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EZ-ECL

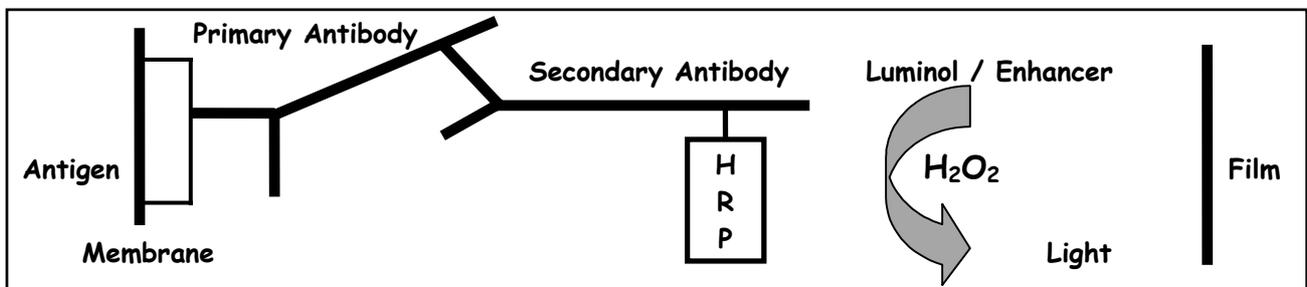
Chemiluminescence Detection Kit for HRP

Cat. No.: 20-500-120
Store at: 2-8°C

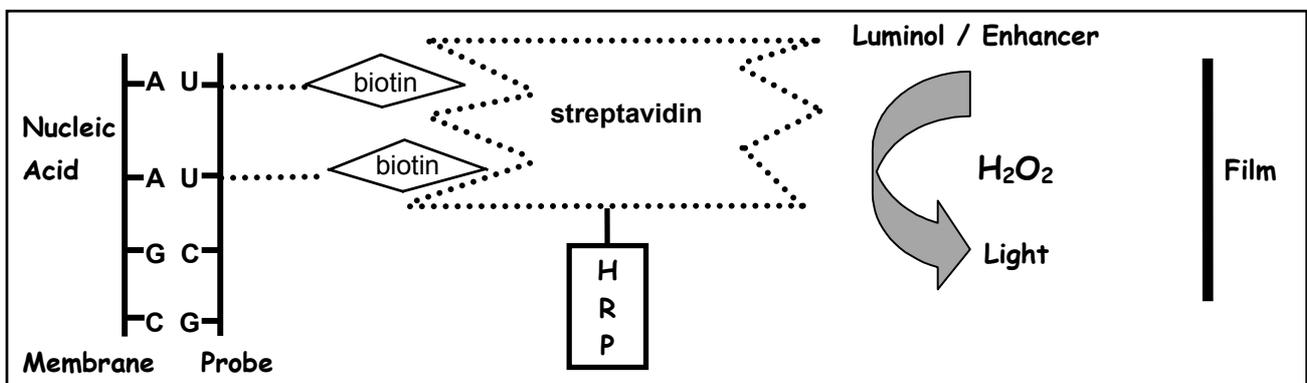
Product Description

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 Reagents

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.

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-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-blot 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)
35.04gr 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 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 $1-2 \times 10^6$ cpm/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:

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- 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)