



BI
Biological Industries

EZ-ECL

Chemiluminescence Detection Kit for HRP

Cat. No.: 20-500-120
Store at: 2-8°C

Instructions for Use

Protocol for Western Blotting and
Chemiluminescence

Protocol for Southern/Northern Blotting and
Chemiluminescence Detection

EZ-ECL Troubleshooting Guide For Nucleic
Acid Blots

EZ-ECL Troubleshooting Guide For Protein
Blots

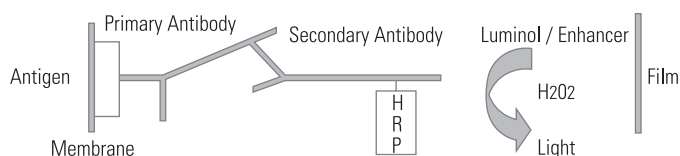
EZ-ECL Protocol-at-a-Glance for Nucleic
Acids

EZ-ECL Protocol-at-a-Glance for Proteins

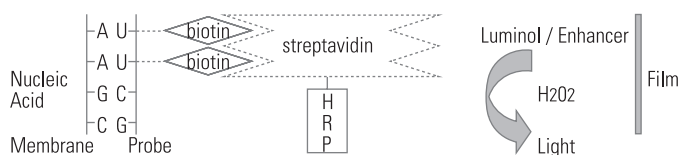
Product Description

EZ-ECL is a complete kit with ready-to-use reagents for chemiluminescent detection of immobilized proteins (Western blotting) or immobilized nucleic acids (Southern or Northern), conjugated with HRP directly or indirectly. The use of enhanced chemiluminescence was introduced by Thorpe and Kricka^[1,2]. In the presence of hydrogen peroxide (H_2O_2), Horseradish peroxidase (HRP) catalyzes the oxidation of cyclic diacylhydrazides, such as luminol. Immediately following the oxidation, the luminol is in an excited state (intermediate reaction product), which decays to the ground state by emitting light. Strong enhancement of the light emission is produced by enhancers, such as phenolic compounds. Using this method, it is possible to detect membrane immobilized specific antigens, or sequences of nucleic acids, labeled directly with HRP or indirectly with HRP-labeled antibodies/streptavidin.

Principles of Protein Detection Procedure



Principles of Nucleic Acid Detection Procedure



Advantages of using the EZ-ECL Detection Kit

- High sensitivity non-radioactive detection system.
- Stable hard copy results on film.
- Only small amounts of antibody required.
- Detection may be achieved in short exposure times (minutes)
- High resolution.

Kit Reagent

Cat. No.:	20-500-120A	EZ-ECL Solution A, 60ml
Contains:	luminol and enhancer	
Store at:	2-8°C	
Cat. No.:	20-500-120B	EZ-ECL Solution B, 60ml
Contains:	stable peroxide solution	
Store at:	2-8°C	

Caution

If above solutions come into contact with eyes or skin, flush with plenty of water and remove contaminated clothing.

1. Preparation of Solutions

1.1 Tris Buffered Saline (TBS)

6.05gr Tris base (50mM)
8.76gr Sodium Chloride (150mM)
Adjust pH to 7.5 with Hydrochloric Acid
Add distilled water up to 1000ml

1.2 phosphate Buffered Saline (PBS) - optional

11.5gr Di-sodium Hydrogen Phosphate, anhydrous (80mM)
2.96gr Sodium Dihydrogen Phosphate (20mM)
5.84gr Sodium Chloride (100mM)
Add distilled water up to 1000ml
Check pH (should be 7.5)

1.3 TBS-Tween (TBS-T) and PBS-Tween (PBS-T)

Dilute 1ml of Tween-20 in 1000ml of buffer (0.1% final concentration).

1.4

A sufficient volume of wash buffer, blocking buffer and antibody solution should be used to cover the blot to ensure that the membrane does not become dry. This will also ensure a reduced non-specific background.

1.5

Do not use sodium azide as a preservative for the secondary antibody dilutions, as azide irreversibly inhibits horseradish peroxidase.

1.6

Wherever use of dried milk is indicated, this can be substituted with low-fat milk.

2. Electrophoresis, Blotting and Membrane Preparation

2.1

Carry out electrophoresis for protein separation. Either non-denaturing gel, SDS-PAGE or two-dimensional gels may be used.

2.2

Transfer proteins from the gel to a membrane. Use nitrocellulose or PVDF membrane. PVDF membranes must be wetted briefly in methanol then soaked in distilled water for 1-3 minutes, followed by equilibration in transfer buffer.

2.3 Membrane Blocking

Block non-specific binding sites by incubating the membrane for 1 hour at room temperature with shaking in TBS-T or PBS-T solution containing 5% dried milk (w/v). This step can be performed overnight at 4°C without shaking.

2.4 Primary Antibody

Dilute the primary antibody in TBS-T or PBS-T with 2% dried milk (w/v). Incubate the membrane in the solution for 1 hour at room temperature with shaking, or overnight at 4°C without shaking.

2.5 Membrane Washing

Wash the membrane three times in TBS-T or PBS-T for 10 minutes each. Use at least 50ml of buffer for 10x10cm membrane.

2.6 Secondary Antibody

Dilute the HRP-labeled secondary antibody in TBS-T or PBS-T with 2% dried milk (w/v). Incubate the membrane in the solution for 1 hour at room temperature with shaking.

2.7 Membrane Washing

Wash the membrane as detailed in 2.5.

3. Enhanced Chemiluminescence Detection

3.1 Preparations

Prepare the following equipment and solutions in a dark room:

- X-ray film cassette
- X-ray film
- Timer
- Developer, fixer and water in tanks
- Transparent plastic bag or saran wrap
- Glass pipettes
- Sterile gloves - to prevent hand contact with membrane, film or reagents

3.2 Detection

- 3.2.1 Mix an equal volume of EZ-ECL Solution A and EZ-ECL Solution B to give sufficient solution to cover the membrane (0.1ml/cm²). Let the detection mix equilibrate for at least 5 minutes.
- 3.2.2 Drain the excess buffer from the washed blots. Do not let the membrane dry out. Add the detection mix directly to the blot (protein side up). Incubate for 1-3 minutes at room temperature.
- 3.2.3 Drain off excess detection mix and wrap the membrane in saran wrap. Gently remove air pockets.
- 3.2.4 Place the blots, protein side up, in the film cassette. Switch off the lights and use red safety light. Place a sheet of film on the blot, close the cassette and expose for 30-60 seconds.
- 3.2.5 Replace the exposed film with a new one, close the cassette and develop the first exposed film.
- 3.2.6 Expose the second film for a suitable time according to the signal intensity on the first film.
- 3.2.7 If signal intensity was too high, wait up to 30 minutes before re-exposing.

4. Optimization of Antibody Concentration for EZ-ECL

It is essential to optimize the immunoblot conditions to achieve maximum signal and minimum background. First optimize the concentration of the primary antibody using a constant amount of secondary-HRP conjugate. Using the optimized primary antibody concentration, adjust the concentration of the secondary antibody-HRP conjugate.

4.1 Dot-Blot for Primary Antibody Optimization

Prepare one piece of nitrocellulose membrane for each primary antibody dilution to be tested.

- 4.1.1 Spot a dilution range of protein onto the membrane.
- 4.1.2 Allow the membrane to air-dry.
- 4.1.3 Block non-specific binding sites by incubating the strip for 1 hour at room temperature with shaking in TBS-T or PBS-T solution containing 5% dried milk (w/v). This step can be performed overnight at 4°C without shaking.
- 4.1.4 Prepare several dilutions of primary antibody in TBS-T or PBS-T with 2% dried milk (w/v), (e.g. 1:100-1:5,000). Incubate one piece of membrane in each dilution for 1 hour at room temperature with constant shaking, or overnight at 4°C without shaking.
- 4.1.5 Wash the membranes three times in TBS-T or PBS-T for 10 minutes each. Use at least 0.5ml of buffer per 1cm² membrane.
- 4.1.6 Dilute the HRP-labeled secondary antibody in TBS-T or PBS-T with 2% dried milk (w/v) to the known optimal dilution. Incubate each strip in the solution for 1 hour at room temperature with shaking.
- 4.1.7 Wash the membranes as detailed in 4.1.5 above.
- 4.1.8 Detection: as detailed in 3.2 above.

4.2 Dot-Blot for Secondary Antibody Optimization

Prepare one piece of nitrocellulose membrane for each secondary antibody dilution to be tested.

- 4.2.1 Prepare dot-blots as detailed in 4.1.1 – 4.1.3 above.
- 4.2.2 Dilute the primary antibody in TBS-T or PBS-T with 2% dried milk (w/v) to the known optimal dilution. Incubate each strip in the solution for 1 hour at room temperature with shaking.
- 4.2.3 Wash the membranes as detailed in 4.1.5 above.
- 4.2.4 Prepare several dilutions of secondary antibody in TBS-T or PBS-T with 2% dried milk (w/v), (e.g. 1:5,000-1:100,000). Incubate one piece of membrane in each dilution for 1 hour at room temperature with constant shaking.
- 4.2.5 Wash the membranes as detailed in 4.1.5 above.
- 4.2.6 Detection: as detailed in 3.2 above.

5. Stripping and Reprobing of Membrane

The immunoblot can be stripped of blocking reagent and antibodies, and then reprobed as required.

5.1

Incubate membrane in stripping buffer for 30 minutes at 50-70°C.
(62.5mM Tris-HCl pH 6.8, 100mM β -mercaptoethanol and 2% (w/v) SDS).

5.2

Wash the membrane twice in TBS-T or PBS-T for 10 minutes each. Use at least 50ml of buffer for 10x10cm membrane. To ensure removal of antibodies, incubate the membrane with EZ-ECL detection reagents and expose against film. Repeat previous steps if a signal is detected.

5.3

Reprobe the blot as detailed in 2.3 - 3.2.7 above.

Protocol for Southern/Northern Blotting and Chemiluminescence Detection

1. Preparation of Solutions

1.1 Tris-Saline Buffer pH 7.5 (Buffer A)

12.114g Tris base (100mM)
35.04g Sodium chloride (600mM)
Adjust pH to pH 7.5 with hydrochloric acid.
Add DEPC-treated water (Cat. No. 01-852-1) up to 1000ml.

1.2 Wash Buffer No. 1

2xSSC
0.1% SDS

1.3 Wash Buffer No. 2

0.1xSSC
0.1% SDS

1.4 0.2% EZ-Block in Buffer A

0.2g EZ-Block (Cat. No. 41-805-10)
100ml Buffer A pH 7.5 (1.1)
Heat in water bath or microwave to 60-65°C.
Mix well.

1.5 0.1% Tween 20 in Buffer A

0.5ml Tween 20
500ml Buffer A pH 7.5
Mix well.

1.6 0.5% EZ-Block in Buffer A

0.5g EZ-Block (Cat. No. 41-805-10)
100ml Buffer A pH 7.5
Heat in a water bath or microwave to 60-65°C.
Mix well.

Notes:

- Do not use Sodium azide as a preservative for the streptavidin-HRP dilution, since azide irreversibly inhibits Horseradish peroxidase.
- Non-fat dry milk inhibits the streptavidin-biotin interaction, due to its content of biotin.
- Probe concentration, which is too high, will often lead to background. Therefore, the probe concentration should not be increased above the recommended concentrations. (The recommended final probe concentration is 2-10ng/ml or 1-2x10⁶cpm/ml for Northern or Southern hybridization).

2. Electrophoresis Blotting and Membrane Preparation

2.1

Carry out electrophoresis for nucleic acid separation.

2.2

Denature the DNA by soaking the gel for 45 minutes in several volumes of 1.5M NaCl; 0.5N NaOH with constant, gentle agitation.

2.3

Rinse the gel briefly in de-ionized water, and neutralize it by soaking for 30 minutes in several volumes of a solution of 1M Tris pH 7.4, 1.5M NaCl at room temperature with constant, gentle agitation. Change the neutralization solution and continue soaking the gel for a further 15 minutes.

Notes:

- Nylon membrane binds small DNA fragments more efficiently than nitrocellulose membranes.
- Fragments of less than 300 nucleotides in length are not retained by 0.45 micron nitrocellulose membranes. (Use a pore size of 0.2 micron).
- Use gloves and blunt-ended forceps to handle the membrane.

2.4

Soak the nitrocellulose membrane in de-ionized water until completely wet. Immerse the membrane in transfer buffer (20xSSC or 20xSSPE).

2.5

Transfer the nucleic acids from the gel to a membrane for 2-24 hours. Mark the positions of the gel slots on the filter with a very soft lead pencil or a ball point pen.

2.6

After the transfers, soak the membrane in 6xSSC for 5 minutes at room temperature (this removes any pieces of agarose sticking to the membrane).

2.7

Remove the membrane from the 6xSSC and allow excess fluid to drain away. Place the membrane flat on a paper towel to dry for at least 30 minutes at room temperature.

2.8

Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter by baking for 30 minutes to 2 hours at 80°C in a vacuum oven.

2.9 Hybridization using EZ-Hybridization Solution with non-radioactively labeled probes

- 2.9.1 Warm the EZ-Hybridization Solution at 68°C for Northern and at 60°C for Southern, and stir well to completely dissolve any precipitate.
- 2.9.2 Pre-hybridize membranes in a minimum of 0.1ml/cm² of EZ-Hybridization Solution with continuous shaking at 68°C for Northern and at 60°C for Southern for 30-90 minutes. The volume of solution must be sufficient to completely cover the membrane, or high backgrounds may result.
- 2.9.3 Denature the non-radioactively labeled DNA probe at 95-100°C for 2-5 minutes. Chill quickly on ice.

- 2.9.4 Add non-radiolabeled probe to a sufficient volume of fresh EZ-Hybridization Solution. Mix gently. For recommended final probe concentrations, see notes above.
- 2.9.5 Replace the EZ-Hybridization Solution with the fresh solution containing the non-radiolabeled DNA probe. Remove all air bubbles from the container, and make sure the EZ-Hybridization Solution is evenly distributed over the entire blot.
- 2.9.6 Hybridize with continuous shaking at 68°C for Northern and at 60°C for Southern for 1-2.5 hours. (For high target applications, shorter hybridization times can be used. For single-gene sequences, hybridization can be performed overnight).
- 2.9.7 Wash the membranes at room temperature twice, 15 minutes each time, with at least 0.5ml/cm² of 2xSSC, 0.1% SDS (Wash No. 1).
- 2.9.8 Wash the membrane twice at 68°C for Northern and at 60°C for Southern, 15 minutes each time, with at least 0.5ml/cm² of 1-0.1xSSC, 0.1% SDS, with continuous agitation.

Note: These washing conditions may be too stringent for probes that are not completely homologous to the target. If this is the case, lower the temperature to 50°C.

- 2.9.9 Remove the blot with forceps and shake off excess wash solution. Rinse the blot in a large amount (2ml/cm²) of Buffer A pH 7.5.
- 2.9.10 Incubate the blot in 0.2% EZ-Block Solution in Buffer A for 30 minutes at room temperature with gentle agitation.
- 2.9.11 Incubate the blot in diluted streptavidin-HRP (1:200-1:3000) in 0.5% EZ-Block in Buffer A for 30 minutes at room temperature with gentle agitation (minimum 0.125ml/cm²).
- 2.9.12 Wash the blot three times in 0.1% Tween 20 in Buffer A, for 10 minutes each time. Use at least 2ml/cm² of buffer.

3. Enhanced Chemiluminescence Detection

3.1 Preparations

Prepare the following equipment and solutions in a dark room:

- X-ray film cassette
- X-ray film
- Timer
- Developer, fixer and water in tanks
- Transparent plastic bag or saran wrap
- Glass pipettes
- Sterile gloves - to prevent hand contact with membrane, film or reagents

3.2 Detection

- 3.2.1 Mix an equal volume of EZ-ECL Solution A and EZ-ECL Solution B to give sufficient solution to cover the membrane (0.1ml/cm²). Let the detection mix equilibrate for at least 5 minutes.
- 3.2.2 Drain the excess buffer from the washed blots. Do not let the membrane dry out. Add the detection mix directly to the blot (nucleic acid side up). Incubate for 1-3 minutes at room temperature.
- 3.2.3 Drain off excess detection mix and wrap the membrane in saran wrap. Gently remove air pockets.
- 3.2.4 Place the blots (nucleic acid side up) in the film cassette. Switch off the lights and use red safety light. Place a sheet of film on the blot, close the cassette and expose for 30-60 seconds.
- 3.2.5 Replace the exposed film with a new one, close the cassette and develop the first exposed film.
- 3.2.6 Expose the second film for a suitable time according to the signal intensity on the first film.
- 3.2.7 If signal intensity was too high, wait up to 30 minutes before re-exposing.

References

- [1] Thorpe, G.H.G. and Kricka, L.J., Methods in Enzymology, 133:331-353 (1986)
- [2] Thorpe, G.H.G., Kricka, L.J., Moseley, S.B. and Whitehead, T.P., Clin. Chem., 31(8):1335-1341 (1985)
- [3] Riko, I., et al, Analytical Biochemistry, 231:170-174 (1995)

Problem	Possible Cause	Suggested Solution
No Signal Or Weak Signal	No, or inefficient transfer	<ul style="list-style-type: none"> • Transfer conditions are improper. • After transfer, stain gel with Ethidium bromide to check transfer efficiency.
	Low target concentration	Increase probe concentration during hybridization, and/or expose blot to film for longer period.
	Probe not completely denatured	Heat probe to 100°C for 10 minutes and chill on ice for 5 minutes.
	Wash condition too stringent	Reduce wash stringency if needed.
	Improper hybridization conditions	Check hybridization buffer and conditions.
	Concentration of HRP-conjugate is too low	<ul style="list-style-type: none"> • Optimize HRP-conjugate concentration. • Prolong incubation with HRP-conjugate to 3 hours. • Shorten washing times and use washing buffer without Tween 20. • Incubate HRP-conjugate in buffer without blocking reagent (background may be increased). • Prolong detection time.
	HRP-conjugate activity dropped	Dot different dilutions of HRP-conjugate onto membrane and detect directly. If no signal appears, use fresh HRP-conjugate and test in the same way. If still no signal appears, check EZ-ECL detection reagents.
	EZ-ECL detection reagents give no signal	<ul style="list-style-type: none"> • EZ-ECL detection reagents may have become contaminated. • Incorrect storage of the EZ-ECL reagents may cause a loss of signal. <p>Check detection reagents: pre-mix equal volumes of EZ-ECL solution A and B (0.5ml each) and, in the dark, add 1µl HRP-conjugate. Visible blue light should be produced.</p>
	Film exposure time is too short	Expose film for extended period (1-2 hours).
High Background	Probe concentration was too high during hybridization	Reduce probe concentration to below 5ng/ml during hybridization.
	Insufficient pre-hybridization and/or blocking	Increase pre-hybridization and blocking time. Consider adding 100mg/ml heterologous DNA (e.g. sheared salmon sperm DNA) to your hybridization buffers.

Problem	Possible Cause	Suggested Solution
Large “Blotchy” Areas of Background	Undiluted probe connected to the membrane	Thoroughly mix the probe, diluted in hybridization solution before pouring it onto the membrane.
	Improper blocking	<ul style="list-style-type: none"> • Check that blocking agent solution has been made properly. • Use a freshly prepared solution of blocking agent. • Increase concentration of blocking agent. • Include blocking agent in HRP-conjugate solutions. • Increase Tween 20 concentration. • Increase incubation time and/or temperature of blocking incubation. • Try alternative blocking agents (freshly prepared): <ul style="list-style-type: none"> - 1-10% bovine serum albumin in TBS-T or PBS-T. - 0.5-3% gelatin in TBS-T or PBS-T. - 1% Polyvinylpyrrolidone (PVP) in TBS-T or PBS-T.
	Improper washing	<ul style="list-style-type: none"> • Increase washing times and volumes of wash buffers. • Add Tween 20 to solutions if not already included • Increase wash stringency if needed.
	Problems with membranes	<ul style="list-style-type: none"> • Check that membranes are completely immersed in all solutions, especially during washing. • Use a fresh supply of membranes. • The type of membrane used was not compatible with non-radioactive systems. • Handle blots carefully with gloves and blunt non-serrated forceps. Damage to the membrane can cause non-specific binding of the probe or the detection reagents. • Use clean forceps to handle blots after washing.
	HRP-conjugate concentration is too high	Optimize HRP-conjugate concentration to reduce the background.
	Detection reagents	<ul style="list-style-type: none"> • Rewash blots twice for 10 minutes in wash buffer and repeat detection steps. • Excess detection reagents in blots. Drain well by absorbing the excess on tissue paper before placing the blots in film cassettes.
	Overexposure	<ul style="list-style-type: none"> • Expose the film for a minimum period (5-30 seconds). • Leave blots in the cassette for 5-10 minutes before re-exposing to film.
	Contaminated buffers	Use fresh buffers.
	Contaminated blotting equipment	Clean or replace equipment

Problem	Possible Cause	Suggested Solution
Uneven Spotted Blot	Improper blotting technique	Check that gel and membrane make proper contact during blotting.
	Unevenly hydrated membrane	<ul style="list-style-type: none"> • Use new membranes. • Make sure that membrane is fully covered and wetted during incubations.
	HRP conjugate solution is not completely clear	Centrifuge the HRP-conjugate solution and use supernatant before diluting in blocking solution. Alternatively, filter through a 0.2µm filter with low protein absorption.
	Fingerprints and/or keratin contamination	Avoid touching membrane. Use gloves and blunt forceps.

Problem	Possible Cause	Suggested Solution
No Signal Or Weak Signal	No, or inefficient protein transfer	Transfer conditions were improper. Check that gel and blotting membrane are correctly oriented with respect to the anode. After transfer, stain gel and/or membrane to check transfer efficiency.
	Increase amount of protein applied to the gel.	Insufficient protein was loaded onto the gel
	Protein degradation on blots stored before detection	<ul style="list-style-type: none"> • Check storage conditions of membrane: target protein degradation may occur if the blots are stored incorrectly. • Use fresh blots.
	Primary antibody does not detect denatured proteins on blots (in denaturing gels containing SDS or urea)	Perform a dot blot with denatured protein and native protein in parallel. If the primary antibody only binds to native protein, try to use a non-denaturing gel system.
	Affinity of primary antibody is low	<ul style="list-style-type: none"> • Optimize antibody concentration. • Prolong incubation with primary antibody to overnight at 4°C. • Shorten washing times and use washing buffer without Tween 20. • Incubate primary antibody in buffer without blocking reagent (background may be increased).
	Concentration of secondary antibody is too low	<ul style="list-style-type: none"> • Optimize antibody concentration. • Prolong incubation with secondary antibody to 3 hours. • Shorten washing times and use washing buffer without Tween 20. • Incubate secondary antibody in buffer without blocking reagent (background may be increased). • Prolong detection time.
	HRP activity of the secondary antibody has dropped	Dot different dilutions of HRP-conjugate onto membrane and detect directly. If no signal appears, use fresh HRP-conjugate and test in the same way. If still no signal appears, check EZ-ECL detection reagents.

Problem	Possible Cause	Suggested Solution
No Signal Or Weak Signal (cont.)	EZ-ECL detection reagent gives no signal	<ul style="list-style-type: none"> • EZ-ECL detection reagents may have become contaminated. • Incorrect storage of the EZ-ECL reagents may cause loss of signal. <p>Check detection reagents: pre-mix equal small volumes of EZ-ECL solution A and B (0.5ml each) and, in the dark, add 1µl HRP-labeled antibody. Visible blue light should be produced.</p>
	Film exposure time is too short	Expose film for extended period (1-2 hours).
High Background	Improper blocking	<ul style="list-style-type: none"> • Check that blocking agent solution has been made properly. • Use a freshly prepared solution of blocking agent. • Increase concentration of blocking agent. • Include blocking agent in antibody solutions. • Increase Tween 20 concentration (Tween 20 may reduce the binding of antibodies, especially of low affinity primary antibodies). • Increase incubation time and/or temperature of blocking incubation. • Try alternative blocking agents (freshly prepared): <ul style="list-style-type: none"> - 1-10% bovine serum albumin in TBS-T or PBS-T. - 0.5-3% gelatin in TBS-T or PBS-T. - 1% Polyvinylpyrrolidone (PVP) in TBS-T or PBS-T.
	Improper washing	<ul style="list-style-type: none"> • Increase washing times and volumes of wash buffers. • Add Tween 20 to solutions if not already included
	Problems with membranes	<ul style="list-style-type: none"> • Check that membranes are completely immersed in all solutions, especially during washing. • Use a fresh supply of membranes. • Handle blots carefully with gloves and blunt non-serrated forceps. Damage to the membrane can cause non-specific binding of the detection reagents. • Use clean forceps to handle blots after washing.
	Antibody concentration is too high	Optimize antibody concentration to reduce the background.
	Detection reagents	<ul style="list-style-type: none"> • Rewash blots twice for 10 minutes in wash buffer and repeat detection steps. • Excess detection reagents in blots. Drain well by absorbing the excess on tissue paper before placing the blots in film cassettes.

Problem	Possible Cause	Suggested Solution
High Background (Cont.)	Overexposure	<ul style="list-style-type: none"> • Expose the film for a minimum period (5-30 seconds). • Leave blots in the cassette for 5-10 minutes before re-exposing to film.
	Contaminated buffers	Use fresh buffers.
	Contaminated blotting equipment	Clean or replace equipment
Uneven Spotted Blot	Improper blotting technique	<ul style="list-style-type: none"> • Check that gel and membrane make proper contact during blotting. • Check that excess temperatures are not reached during electroblotting, producing bubbles, gel/membrane distortion, etc.
	Unevenly hydrated membrane	<ul style="list-style-type: none"> • Use new membranes. • Make sure that membrane is fully covered and wetted during incubations.
	Primary antibody and/or HRP conjugate solutions are not completely clear	Centrifuge the antibodies and use supernatant before diluting in blocking solution. Alternatively, filter through a 0.2µm filter with low protein absorption
	Fingerprints and/or keratin contamination	Avoid touching membrane. Use gloves and blunt forceps.
Excessive Diffuse Signal	Overloading of protein	Load less protein on gel.
	Improper gel conditions	Optimize gel, electrophoresis and blotting conditions: <ul style="list-style-type: none"> - Increase acrylamide concentration of gel. - Check gel and buffer recipes. - Check that no bubbles interfere with transfer from gel to membrane.

Step	Action	Volume	Time	Remarks
Electrophoresis	According to the usual protocol			
Blotting	Immerse the membrane in ddH ₂ O and then in transfer buffer Transfer according to the usual protocol			Use Nitrocellulose or nylon membrane
Membrane Washing and Fixation	soak the membrane in 6xSSC dry for at least 30 minutes fixation by baking or by U.V. crosslinking	0.5ml/cm ²	5 minutes at room temperature room temperature 0.5-2 hours at 80°C or 32 seconds 1200 erg.	
Pre-hybridization and Hybridization	According to the manufacturer's instructions Use denatured biotin-labeled DNA probe	0.1ml/cm ²	1-2.5 hours at 68°C or 60°C	
Washing (I)	Twice with 2xSSC, 0.1% SDS (Wash No. 1)	0.5ml/cm ²	2 x 15 minutes at room temperature	
Washing (II)	Twice with 1-0.1xSSC, 0.1% SDS (Wash No. 2)	0.5ml/cm ²	2 x 15 minutes at 68°C or 60°C	
Blocking	Immerse the membrane in Buffer A. Incubate the blot in 0.2% EZ-Block (Cat. No. 41-805-10) in Buffer A	2ml/cm ² 2ml/cm ²	30 minutes at room temperature	
Streptavidin-HRP	Dilute the streptavidin-HRP (1:2000-1:10,000) in 0.5% EZ-Block in Buffer A. Incubate the membrane in the solution.	0.125ml/cm ²	30 minutes at room temperature	
Washing	Three times with 0.1% Tween 20 in Buffer A	2ml/cm ²	3 x 10 minutes	
Equilibration	Mix equal volumes of EZ-ECL solution A&B	0.1ml/cm ²	5 minutes	
Detection	Incubate membrane in detection mix solution	0.1ml/cm ²	1-3 minutes	With gentle shaking
Exposure	Remove excess detection mix, wrap in saran wrap and expose to film		0.5-60 minutes	Remove air pockets

Step	Action	Volume	Time	Remarks
Electrophoresis and Blotting	According to the usual protocols			Use Nitrocellulose or PVDF membrane
Membrane Blocking	Block membrane with blocking solution, TBS-T or PBS-T with 5% dried milk (w/v), under constant shaking	0.5ml/cm ²	1 hour at room temperature	Alternatively: overnight at 4°C without shaking
Primary Antibody	Dilute the primary antibody in TBS-T or PBS-T with 2% dried milk (w/v). Incubate the membrane in solution with shaking	0.1ml/cm ²	1 hour at room temperature	Alternatively: overnight at 4°C without shaking
Washing	Three times with TBS-T under constant shaking	0.5ml/cm ²	3 x 10 minutes	
Secondary Antibody	Dilute the HRP-labeled secondary antibody (1:10,000-1:60,000) in TBS-T or PBS-T with 2% dried milk (w/v). Incubate the membrane in the solution	0.1ml/cm ²	1 hour at room temperature	
Washing	Three times with TBS-T under constant shaking	0.5ml/cm ²	3 x 10 minutes	
Equilibration	Mix equal volumes of EZ-ECL solution A&B	0.1ml/cm ²	5 minutes	
Detection	Incubate membrane in detection mix solution	0.1ml/cm ²	1-3 minutes	With gentle shaking
Exposure	Remove excess detection mix, wrap in saran wrap and expose to film		0.5-60 minutes	Remove air pockets

