles with water should be used to wash out cleaning solutions. Do not use alcohol, strong acids or strong base solutions to clean the unit.

All 9 drain tubes are placed into container of a cleaning or rinse solution. The programme draws solution through each reagent line in succession— 1, 2, 3, 4, 5, 6, 7, 8, and 9—and empties the tray through the drain tube. Line 0, the air vent, is not washed.

3 Automating manual processing protocols

For best results, manual or automated membrane processing protocols should be optimized for the desired balance of positive signal and minimal background. Lot-to-lot differences in immuno-reagents, antigens, membranes and other materials may affect reproducibility and should be controlled appropriately. In converting from a manual to an automated protocol, the following points address the most common causes of variations in the results.

1 Primary antibody incubation

If antibody concentrations and volumes per cm^2 are held constant in the conversion, incubation times should remain approximately the same as used for manual methods. However, if the primary antibody is diluted further to reach the required minimum volume for automated processing, extending the incubation times should restore any decrease in sensitivity. Alternatively, decrease subsequent wash times, as long as background does not increase, or use a more sensitive method of detection such as ECL Plus or an avidin-biotin-enzyme conjugate to enhance the signal.

2 Washes

Washes should initially be performed over the same times and using the same volume as manual. If a volume of wash is required that exceeds the processor chamber volume capacity, add more wash steps so that the same total volume is used. Alternatively, try longer wash steps if background is the problem, or shorter wash steps if sensitivity is the problem.

3 Minimum volume

In order to have sufficient fluid coverage across the membrane to allow rocking, a minimum of 10 ml must be used for each of the mini tray chambers and 25 ml for the standard tray chambers. For smaller volumes, the Processor Plus should be programmed to stop and Hold, which leaves the tray in a level position. The required volume of antibody is manually pipetted onto the membrane surface and the incubation done with the tray remaining level. In some cases, the lack of agitation may lead to reduced sensitivity.

4 Temperature

Antibody/antigen binding and detection enzyme kinetics are affected by the temperature. If lower or higher temperatures are required, the Processor Plus may be operated in a cold room or warm room/incubator within the operating limits specified for the instrument. If a reagent must be kept cool prior to use, solutions may be placed on ice or in a thermostatted bath adjacent to the instrument.

Note: The pump must be recalibrated for operation at each temperature.

4 Troubleshooting tips

Table 4-1includes some common problems encountered with the blot processing procedure.

Table 4-1. Immunoblotting troubleshooting tips

Symptom	Possible cause	Recommended action
Weak or no signal	Insufficient protein loaded on the gel.	Load more protein.
	Antibody concentration too low.	If commercially prepared, follow manufacturer's suggested dilutions, titer up and down from starting point. Concentration and titer of antibodies are both important.
	Detection reagents degraded or not properly stored.	Prepare fresh reagents.
	Membrane degraded.	Keep nitrocellulose in a closed bag away from heat, light and moisture. It will deteriorate if not stored properly. Check membrane wetting. Nitrocellulose should quickly absorb water. PVDF should be wet in methanol prior to equilibration in transfer buffer.
	Excessive washing.	Reduce number or duration of wash steps. Reduce concentration of detergents, such as Tween 20, if used.
Excessive signal	Too much protein loaded on the gel.	Load less protein.
	Antibody concentrations too high.	Try lower concentrations of antibody.
	Inadequate washing.	Increase the number of washes or extend the wash times.
Non-specific binding	Impure (mixed) antibodies or other proteins binding to other targets on membrane.	Try lower concentrations of antibody or further purify antibody.
Uneven, splotchy background	Membrane not fully or properly rehydrated.	Pre-wet membranes prior to incubation. Wet nitrocellulose membranes in water, then equilibrate with wash buffer. Wet PVDF first in methanol, then equilibrate with wash buffer.
	Fingerprints, marks or damage to the membrane can cause non-specific binding artifacts.	Wear gloves and use forceps when handling membranes. Membranes are very fragile. Avoid tearing, folding or creasing membranes to minimize processing artifacts.
	Areas of the blot were not fully coated with blocking agent or the membrane partially dried during incubation.	Ensure proper volumes used for each tray position. Ensure reagent lines are secured into reagent vessels and that the opaque reagent tubing reaches to the bottom of the container.

Symptom	Possible cause	Recommended action
High background	Inadequate blocking.	Blocking agent contaminated or too dilute.
	Inadequate washing between antibody steps.	Extend the time, volume or number of washes.
	Choice of membrane.	Avoid using nylon membranes.
	Contaminated buffers.	Prepare fresh buffers.
	Cross-reactivity with blocking reagents.	Try a different blocking agent, such as 0.1% Tween 20, 3% BSA, 5% non-fat dry milk, 1–2% casein.

(See other references for problems with gel electrophoresis and transfer protocols)

5 Useful references

Towbin, H. et al., *Proc. Natl. Acad. Sci. 76*, 4350-4354 (1979).

Harlow, E. and Lane, D. *Immunoblotting. In Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, pp. 471–510 (1988).

Beisiegel, U., *Electrophoresis 7*, 1–18 (1986)

Bjerrum, O.J. et al., *CRC Handbook of Immunoblotting of Proteins*, **Vol. 1**, pp. 227–254 (1988).

Dunbar, B. ed., *Protein Blotting: A Practical Approach*, IRL/Oxford University Press (1994).

Coligan J. E. et al., eds., *Current Protocols in Immunology*, John Wiley & Sons, Inc. (1994).

6 Ordering information

See www.gelifesciences.com for additional ordering information for membranes, stains, film, detection chemicals, and instruments.

Processor Plus—Blot processing system	Code no.
Processor Plus Base unit, reagent tubing, protocol key	80-6444-04
Blot Processing Tray Pack Complete with tray base, disposable mini and standard tray, lid, reagent bottles and rack	80-6444-23

Accessories for blot processing	Code no.
Blot Processing Mini Tray Disposable mini trays (3/pk)	80-6444-42
Blot Processing Standard Tray Disposable standard tray	80-6444-61

ECL Western blotting reagents	Code no
For 1,000 cm^2 membrane	RPN2109
For 2,000 cm^2 membrane	RPN2209
For 4,000 cm^2 membrane	RPN2106
For 6,000 cm^2 membrane	RPN2134
ECL Western Blotting Analysis System	RPN2108

ECL Plus Western blotting reagents	Code no
For 1,000 cm^2 membrane	RPN2132
For 3,000 cm^2 membrane	RPN2133

Rainbow Molecular Weight Markers	Code no
Full-Range Rainbow Molecular Weight Markers	RPN800E
High-Range Rainbow Molecular Weight Markers	RPN756E
Low-Range Rainbow Molecular Weight Markers	RPN755E

Nitrocellulose membranes	Quantity	Code no
Hybond-ECL (6 × 8 cm sheets)	50 sheets	RPN68D
Hybond-ECL (20 × 20 cm)	10 sheets	RPN2020D
Hybond-ECL (30 cm × 3 m)	1 roll	RPN303D

PVDF (polyvinylidene difluoride) membranes	Quantity	Code no
Hybond-P (20 × 20 cm)	10 sheets	RPN2020F
Hybond-P (30 cm × 3 m)	1 roll	RPN303F

For local office contact information, visit
www.gelifesciences.com/contact

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare UK Limited
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327, USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1, Hyakunincho Shinjuku-ku, Tokyo 169-0073, Japan

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