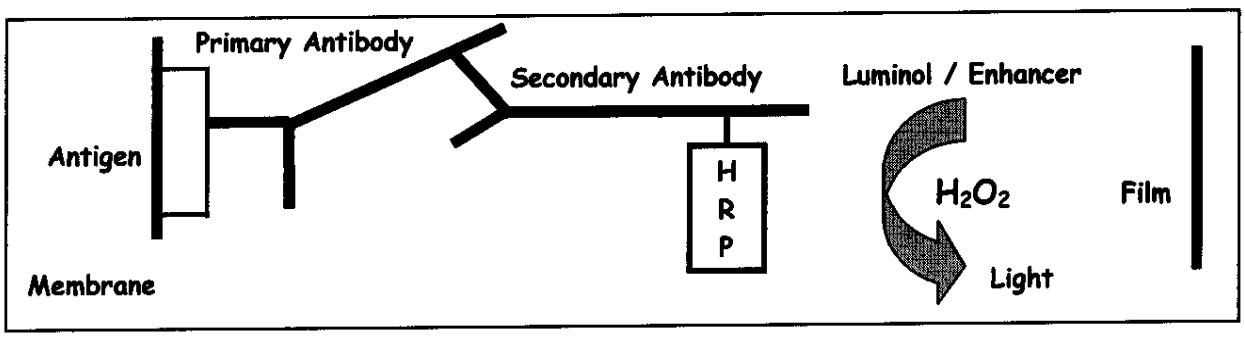
EZ Chemiluminescence Detection Kit for HRP

Cat. No.: **MDMB6**
Lot No.:
Expiry Date:
Store at: **2-8°C**

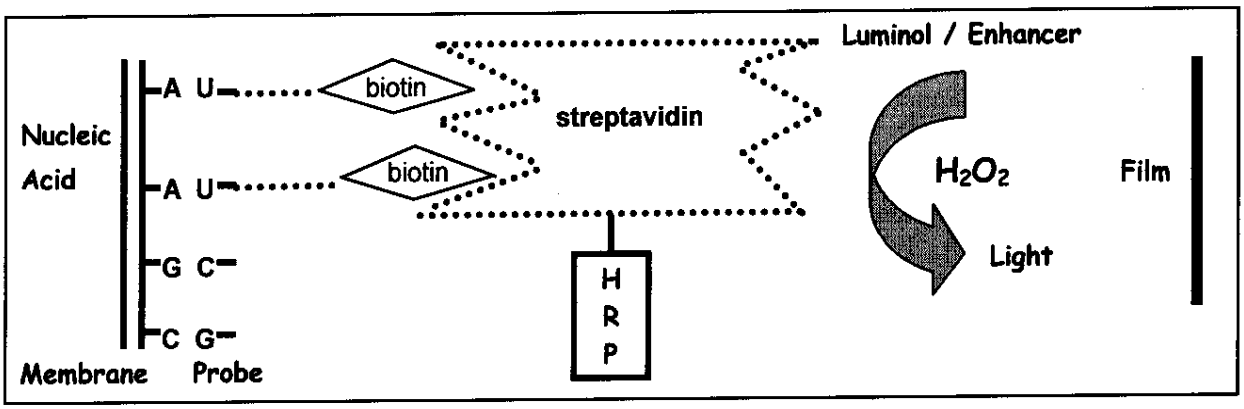
Product Description

EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP 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



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Advantages of using the EZ Chemiluminescence Detection Kit for HRP 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 Reagents

Cat. No.: 20-500-120A EZ Chemiluminescence Detection Kit for HRP Solution A, 60ml
Contains: luminol and enhancer
Store at: 2-8°C

Cat. No.: 20-500-120B EZ Chemiluminescence Detection Kit for HRP 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.

Protocol for Western Blotting and Chemiluminescence Detection

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 Hydrochloride 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 Chemiluminescence Detection Kit for HRP Solution A and EZ Chemiluminescence Detection Kit for HRP Solution B to give sufficient solution to cover the membrane (0.1ml/cm^2). 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 Chemiluminescence Detection Kit for HRP

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 1cm^2 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 Chemiluminescence Detection Kit for HRP 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)
35.04gr 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 straptavidin-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 1-2x10^6cpm/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/cm^2 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/cm^2 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/cm^2 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/cm^2) 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/cm^2).

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/cm^2 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 Chemiluminescence Detection Kit for HRP Solution A and EZ Chemiluminescence Detection Kit for HRP Solution B to give sufficient solution to cover the membrane (0.1ml/cm^2). 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.

3.3.3 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)

EZ Chemiluminescence Detection Kit for HRP

Cat. No.: MDMB6

Troubleshooting Guide

COSMO BIO CO., LTD.

EZ Chemiluminescence Detection Kit for HRP Troubleshooting Guide

For Protein Blots

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.
	Insufficient protein was loaded onto the gel	Increase amount of protein applied to the gel.
	Protein degradation on blots stored before detection	▪ 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	▪ 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	▪ 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 CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagents.

Problem	Possible Cause	Suggested Solution
No Signal Or Weak Signal (cont.)	EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagent gives no signal	▪ EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagents may have become contaminated. ▪ Incorrect storage of the EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP reagents may cause loss of signal. Check detection reagents: pre-mix equal small volumes of EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP solution A and B (0.5ml each) and, in the dark, add 1µl HRP-labeled antibody. Visible blue light should be produced.
	Film exposure time is too short	Expose film for extended period (1-2 hours).
High Background	Improper blocking	▪ 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): ◆ 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	▪ Increase washing times and volumes of wash buffers. ▪ Add Tween 20 to solutions if not already included
	Problems with membranes	▪ 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.

Problem	Possible Cause	Suggested Solution
High Background (cont.)	Antibody concentration is too high	Optimize antibody concentration to reduce the background.
	Detection reagents	▪ 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	▪ 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	▪ 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	▪ 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: ◆ Increase acrylamide concentration of gel. ◆ Check gel and buffer recipes. ◆ Check that no bubbles interfere with transfer from gel to membrane.

For Nucleic Acid Blots

Problem	Possible Cause	Suggested Solution
No Signal Or Weak Signal	No, or inefficient transfer	▪ 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	▪ 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 CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagents.
	EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagents give no signal	▪ EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP detection reagents may have become contaminated. ▪ Incorrect storage of the EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP reagents may cause a loss of signal. Check detection reagents: pre-mix equal volumes of EZ CHEMILUMINESCENCE DETECTION KIT FOR HRP solution A and B (0.5ml each) and, in the dark, add 1µl HRP-conjugate. Visible blue light should be produced.
	Film exposure time is too short	Expose film for extended period (1-2 hours).

Problem	Possible Cause	Suggested Solution
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 hetrologous DNA (e.g. sheared salmon sperm DNA) to your hybridization buffers.
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	▪ 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): ◆ 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	▪ 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	▪ 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.

Problem	Possible Cause	Suggested Solution
Large "Blotchy" Areas of Background (cont.)	HRP-conjugate concentration is too high	Optimize HRP-conjugate concentration to reduce the background.
	Detection reagents	▪ 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	▪ 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	Check that gel and membrane make proper contact during blotting.
	Unevenly hydrated membrane	▪ 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.