Automated Colorimetric Immunodetection on Western Blots

Application Note #1
Hoefer® Automated Gel Stainer

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Introduction

Immunoblotting or western blotting is a powerful technique for the detection, quantification and characterization of individual proteins in complex mixtures. This technique involves: 1) electrophoretically separating the protein mixture on a polyacrylamide gel, 2) transferring (blotting) the separated proteins onto a solid support such as nitrocellulose membrane, 3) probing the resulting blot with an antibody specific to the protein of interest, and 4) developing the blot by treatment with reagents which allow visualization of zones to which antibody has bound. This last step involves exposing the blot to a series of reagents, and since it is normally performed manually, can be quite time-consuming. A version of this procedure has been developed for use in the Hoefer Automated Gel Stainer. An additional benefit of automation is enhanced reproducibility due to precise control of the timing of the development steps.

The following procedure is an adaptation of a widely utilized technique that uses an alkaline phosphatase-conjugated secondary detection reagent and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in the developing solution [1]. To limit the volume of primary antibody used, probing with the primary antibody is performed manually. Subsequent steps are performed in the Hoefer Automated Gel Stainer. In the case described here, *E. coli* extract was probed with a polyclonal antibody raised against GroEL. The concentration of the antibody and the time of development were optimized for this combination. Detection of other proteins with other antibodies will require independent optimization of these parameters.

Results

As indicated in Figure 1, results obtained using the Hoefer Automated Gel Stainer for colorimetric immunodetection on a western blot are equivalent to results obtained by processing the same blot manually. Some variations of the basic protocol described here were tested as well. There was little difference in the quality of the results when BSA Blocking Solution replaced Non-fat Dry Milk Blocking Solution, or when Protein A-alkaline phosphatase conjugate replaced goat anti-rabbit IgG alkaline phosphatase conjugate (data not shown).

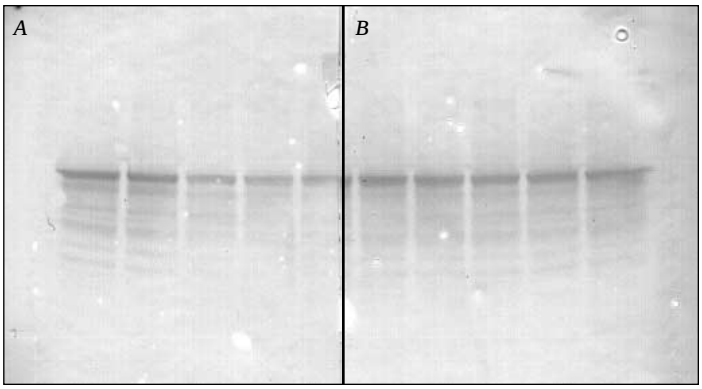

Figure 1 Comparison of manual and automated blot processing. A blot containing several identical samples of E. Coli extract was probed with a 1:10,000 dilution of rabbit anti-GroEL. The left half (A) was processed manually according to the protocol described in "Methods" and the right half (B) was processed in the Hoefer Automated Gel Stainer according to the identical protocol.

Methods and Materials

Sample Preparation

E. coli strain B extract was prepared by suspending 4 mg of lyophilized cells in 1 ml of 1X Laemmli sample buffer [2]. The extract was heated at 95 °C for 3 min, vortexed for 1 min, heated at 95 °C for an additional 3 min and centrifuged at 12,000 x g for 10 min. The supernatant was stored at -40 °C.

Electrophoresis and Electrotransfer

Samples of *E. coli* extract (10 µl) were run on standard 1 mm thick 12%T, 2.6%C Laemmli SDS-polyacrylamide gels in the Hoefer SE 260 Mighty Small™ II Mini Vertical Unit at 25 mA per gel. Transfers were carried out in the Hoefer TE 22 Mighty Small TransPhor™ Tank Transfer Unit as described in [2]. Amersham Biosciences 9 x 10.5 cm

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sheets of blotter paper (80-6205-40), and 9 x 10.5 cm nitrocellulose transfer membranes (80-6221-17) were used for all transfers. Ponceau S staining was used to verify electrophoresis and transfer [3]. The blots were air-dried and stored in a dark drawer at room temperature.

Reagents

Electrophoresis chemicals used (Tris (17-1321-01), glycine (17-1323-01), SDS (17-1313-01), acrylamide PAGE (17-1302-02), *N,N′*-methylenebisacrylamide (17-1304-02) and Tween 20 (17-1316-01)) were Amersham Biosciences PlusOne™. The non-fat dry milk was from Carnation. Sodium azide, bovine serum albumin (BSA, catalog #A-3912), Ponceau S, NaCl, $MgCl_2$, dimethyl formamide, NBT (catalog # N-6876), BCIP (p-toluidine salt, catalog #B-8503) were purchased from Sigma.

The primary antiserum used was rabbit anti-GroEL, IgG fraction from Sigma (catalog # G-6532). Secondary reagent was anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate from Sigma (catalog # A-3687) or protein A-alkaline phosphatase conjugate (catalog #P-9650) from Sigma.

Solutions

10X Tris-Buffered Saline (TBS)
(1.5 M sodium chloride, 500 mM Tris-HCl, pH 7.5, 1 liter)

NaCl (FW 58.44)	1.5 M	87.66 g
Tris (FW 121.1)	500 mM	60.55 g
Concentrated HCl		to pH 7.5
Distilled H_2O		to 1000 ml

Store at room temperature.

1X Tris-Buffered Saline (TBS)
(150 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, 4 liters)

10X TBS	400 ml
Distilled H_2O	3600 ml

Store at room temperature.

Non-Fat Dry Milk Blocking Solution
(5% non-fat dry milk, 0.02% sodium azide in 1X TBS, 1 liter)

Non-fat dry milk	50 g
NaN_3	0.2 g
10X TBS	100 ml
Distilled H_2O	to 1000 ml

Store at 4 °C.

BSA Blocking Solution
(1% bovine serum albumin, 0.02% sodium azide in 1X TBS, 1 liter)

Bovine serum albumin (BSA)	10 g
NaN_3	0.2 g
10X TBS	100 ml
Distilled H_2O	to 1000 ml

Store at 4 °C.

Primary Antibody Solution
(1:10,000 dilution of rabbit anti-GroEL plus 0.02% Tween 20 in Blocking Solution, 25 ml)

Rabbit anti-GroEL (Sigma G-6532)	2.5 µl
Tween 20	5 µl
Blocking Solution (either non-fat dry milk or BSA)	25 ml

Make fresh each time used.

Secondary Solution
(1:10,000 dilution of goat anti-rabbit alkaline phosphatase conjugate in Blocking Solution
or
2 µg/ml protein A-alkaline phosphatase conjugate in Blocking Solution, 125 ml)

Goat anti-rabbit alkaline phosphatase	12.5 µl
Blocking Solution (either non-fat dry milk or BSA)	125 ml
or	
Protein A-alkaline phosphatase conjugate (resuspended to 2 mg/ml)	125 µl
Blocking Solution (either non-fat dry milk or BSA)	125 ml

Store at 4 °C.

5% NBT
(5% nitro blue tetrazolium chloride in 70% dimethyl formamide, 2 ml)

Nitro blue tetrazolium chloride (NBT)	100 mg
Dimethyl formamide (DMF)	1.4 ml
Distilled H_2O	600 µl

Store at -20 °C.

5% BCIP
(5% 5-bromo-4-chloro-3-indolyl phosphate in dimethyl formamide, 2 ml)

5-Bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP)	100 mg
Dimethyl formamide (DMF)	2 ml

Store at -20 °C.

Alkaline Phosphatase Buffer
(100 mM sodium chloride, 100 mM Tris-HCl, 5 mM magnesium chloride, pH 9.5, 1 liter)

NaCl (FW 58.44)	100 mM		5.84 g
Tris (FW 121.1)	100 mM		12.11 g
$MgCl_2 \cdot 6H_2O$ (FW 203.30)	5 mM		1.02 g
Concentrated HCl		to	pH 9.5
Distilled H_2O		to	1000 ml

Store at room temperature.

Developer
(0.033% nitro blue tetrazolium chloride, 0.017% 5-bromo-4-chloro-3-indolyl phosphate in Alkaline Phosphatase Buffer, 125 ml)

5% NBT	825 µl
5% BCIP	413 µl
Alkaline Phosphatase Buffer	125 ml

Prepare just before use. Do not store. This solution should be handled in an opaque bottle as it is light sensitive.

Stop Solution
(2 mM disodium EDTA in 1X TBS, 1 liter)

Na_2EDTA (FW 372.2)	2 mM	744 mg
1X TBS		1000 ml

Store at room temperature.

Blot Processing

The blot is placed in enough Blocking Solution to fully submerge the blot and incubated for thirty minutes at room temperature in with gentle shaking on a Hoefer PR70 Red Rotor Orbital Shaker. It is then incubated in primary solution at 4°C for two hours with gentle shaking on a on a Hoefer PR70 Red Rotor Orbital Shaker (this step has been extended to overnight without any detrimental effects). A Hoefer PR150 Mighty Small Deca-Probe Incubation Manifold is useful when testing multiple antibodies, or multiple dilutions of antibody on the same blot. Subsequent steps are performed at room temperature in the Hoefer Automated Gel Stainer using the solutions listed in Table 1, according to the protocol in Table 2. After processing, the blot is air-dried and stored in the dark at room temperature. In the experiment presented here, Non-Fat Dry Milk Blocking solution and Goat Anti-Rabbit IgG Alkaline Phosphatase Conjugate Secondary Solution were used.

Table 1. Solutions Required for Colorimetric Development of Western Blots in the Hoefer Automated Stainer

Solution	Volume (ml) for 125 ml tray	Volume (ml) for 250 ml tray
1X TBS (Wash)	875	1750
Secondary Solution	125	250
Developer*	125	250
Stop Solution	125	250

*Prepare as close as possible to the time needed in an opaque bottle.

Table 2. Automated Blot Processing Protocol

Step	Solution	IN-port	OUT-port	Time (min)
1	1X TBS	1	9	10
2	1X TBS	1	9	10
3	1X TBS	1	9	10
4	Secondary Solution	2	2*	60
5	1X TBS	1	9	10
6	1X TBS	1	9	10
7	1X TBS	1	9	10
8	1X TBS	1	9	10
9	Developer	3	9	40†
10	Stop Solution	4	9	10

* Note that the in and out port are the same for this step. This allows re-use of the Secondary Solution. This solution can be re-used up to three times.

† The time required for optimal development differs depending on the primary antibody used and the protein that is being analyzed. Optimal development time must be determined on a case-by-case basis by visual monitoring of this step.

Blot Analysis

The blots were scanned in reflectance mode using a Sharp Scanner JX-330. The resultant image was analyzed using ImageMaster VDS software.

References

1. Harlow, E., Lane, D. (1988). in *Antibodies, a Laboratory Manual* pp. 493-505, Cold Spring Harbor Laboratory.
2. *Protein Electrophoresis Applications Guide* (1994) pp. 77-81, Hoefer Scientific Instruments, San Francisco.
3. Coligan, J.E., Dunn, B.M., Ploegh, H.L., Speicher, D.W., Wingfield, P.T. (1995) in *Current Protocols in Protein Science* p. 10.8.3, John Wiley & Sons, Inc.

Ordering Information

Code No.	Item
Equipment:	
80-6149-35	Hoefer SE260 Mighty Small™ II Mini Vertical Unit
80-6204-26	Hoefer TE22 Mighty Small™ Transphor Tank Transfer Unit
80-6087-98	Hoefer PR150 Mighty Small Deca-Probe Incubation Manifold
80-6096-91	Hoefer PR70 Red Rotor Orbital Shaker (115 V)
80-6097-10	Hoefer PR70 Red Rotor Orbital Shaker (230 V)
80-6330-23	Hoefer Automated Gel Stainer (115 V)
80-6330-04	Hoefer Automated Gel Stainer (230 V)
18-1108-33	Sharp Scanner JX-330 (110 V)
18-1108-95	Sharp Scanner JX-330 (220 V)
80-6309-52	ImageMaster VDS software
PlusOne Reagents and consumables:	
80-6205-40	Blotting Paper, 9 X 10.5 cm, 50 sheets
80-6221-17	Nitrocellulose membranes , 9 X 10.5 cm, 0.45 mm, 10 sheets
17-1321-01	Tris, 500 g
17-1323-01	Glycine, 500 g
17-1313-01	SDS, 100 g
17-1302-02	Acrylamide PAGE, 1 kg
17-1304-02	N,Ní-Methylenebisacrylamide, 100 g
17-1316-01	Tween 20, 500 ml
17-1311-01	Ammonium persulfate, 25 g
17-1312-01	TEMED, 25 ml

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