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Introduction

Western blotting is a protein detection method performed on nitrocellulose or PVDF membranes. After electrophoresis, proteins are transferred onto the membrane where they are reacted with specific antibodies. Proteins of interest are then identified using a detection reagent. The blotting method itself was first developed by Edwin Mellor Southern, who lent his name to the "southern blot" DNA detection test. Later, an RNA detection method was named "northern blot", and the term "western blot" was chosen and accepted for protein detection.

Western Blotting Procedure

The procedure for Western blotting is as follows:

1. Protein transfer
2. Membrane blocking
3. Antigen-Antibody reaction
4. Detection

There are numerous ways to perform each step. Choosing the appropriate methods and reagents is important for obtaining a good result. This protocol primarily discusses the particularities of each method and provides precautions for each step.
Transfer

There are two blotting method types, semi-dry and tank blotting. The semi-dry method is more likely to be preferred because the blotting time is shorter. As for the membrane, PVDF membranes tend to be used more frequently because their protein binding strengths exceed those of nitrocellulose membranes. Furthermore, they allow direct application of the amino acid sequencer. Please see the details below.

Blotting Methods Comparison

<table>
<thead>
<tr>
<th></th>
<th>Tank Blotting</th>
<th>Semi-dry Blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer buffer volume</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>Treatment temperature</td>
<td>Low because of the cooling provided by large amounts of transfer buffer during blotting.</td>
<td>Slightly increased, but it is still unnecessary to provide cooling during blotting.</td>
</tr>
<tr>
<td>Blotting time</td>
<td>More than 4 hours</td>
<td>Up to 2 hours</td>
</tr>
<tr>
<td>Expected transfer result</td>
<td>Relatively uniform</td>
<td>Tends to lack uniformity</td>
</tr>
<tr>
<td>Transfer efficiency</td>
<td>The long, low voltage blotting period provides sufficient time for each protein, regardless of molecular weight, to be efficiently transferred to the membrane.</td>
<td>The short, high voltage blotting period can cause difference in transfer efficiency between high and low molecular weight proteins.</td>
</tr>
</tbody>
</table>

Authority: Baiozikken de shippai shinha! Kensyutsu to teiryō no kotsu. Naoya Moriyama p.41

Blotting Membranes Comparison

<table>
<thead>
<tr>
<th></th>
<th>PVDF (Polyvinylidene Difluoride) Membrane</th>
<th>Nitrocellulose Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Property</td>
<td>Hydrophobic</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Strength of membrane</td>
<td>Strong</td>
<td>Weak</td>
</tr>
<tr>
<td>Retaining amount of proteins</td>
<td>Approx. 250 μg / cm²</td>
<td>Approx. 100 μg / cm²</td>
</tr>
<tr>
<td>Cost</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

Authority: Baiozikken de shippai shinha! Kensyutsu to teiryō no kotsu. Naoya Moriyama p.41

Please note:
PVDF membranes have recently come to be preferred by researchers.

Protocol for Semi-dry Transfer with PVDF Membrane

Required Reagents

1. Preparation of PVDF Membrane

1-1. Pour about 50 ml of 100% methanol into a clean tray (disposable tray or similar), hereafter referred to as Tray 1. Next, starting from the edge of the tray, slowly slide the membrane into the methanol until it is completely immersed. Then, gently agitate the tray with a shaker for 1 minutes.

Please note:
1. It is not necessary to immerse nitrocellulose membranes in methanol.
2. When cutting the PVDF membrane and filter paper, wear gloves to prevent stray proteins from adhering.

1-2. Remove the methanol completely from the Tray 1. Next, pour 50 ml of Semi-dry Blotting Solution for Western blotting (Product No.30650-31) into the Tray 1, then agitate the tray with a shaker for 10-20 minutes.
2. Preparation of Polyacrylamide Gel

2-1. Pour 50 ml of Semi-dry Blotting Solution for Western blotting (Product No. 30650-31) into another clean tray, hereafter referred to as Tray 2. Immerse the polyacrylamide gel in the Tray 2, and then agitate with a shaker for 10-20 minutes to ensure the gel is completely saturated.

3. Set on Electrode Plate

3-1. Cut 12 pieces of filter paper at a size slightly larger than the PVDF membrane.

*Please note:*
When cutting the PVDF membrane and filter paper, wear gloves to prevent stray proteins from adhering.

3-2. Pour 50 ml of Semi-dry Blotting Solution for Western Blotting (Product No. 30650-31) to new clean tray (hereafter Tray 3).

3-3. Immerse the filter papers for a second, one by one, in the Tray 3, and then use the wall of the tray (if necessary) to remove excess liquid. Place the filter papers in a row on the positive electrode plate, beginning from the edge. The six pieces of filter paper should overlap exactly.

3-4. Overlap with PVDF membrane, beginning from the edge.

*Please note:*
When placing the membrane on the six pieces of filter paper, work carefully to prevent air bubbles from forming because they will interrupt membrane transfer wherever they exist. For more information, refer to page 6, troubleshooting.

3-5. Place the polyacrylamide gel equilibrated according to the step 2, preparation of polyacrylamide gel, onto the PVDF membrane. Mark the edge of the gel on the membrane with a ballpoint pen in order to see the side of transferred protein and area easily. Then, stack the remaining six pieces of filter paper by repeating the above from step 3-3.

3-6. Set the negative electrode plate on the filter papers, as shown in the image below, then connect the power supply and the blotting equipment.

*Please note:*
The constant electric current is about 0.8 - 1 mA/cm² for 1-1.5 hours when transferring a mini-gel (size 10 cm x 10 cm).

*Please note:*
Two pieces of gel can be transferred at one time. We recommend that the current value be set to 1.5 mA / cm². To ensure good transfer efficiency, we also recommend pretesting the current and time as those values may vary depending on the blotting equipment and filter paper used.

3-7. After step 3-6, disconnect the power supply from the blotting equipment, and then remove each filter paper, one by one, from the side of negative electrode plate. Next, immerse the PVDF membrane in PBS Tween® 20 or TBS Tween® 20. If it is necessary to stop this work temporarily, re-immers the PVDF membrane in the TBS Tween® 20 to prevent air drying.

*Please note:*
When using an antibody for phosphorylated proteins detecton, wash the membrane with TBS Tween® 20.
4. PVDF Membrane Washing

4-1. After placing the PVDF membrane into a disposable tray, add TBS Tween® 20 solution and agitate the tray with the a shaker for 5 minutes. Then, replace with fresh solution and repeat the entire process.

Please note:
When confirming the transfer efficiency, we recommend the use of a pre-stained protein marker such as Protein Ladder One, Triple-color (Product No. 09547-74) and Pre-stained Protein Marker (Product No. 02525-35). Additionally, for confirming the transfer efficiency of whole proteins, we recommend staining the transferred membrane with CBB dyes. Refer to page 8 for details.

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransferred spots are present.</td>
<td>Air bubbles were captured between the gel and the membrane.</td>
<td>Emplace the gel on the membrane gently and carefully to avoid trapping air bubbles.</td>
</tr>
<tr>
<td>Insufficient transfer for low molecular weight proteins.</td>
<td>A transfer efficiency gap occurred between high and low molecular weight proteins due to the high voltage used and the short blotting time.</td>
<td>Optimize the transfer time for your target proteins. Switch to tank blotting to ensure uniform target protein transfers.</td>
</tr>
<tr>
<td>Insufficient transfer for high molecular weight proteins.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E.g., Locations where protein transfer failed due to air bubbles are shown with pink arrows.
Confirming Transfer

Nitrocellulose and PVDF membranes transferred from a polyacrylamide gel are sometimes stained using dyes that show the locations and transfer efficiency of whole proteins on the membrane. For example, when comparing the expression level of target proteins via western blotting, transfers should be uniform on each lane. Therefore, the use of dyes such as CBB is recommended after blotting to check for the presence of non-uniform transfers. For an N-terminal sequence of target proteins, the transferred membrane will be stained using Ponceau S. In addition, relativity of detection level of the interested protein on western blot and its transfer efficiency can be compared by staining the transferred membrane with CBB dyes.

Authority: Baizozekke de shippaishinai! Kensyutsu to teiryo no kotsu. Naoya Moriyama p.88

There are two methods for staining the transferred membrane with CBB dyes. One method uses organic solvents like acetic acid, methanol, and ethanol, while the other does not. Accordingly, we offer a support product for each: CBB Stain One and Rapid Stain CBB Kit. CBB Stain One makes it easier to proceed to the western blotting analysis because just one step is needed to destain CBB dyes from the membrane when using a Rapid CBB Destain Kit.

Comparison of Coomassie Brilliant Blue Dye Staining Kit

<table>
<thead>
<tr>
<th></th>
<th>CBB Stain One</th>
<th>Rapid Stain CBB Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining Solution</td>
<td>No methanol or acetic acid</td>
<td>Contains organic solvents</td>
</tr>
<tr>
<td>Staining Time</td>
<td>25 minutes, including two wash steps</td>
<td>1 minute</td>
</tr>
<tr>
<td>Background Destaining Step</td>
<td>Unnecessary</td>
<td>Necessary</td>
</tr>
<tr>
<td>Protein Band Destaining Step</td>
<td>5 minutes</td>
<td>5-10 minutes</td>
</tr>
</tbody>
</table>

Protocol for Membrane Staining with CBB Stain One

**Required Reagents**

- CBB Stain One (Ready To Use) (Product No. 04543-51)
- Rapid CBB Destain Kit (Product No. 30046-74)
- Protein Markers (Product No. 29458-24)
- Pre-stained Protein Markers (Broad Range) (Product No. 02525-35)
- Disportray (Product No. 06563-44)

1. **Staining**

   1-1. Pour CBB Stain One into a clean tray, and immerse the membrane for 15 minutes.
   1-2. Wash the membrane with water for 5 minutes. Repeat.

   ![Albumin (66kDa)]

   [Condition]
   - SDS-PAGE: 12% gel
   - Sample: 1. Protein Markers (Product No. 29458-24)
   - 2. Human Serum
   - 3. Pre-stained Protein Markers (Broad Range) (Product No. 02525-35)
   - Membrane: PVDF Membrane

2. **Destaining Stained Protein Bands for Western Blot Analysis**

   2-1. Pour 20 ml of solution A, 10 ml of solution B, and 30 ml of deionized water (included in Rapid CBB Destain Kit) into a disposable tray. This solution volume is suitable for one 10 cm x 10 cm piece of membrane.

   ![Albumin (66kDa)]

   2-2. Immerse the stained membrane in the above mixed solution for 5 minutes.

3. **Western Blotting**

   Detect reactions to the primary and peroxidase-linked secondary antibodies, and thus the target protein, using the appropriate detection kit for the destained membrane. The figure on the left shows that staining with CBB Stain One and destaining with Rapid CBB Destaining has no affect on the antigen-antibody reaction.

   ![Albumin (66kDa)]
Protocol for Membrane Staining with Rapid Stain CBB Kit

1. Staining

1-1. To prepare working solution, mix 30 ml of deionized or distilled water with 10 ml of solution A, then add 10 ml of solution B. This solution volume is suitable for one 10 cm x 10 cm piece of membrane. Prepare this staining solution fresh for each use.

1-2. Pour the above working solution into a disposable tray, then immerse the transferred membrane in the tray. Agitate the tray in a shaker for exactly 1 minute.

1-3. Thoroughly wash the stained membrane with PBS Tween® 20.

2. Destaining the Background to Visualize the Target Proteins

2-1. Pour 20 ml of solution A, 10 ml of solution B (included in the Rapid CBB Destain Kit) and 30 ml of deionized water into a disposable tray. This solution volume is suitable for one 10 cm x 10 cm piece of membrane.

2-2. Immerse the stained membrane in the destaining solution, then agitate the tray with a shaker for 3-5 minutes, or until each band is clearly visible.

2-3. Thoroughly wash the destained membrane with PBS Tween® 20.

2-4. Photograph or record data, as necessary.

3. Protein Band Destaining for Western Blot Analysis

3-1. Pour 25 ml of solution A and 25 ml of solution B (included in the Rapid CBB Destain Kit) into a disposable tray. This solution volume is sufficient for one 10 cm x 10 cm piece of membrane.

3-2. Immerse the membrane in the above destaining solution, and then agitate the tray with a shaker for 5-10 minutes.

3-3. Remove the destaining solution and thoroughly wash the destained membrane with PBS Tween® 20.

Please note:
To avoid interference with the antigen-antibody reaction of the western blotting analysis, completely remove the CBB dyes from the entire membrane using the above procedure.
Blocking

After transferring proteins to a membrane, the membrane should be masked with blocking reagents to prevent nonspecific antibody reactions. Failure to block the membrane sufficiently can cause high background. Blocking reagents include BSA, skim milk, casein, gelatin and high molecular weight compounds. Therefore, determining the appropriate agent for use depends on the target proteins, costs, and other related factors.

Comparison of Popular Blocking Reagents

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Procedure</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking One</td>
<td>This reagent is based on synthesized high molecular weight compounds so it can provide fast and strong blocking efficiency.</td>
<td></td>
<td>Approx. 30 minutes</td>
</tr>
<tr>
<td>Blocking One-P</td>
<td>For phosphorylated protein detection. This product is related to Blocking One, but is free from both the phosphate group and endogenous phosphates.</td>
<td></td>
<td>Approx. 30 minutes</td>
</tr>
<tr>
<td>BSA</td>
<td>When detecting phosphorylated tyrosine proteins, use 1-5% BSA solution.</td>
<td></td>
<td>Approx. 1 hour</td>
</tr>
<tr>
<td>Skim milk</td>
<td>The most popular blocking agent, commonly used in a 5% skim milk / TBS-Tween® 20 solution.</td>
<td></td>
<td>Approx. 1 hour</td>
</tr>
</tbody>
</table>

Protocol for Blocking One

Required Reagents

- Blocking One (Product No.03953-95)
- Dipotray (Product No. 06563-44)

Protocol

1. Pour the Blocking One solution into a disposable tray and immerse the membrane thoroughly.
2. Agitate the tray in a shaker for 30 minutes at room temperature.

Application Data

Detection of IgG from mouse serum

<Condition>
Marker: Protein marker (Biotin conjugated)
Sample: Mouse serum
Lane 1: 2.5 μl
Lane 2: 5 μl
Lane 3: 10 μl

Mouse IgG (H+L) in Mouse Serum detected with Biotinylated anti-Mouse IgG (H+L)
Protocol for Blocking One-P

Required Reagents

- Blocking One-P (Product No. 05999-84)
- Dispotray (Product No. 06563-44)

Protocol

1. Pour Blocking One-P into a disposable tray and immerse the membrane.
2. Agitate the tray in a shaker for 20 minutes at room temperature.

Please note:
The product cannot be used as buffer solution for alkaline phosphatase labeled antibodies. If using alkaline phosphatase labeled antibody, wash the membrane thoroughly and carefully after blocking with Blocking One-P.

Applications

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sample: HeLa extract cells (1.0x10^7) with RIPA Buffer (Product No. 08714-04)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1. 5 μl</td>
<td>Lane 2. 10 μl</td>
</tr>
<tr>
<td>Primary Abs: Anti-p-Tyr (PY20) mouse (SantaCruz #SC-508)</td>
<td></td>
</tr>
<tr>
<td>Secondary Abs: Anti-mouse IgG-HRP (SantaCruz #SC-2005)</td>
<td></td>
</tr>
<tr>
<td>Staining of membrane: CBB Stain One (Product No. 04543-51)</td>
<td></td>
</tr>
<tr>
<td>Detection: Chemi-Lumi One L (Product No. 07880-70)</td>
<td></td>
</tr>
</tbody>
</table>

FAQ

<table>
<thead>
<tr>
<th>Question</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is Blocking One or Blocking One-P suitable for use when diluting the primary and secondary Abs?</td>
<td>It is not always appropriate to dilute the Abs with blocking reagents, but it is sometimes useful in order to reduce background, as well as to avoid non-specific interaction with tubes and trays and to utilize costly Abs in the most economic manner. When dilution is advisable, we recommend adding Blocking One or Blocking One–P reagents to Abs at a 20:1 ratio. If undiluted Blocking One or Blocking One–P reagent is used for Abs dilution, it has the potential to interfere with antigen-antibody reactions due to its robust blocking efficiency.</td>
</tr>
</tbody>
</table>

Please note:
Blocking One-P should not be used to dilute alkaline phosphatase-linked antibodies.
Detection

Chemiluminescence Detection on Western Blot

We offer three types of chemiluminescence detection kit as follows.

Selection Guide

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Chemi-Lumi One L</th>
<th>Chemi-Lumi One Super</th>
<th>Chemi-Lumi One Ultra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product No.</td>
<td>07880</td>
<td>02230</td>
<td>11644</td>
</tr>
<tr>
<td>Usage</td>
<td>General Use</td>
<td>High Sensitivity</td>
<td>Ultrahigh Sensitivity</td>
</tr>
<tr>
<td>Lower Detection Limit</td>
<td>Low-picogram</td>
<td>Mid-femtogram</td>
<td>Low-femtogram</td>
</tr>
<tr>
<td>Required Working Solution</td>
<td>Approx. 0.125 ml / cm²</td>
<td>Approx. 0.1 ml / cm²</td>
<td>Approx. 0.1 ml / cm²</td>
</tr>
<tr>
<td>Suggested Antibody Dilution</td>
<td>Primary: 1:1,000-1:5,000</td>
<td>Secondary: 1:20,000-1:100,000</td>
<td>Primary: 1:1,000-1:20,000</td>
</tr>
<tr>
<td>Reaction Period</td>
<td>1 min.</td>
<td>1 min.</td>
<td>5 min.</td>
</tr>
<tr>
<td>Comparable to</td>
<td>ECL Pico</td>
<td>ECL Prime</td>
<td>ECL Select</td>
</tr>
</tbody>
</table>

Sensitivity

General Use High Sensitive Ultra high Sensitive

Conc. of IgG HRP-linked 900 300 100 33.3 11.1 3.7 1.2 412 46 15 3

<Condition>
Antigen: Anti-Mouse IgG (Goat), HRP Conjugated (Santa Cruz, sc-2005)
Detection: L (1 min.), Super (1 min.) and Ultra (5 min.)
Detector: LAS-3000 Super mode (Analyze 3 min. later after reaction with each substrate)
Expose time: 30 min.

Please note:
Detection limit differs depending on condition, such as transfer method, membrane type, concentration of Abs and more. The result above is performed by blotting IgG HRP-linked directly on PVDF membrane and detected by each substrate.

Protocol for three types of chemiluminescence detection kit is almost the same. The followings is the protocol of most widely used kit, Chemi-Lumi One Super. (Specific conditions of Chemi-Lumi One L and Chemi-Lumi One Ultra are also described.)
Detection

Chemi-Lumi One Super Protocol

Required Reagents

1. Antibody Reaction

Primary antibody reaction

1-1. Dilute the primary antibody with an appropriate buffer such as Signal Enhancer HIKARI, TBS-Tween® 20 or PBS Tween® 20, and then agitate the transferred membrane at room temperature for 1 hour using a shaker. Dilution ratio of primary antibody is 1 : 1,000 - 1 : 20,000. The sensitivity and specificity of the antibody can be improved by diluting it with Signal Enhancer HIKARI Solution A. Refer to page 23.

Dilution ratio of primary antibody for each product
- Chemi-Lumi One L = 1 : 1,000 - 1 : 5,000
- Chemi-Lumi One Ultra = 1 : 5,000 - 1 : 100,000

1-2. Immerse the membrane in a tray filled with PBS Tween® 20 and agitate the tray for 5 minutes using a shaker. Repeat.

Secondary antibody reaction

1-1. Dilute the secondary antibody with an appropriate buffer such as Signal Enhancer HIKARI, TBS-Tween® 20 or PBS Tween® 20, and then agitate the transferred membrane at room temperature for 1 hour using a shaker. Dilution ratio of secondary antibody is 1 : 20,000 - 1 : 200,000. The sensitivity and specificity of the antibody can be improved by diluting it with Signal Enhancer HIKARI Solution B. Refer to page 23.

Dilution ratio of secondary antibody for each product
- Chemi-Lumi One L = 1 : 20,000 - 1 : 100,000
- Chemi-Lumi One Ultra = 1 : 100,000 - 1 : 500,000

1-2. Immerse the membrane in a tray filled with PBS Tween® 20 and agitate the tray for 5 minutes using a shaker. Repeat.

Please note:
Pretest suitable antibody concentrations by referencing the datasheet attached to the product.

2. Procedure

2-1. For working solution preparation, mix Solutions A and Solution B (included in the Chemi-Lumi One Super Kit) at a one to one ratio.

2-2. Remove excess secondary antibody solution buffer by touching the edge of the membrane with Kimwipe® laboratory wipes.
2-3. Carefully place the membrane on the plastic wrap with the transferred proteins side facing up, then apply the working solution to the membrane. Allow to stand at room temperature for 1 minute. Add approximately 0.1 ml of working solution for each cm$^2$ of membrane.

Transferred proteins side facing up

Required working solution and reaction time for each product
Chemi-Limi One L = required working solution : approximately 0.125 ml/cm$^2$, reaction time : 1 min.
Chemi-Limi One Ultra = required working solution : approximately 0.1 ml/cm$^2$, reaction time : 5 min.

Please note:
Use care to prevent air bubbles from forming between the membrane and the plastic wrap.

2-4. Grasp the membrane with tweezers and remove excess working solution by touching the edge of membrane to Kimwipe® laboratory wipes.

2-5. Place the membrane on a new larger sheet of plastic wrap with the transferred protein side facing down, and wrap carefully.

Transferred proteins side facing down

Please note:
Use care to prevent air bubbles from forming between the membrane and the plastic wrap, and avoid wrinkling the plastic when wrapping.

2-6. Place the wrapped membrane on the film cassette with the transferred proteins side facing upward.
(Please proceed to the darkroom steps described below)

Please note:
Pretest exposure times because good results depend on a proper balance of sample, method and condition.

2-7. Place an X-ray film on top of the wrapped membrane, close the film cassette, then expose for 1 min. or longer if necessary.

Exposer time for each product
Chemi-Limi One L = 3 min. or longer if necessary.
Chemi-Limi One Ultra = 1 min. or longer if necessary.
2-8. Remove the X-ray film from the film cassette, and then immerse it in developer until the band image appears.

**Please note:**
*Always be sure to follow the package directions of developer solutions.*

2-9. Immerse the X-ray film in the stop solution (normally, 0.3% acetic acid aqueous solution).

**Please note:**
*When it is necessary to use a 0.3% acetic acid aqueous solution, we recommend preparing a fresh stop solution each time to prevent solution degradation.*

2-10. Immerse the X-ray film in the fixer solution.

**Please note:**
*Always be sure to follow the package directions of fixer solution.*

2-11. Wash the X-ray film with running water to remove fixer, then hang X-ray to dry.

---

**Applications**

1. **[Chemi-Lumi One L] Comparison data of sensitivity with competitors**

   Chemi-Lumi One L offers the similar sensitivity as T and W company's products and higher sensitivity than G company's products.

   - **Chemi-Lumi One L**
   - **Competitor G**
   - **Competitor T**
   - **Competitor W**

   <Condition>
   - Gel: 10% PAGE gel
   - Wash: 0.1% t-TBS(1x), pH7.4
   - Blocking: Blocking One
   - 1st Ab: Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruz sc-47778), 1:1,000
   - 2nd Ab: Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruz sc-2005), 1:20,000
   - Detection period: Chemi-Lumi One L, 1 min.
   - Detector: LAS-3000 High mode
   - Expose time: 5 min.

2. **[Chemi-Lumi One L] Comparison data of detection limit**

   Chemi-Lumi One L is suitable for detection of unknown protein concentration or optimization conditions.

   <Condition>
   - Gel: 10% PAGE gel
   - Wash: 0.1% t-TBS(1x), pH7.4
   - Blocking: Blocking One
   - 1st Ab: Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruz sc-47778), 1:1,000
   - 2nd Ab: Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruz sc-2005), 1:20,000
   - Detection period: Chemi-Lumi One L, 1 min.
   - Detector: LAS-3000 High mode
   - Expose time: 30 min.
3. [Chemi-Lumi One L] Detection of transcription factor of SREBP-1 and ChREBP

Transcription factor of SREBP-1 and ChREBP are detected after extraction of proteins such as nucleus or cytoplasm with RIPA Buffer.

**Western blotting**
(Blotting: Blocking One, Detection: Chemi-Lumi One L)

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RIPA (w/ SDS), Nacalai</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>2</td>
<td>RIPA (w/o SDS, Nacalai)</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>3</td>
<td>RIPA (w/o SDS, A company)</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>4</td>
<td>Mouse Liver (RIPA w/ SDS, Nacalai)</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>5</td>
<td>Mouse Kidney</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>6</td>
<td>Mouse Stomach</td>
<td>CBB Stain One</td>
</tr>
<tr>
<td>7</td>
<td>Mouse Brain</td>
<td>CBB Stain One</td>
</tr>
</tbody>
</table>

Extraction condition:
Incubate 100 mg of tissue, which is washed by cold-PBS, with 300 ml of RIPA Buffer on ice for 30 min.

Data courtesy of associate professor Tatsuya Moriyama, Faculty of Agriculture, Kinki University

4. [Chemi-Lumi One L] Detection after immunoprecipitation

The Applications below are detected with Chemi-Lumi One L after immunoprecipitation of sample which are extracted by RIPA Buffer for lysing cultured mammalian cells.

**Figure 1 : Gel staining after extraction**
Figure 2: Gel staining and western blotting after immunoprecipitation

Sample: Jurkat Cell
Primary Antibody: Anti-β-Actin Mouse Monoclonal Antibody (Santa Cruz, sc-47778)
Secondary Antibody: Anti-Mouse IgG (Goat), HRP Conjugated (Santa Cruz, sc-2005)
Left: Stained with Sil-Best Stain One
Right: Detected with Chemi-Lumi One L

Lane 1: Protein extracted solution with RIPA (w/o SDS)
2: Protein extracted solution with RIPA (w/ SDS)
3: Protein Markers (10x) (#29458-24)
4: Immunoprecipitated protein extracted solution with (w/o SDS)
5: Immunoprecipitated protein extracted solution with (w/ SDS)
6: Agarose control

Extraction condition:
Incubate Jurkat Cell 1.0 x 10^7 containing 1 ml of RIPA buffer on ice for 15 min.

According to the lane 1 and 2 on the right gel image in the figure 1, the extraction efficiency of the cell which was treated under SDS condition was higher than that of cell under non-SDS condition, while immunoprecipitation efficiency of beta-actin which were treated under non-SDS condition is higher than that of beta-actin under SDS condition according to the lane 4 and 5 in the figure 2 as SDS tends to cause lower immunoprecipitation efficiency due to denature and interfere with antigen-antibody as well as antigen-antigen.

5. [Chemi-Lumi One Super] Comparison data of sensitivity with competitors

Chemi-Lumi One Super is the most sensitive of competitors under the above condition, and its expose time is only 1 min., while others are required 5 min.

6. [Chemi-Lumi One Super] Comparison data of background

Chemi-Lumi One Super performs under lower background, so users enable expose time to be longer for achieving more sensitive analysis.
7. [Chemi-Lumi One Ultra] Comparison data of sensitivity with competitors 1

Chemi-Lumi One Ultra is an extremely sensitive chemiluminescent substrate. Western blot of identical transferrin samples. The membranes were incubated with substrate that was prepared according to the manufacturers’ instructions. The membranes were exposed to film for 2 minutes.

8. [Chemi-Lumi One Ultra] Comparison data of sensitivity with competitors 2

Chemi-Lumi One Ultra shows equal to or higher sensitivity compared to competitors'.

9. [Chemi-Lumi One Ultra] Comparison data of Abs concentration

Due to low background, Chemi-Lumi One Ultra is applicable to wide range of primary antibodies, and makes it easy to optimize condition.
## Troubleshooting

<table>
<thead>
<tr>
<th>Case</th>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
</table>
| **Case 1** | The expected band is white in color. | 1. Excessive sample volume when using SDS-PAGE.  
2. High concentration of primary antibody.  
3. High concentration of secondary antibody. | Repeat the procedure using reduced amounts of sample volume, primary and secondary antibodies. |
| **Case 2** | There are non-specific bands. | 1. Excess sample volume when using SDS-PAGE.  
2. High concentration of primary antibody. | Repeat the procedure using reduced amounts of sample volume and primary antibody. |
| **Case 3** | High background | Insufficient blocking. | Check the blocking time and concentration of the blocking agents. Note that when detecting phosphorylated proteins, it is necessary to use a casein-free agent such as Blocking One-P (Product No. 05999-84) because casein is present in many phosphorylated residues.  
High concentration of secondary antibody. | Reduce secondary antibody and retry.  
Insufficient washing. | Wash the membrane in copious amounts of TBS-Tween® 20, especially after the secondary antibody reaction procedure. |
| **Case 4** | No band is visible | Incorrect combination a primary and secondary antibodies. (e.g., Primary Abs: Mouse monoclonal and Secondary Abs: Anti-rabbit.) | Ensure correct combination of primary and secondary antibody are used.  
Developer solution has deteriorated. | When the developer color turns bright yellow the solution has become oxidized. However, if results are unsatisfactory even though the color has not changed, it still may have become depleted. Use a fresh developer solution.  
Low concentration of primary and secondary antibodies, or insufficient amount of sample volume on SDS-PAGE. | Increase primary and secondary antibodies and SDS-PAGE sample volume. |
Detection

Colorimetric Detection 1-1 : Peroxidase Stain Kit

Required Reagents

- Peroxidase Stain Kit (Product No. 26652-70)
- Dispotray (Product No. 06563-44)

Components

<table>
<thead>
<tr>
<th>Regent Name</th>
<th>Main Composition</th>
<th>Volume</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining Stock Solution</td>
<td>Naphthol / Benzidine derivative solution</td>
<td>10 ml x 1 btl</td>
<td>Freezer</td>
</tr>
<tr>
<td>Buffer Solution</td>
<td>Phosphate-Citrate Buffer Solution, Hydrogen Peroxide</td>
<td>200 ml x 1 btl</td>
<td>Refrigerator</td>
</tr>
</tbody>
</table>

1. Working Solution Preparation

Prepare working solutions just before use and commence membrane staining process within 10 minutes. Staining with working solution that was prepared more than 30 minutes prior might not yield good results.

For a 10 cm x 10 cm membrane, use the following procedure:

1-1. Check that the lid is tightly closed, then ensure that the Buffer Solution (included in the kit) is well mixed by turning the bottle upside down and then back upright.

1-2. Transfer 50 ml of Buffer Solution to a clean measuring cylinder, add 2.5 ml of Staining Stock Solution (included in the kit), and mix well.

Please Note:

1. Prior to commencing the above procedure, wash the measuring cylinder and plastic tray with water, then immerse in a (1 mol/l) sulfuric acid solution for at least 30 minutes. Then wash again with deionized water before drying.

2. If the Staining Stocking Solution comes into contact with skin, wash with copious amounts of water immediately as it contains a mutagenic substance. Wear gloves and goggles whenever handling.

2. Procedure

2-1. Apply primary antibody to the antigen on a membrane, and then wash membrane well with TBS-Tween® 20. When using a peroxidase-linked primary antibody, proceed to step 2-3.

2-2. Apply the peroxidase-linked secondary antibody, and wash the membrane well with TBS-Tween® 20.

2-3. Pour the above working solution into the clean plastic tray, then immerse the membrane into the solution. Leave in place or agitate with a shaker at room temperature.

Please Note:

Use of a disposable plastic tray is recommended as dyes might be absorbed into stainless steel trays.

2-4. After an image appears, remove the membrane from the plastic tray and wash it in running water for more than 10 minutes to ensure the staining reaction has stopped.

Please Note:

1. If the protein bands are diffused, use PBS or TBS without Tween® 20 to wash the membrane after peroxidase-labeled antibody reactions.

2. If the peroxidase-labeled antibody concentration is too high, the background is likely to be high as well. Optimize the appropriate concentration beforehand.

3. If the membrane is washed insufficiently after staining, the entire membrane might appear dark after drying. Be sure to wash the membrane in running water for at least 10 minutes.

4. Keep the stained membrane away from well-lighted area as each protein band may suffer color degradation under strong light.
Application

Detection of human IgG from human serum

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sample</th>
<th>Sample Applying Volume</th>
<th>Primary Antibody</th>
<th>Electrophoresis</th>
<th>Membrane</th>
<th>Staining Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human Serum</td>
<td>Lane 1, 5 μg</td>
<td>POD-linked Goat Anti- Human IgG</td>
<td>12.5% SDS-PAGE (35 mA, 40 minutes)</td>
<td>PVDF Membrane</td>
<td>60 minutes</td>
</tr>
<tr>
<td></td>
<td>Lane 2, 1.7 μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lane 3, 0.55 μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lane 4, 0.2 μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lane 5, 60 ng</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Colorimetric Detection 1-2: Peroxidase Stain DAB Kit (Brown Stain)

Required Reagents

Peroxidase Stain DAB Kit (Brown Stain) (Product No. 25985-50)
Dispotray (Product No. 06563-44)

Components

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Main Composition</th>
<th>Volume</th>
<th>Cap Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB Stock Solution</td>
<td>Diaminobenzidine Solution</td>
<td>10 ml x 1 btl</td>
<td>Red</td>
</tr>
<tr>
<td>Buffer Solution</td>
<td>Imidazole Buffer Solution</td>
<td>10 ml x 1 btl</td>
<td>Yellow</td>
</tr>
<tr>
<td>Substrate Reagent</td>
<td>Hydrogen Peroxide Solution</td>
<td>10 ml x 1 btl</td>
<td>Purple</td>
</tr>
</tbody>
</table>

Please note:
This kit contains mutagenic substances, so wear gloves and goggles whenever handling.

1. Working Solution Preparation

Prepare working solutions just before use and commence membrane staining process within 10 minutes. Staining with a working solution that was prepared more than 30 minutes prior might not yield good results.

Please Note:
1. Prior to commencing the above procedure, wash the measuring cylinder and plastic tray with water, then immerse in a (1 mol/l) sulfuric acid solution for at least 30 minutes. Then wash again with deionized water before drying.
2. If the Staining Stocking Solution comes into contact with skin, wash with copious amounts of water immediately as it contains mutagenic substance. Wear gloves and goggles whenever handling.

For a 10 cm x 10 cm membrane, use the following procedure:

1. Transfer 40 ml of ion-exchanged water to a freshly cleaned measuring cylinder.
2. Open the lid and inside cover of the Buffer Solution, DAB Stock Solution and Substrate Solution. Add 900 μl of each solution to the measuring cylinder and well mix.
2. Procedure

2-1. Apply primary antibody to antigen on a membrane, and wash the membrane with TBS Tween® 20 or PBS Tween® 20. When using a peroxidase-linked primary antibody, proceed to step 2-3.

2-2. Apply the peroxidase-linked secondary antibody, and wash the membrane well with TBS Tween® 20 or PBS Tween® 20.

2-3. Pour the above working solution into a clean plastic tray and immerse the membrane into the solution. Agitate the tray with a shaker at room temperature for 10-60 minutes.

Please Note:
Due to its high detection sensitivity, a high background may occur. Accordingly, optimize conditions such as reaction time and antibody concentrations beforehand.

2-4. After the image appears, remove the membrane from the plastic tray and wash it in running water for more than 10 minutes to ensure the staining action has stopped.

Please Note:
Be sure to wash the stained membrane well as any staining solution residue may cause increase background.

Application

Detection of β-actin from HL-60 cell line

<Condition>
Sample: HL-60 cell extract (1.6 μg)
Membrane: PVDF membrane
Blocking: Blocking One for 30 minutes. at room temperature
Primary Abs: Anti-β–Actin, 1 hour, at room temperature (1:5,000 Santa Cruz)
Secondary Abs: Anti-Mouse IgG HRP conjugated, 1 hour, at room temperature (1:10,000 Santa Cruz)
Detection Kit: Peroxidase Stain DAB Kit (Brown Stain)

Colorimetric Detection 1-3:
High sensitivity detection with Metal Enhancer for DAB Stain

Capable of staining a target protein with gray color using Peroxidase Stain DAB Kit (Brown Stain) (Product No. 25985-50) and Metal Enhancer for DAB Stain (Product No. 07388-24). When using this method, detection sensitivity increases more than two times over the use of Peroxidase Stain DAB Kit (Brown Stain) alone.

Required Reagents

Peroxidase Stain DAB Kit (Brown Stain) (Product No. 25985-50)
Metal Enhancer for DAB Stain (Product No. 07388-24)
Dispotrays (Product No. 06563-44)

Components

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Main composition</th>
<th>Volume</th>
<th>Cap Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB Stock Solution</td>
<td>Diaminobenzidine Solution</td>
<td>10 ml x 1 btl</td>
<td>Red</td>
</tr>
<tr>
<td>Buffer Solution</td>
<td>Imidazole Buffer Solution</td>
<td>10 ml x 1 btl</td>
<td>Yellow</td>
</tr>
<tr>
<td>Substrate Reagent</td>
<td>Hydrogen Peroxide Solution</td>
<td>10 ml x 1 btl</td>
<td>Purple</td>
</tr>
</tbody>
</table>

Please note:
This kit contains mutagenic substances, so wear gloves and goggles whenever handling.
Detection

1. Working Solution Preparation

Prepare working solutions just before use and commence membrane staining process within 10 minutes. Staining with working solution that was prepared more than 30 minutes prior might not yield good results.

Please Note:
1. Prior to commencing the above procedure, wash the measuring cylinder and plastic tray with water, then immerse in a (1 mol/L) sulfuric acid solution for at least 30 minutes. Then wash again with deionized water before drying.
2. If the Staining Stocking Solution comes into contact with skin, wash with copious amounts of water immediately because it contains mutagenic substances. Wear gloves and goggles whenever handling.

For a 10 cm x 10 cm membrane, use the following procedure:

1. Transfer 40 ml of Metal Enhancer for DAB Stain to a clean measuring cylinder.
2. Open the lid and inside cover of the Buffer Solution, DAB Stock Solution, and Substrate Solution. Add 900 μl of each solution to the measuring cylinder and well mix.

2. Procedure

2-1. Apply primary antibody to antigen on a membrane, then wash the membrane well with TBS Tween® 20 or PBS Tween® 20. When using a peroxidase-linked primary antibody, proceed to step 2-3.
2-2. Apply the peroxidase-linked secondary antibody, and then wash the membrane well with TBS Tween® 20 or PBS Tween® 20.
2-3. Pour the working solution into a clean plastic tray and immerse the membrane into the solution. Then agitate the tray with a shaker at room temperature for 10-60 minutes.

Please Note:
Due to its high detection sensitivity, high background may occur. Accordingly, optimize the conditions such as reaction time and antibody concentrations beforehand. Use of a disposable plastic tray is recommended as dyes might be absorbed into stainless steel trays.

2-4. After an image appears, remove the membrane from the plastic tray and wash it with running water for more than 10 minutes to ensure the staining reaction has stopped.

Please Note:
1. Be sure to wash the stained membrane well as any staining solution residue may increase background.
2. Keep the stained membrane away from well-lighted area as each protein band may suffer color degradation under the strong light.

Comparison of Sensitivity with Dot Blot

<table>
<thead>
<tr>
<th>HRP conjugated antibody (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
</tr>
</tbody>
</table>

- Peroxidase Stain DAB Kit is more sensitive than competitors' even though staining with this kit alone.
- Peroxidase Stain DAB Kit together with Metal Enhancer increases sensitivity more than two times.
Colorimetric Detection 2 : BCIP-NBT Solution Kit

Required Reagents

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Main composition</th>
<th>Volume</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining Stock Solution</td>
<td>5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitrotetrazolium blue chloride</td>
<td>2 ml x 1 btl</td>
<td>Freezer</td>
</tr>
<tr>
<td>Buffer Solution</td>
<td>Tris-HCl Buffer</td>
<td>200 ml x 1 btl</td>
<td>Refrigerator</td>
</tr>
</tbody>
</table>

1. Working Solution Preparation

Prepare working solutions just before use and commence membrane staining process within 10 minutes. Staining with working solution that was prepared more than 30 minutes prior might not yield good results.

*Please note:*

1. Prior to commencing the above procedure, wash the measuring cylinder and plastic tray with water, then immerse in a (1 mol/L) sulfuric acid solution for at least 30 minutes. Then wash again with deionized water before drying.
2. If the Staining Stocking Solution comes into contact with skin, wash with copious amounts of water immediately because it has mutagenic substances. Wear gloves and goggles whenever handling.

For a 10 cm x 10 cm membrane, use the following procedure

1. Check that the lid is tightly closed, then ensure that the Buffer Solution (included in the kit) is well mixed by turning the bottle upside down and then back upright.
2. Transfer 50 ml of Buffer Solution into a clean measuring cylinder, and then add 0.5 ml of Staining Stock Solution (included in the kit) and well mix. If preparing working solutions for use at other volumes, mix 100 parts Buffer Solution to 1 part Staining Stock Solution.

2. Procedure

1. Apply primary antibody to antigen on a membrane and then wash the membrane well with TBS Tween® 20. When using an alkaline phosphatase-linked primary antibody, proceed to step 3.
2. Apply the alkaline phosphatase-linked secondary antibody, and wash the membrane well with TBS Tween® 20.

*Please note:*

1. Do not wash the membrane with PBS Tween® 20 because the presence of phosphates that are derived from solution decrease enzymatic activity levels. Make sure to wash with TBS Tween® 20.
2. If the alkaline phosphatase-labeled antibody concentration is too high, the background color is likely to be high as well. Optimize the appropriate concentration beforehand.

3. Pour the working solution into a clean plastic tray, and immerse the membrane into the solution. Agitate the tray with a shaker at room temperature.
4. After an image appears, remove the membrane from the plastic tray, and wash it with running water for more than 10 minutes to ensure staining reaction has stopped.

*Please note:*

Make sure to wash the stained membrane well as any staining solution residue may cause high background color.
**Application**

Detection of human IgG from human serum

<table>
<thead>
<tr>
<th>M</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgG, Heavy Chain</td>
<td>Human IgG, Light Chain</td>
<td>Leading End Marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**<Condition>**

Sample: Human Serum  
Sample Applying Volume:  
Lane 1, 5 μg  
Lane 2, 1.7 μg  
Lane 3, 0.55 μg  
Lane 4, 0.2 μg  
Lane 5, 60 ng  
Lane 6, 20 ng  

Membrane: PVDF Membrane  
Primary Antibody: Goat anti-Human IgG  
Secondary Antibody: ALP-linked rabbit anti-goat IgG  
Staining Time: 30 minutes  
Electrophoresis: 12.5% SDS-PAGE (35mA, 40 minutes)

---

**High Sensitivity Detection with Streptavidin Biotin Complex Peroxidase Kit**

**Required Reagents**

![Streptavidin Biotin Complex Peroxidase Kit](30462-30)  
![Chemi-Lumi One L](07880-70)

1. Preparation of Working Solution from the Streptavidin Biotin Complex Peroxidase Kit

**Components**

<table>
<thead>
<tr>
<th>Reagent of Dispenser Name</th>
<th>Main composition or Purpose of Use</th>
<th>Volume</th>
<th>Package</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>Streptavidin Solution</td>
<td>2 ml x 1 btl</td>
<td>Blue</td>
</tr>
<tr>
<td>Solution B</td>
<td>Biotinylated Peroxidase Solution</td>
<td>2 ml x 1 btl</td>
<td>Green</td>
</tr>
<tr>
<td>Empty Bottle C</td>
<td>Bottle for Mixing Solution A and Solution B</td>
<td>for 10 ml x 1 btl</td>
<td>Purple</td>
</tr>
</tbody>
</table>

1-1. Add 5 ml of PBS Tween® 20 or TBS Tween® 20 to an empty bottle C.  
1-2. Add 2 drops of Solution A and B to bottle C, seal the bottle and mix well by turning it upside down and back upright. Allow the mixture to stand at room temperature for 30 minutes.

**Please note:**  
1. One drop of Solution A and B is approximately 45 μl.  
2. If stored in a refrigerator, the working solution will remain stable for a few days.

2. Prepare of Chemi-Lumi One L working solution

**Components**

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Main composition</th>
<th>Volume</th>
<th>Package</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>Luminol Solution</td>
<td>250 ml x 1 btl</td>
<td>Brown Plastic Bottle</td>
</tr>
<tr>
<td>Solution B</td>
<td>Peroxide Solution</td>
<td>250 ml x 1 btl</td>
<td>White Plastic Bottle</td>
</tr>
</tbody>
</table>

Mix 1 part Solution A with 1 part Solution B.

**Please note:**  
Prepare the working solution just before use. Prepare 0.125 ml of working solution per cm² of membrane.
3. Antigen-Antibody Reaction

**Reaction of primary antibody**

1. Dilute the primary antibody with TBS Tween® 20 or Signal Enhancer HIKAR (refer to p.23 for detail), and apply it to the antigen on a membrane for 1 hour by shaking.

*Please note:*
*Optimize the appropriate antibody concentration and reaction time beforehand.*

2. Immerse the membrane in TBS Tween® 20 and then agitate for 5 minutes using a shaker. Repeat.

**Reaction of secondary antibody**

1. Apply the biotin-labeled secondary antibody to the membrane for 1 hour at room temperature.

2. Immerse the membrane in TBS Tween® 20 and then agitate for 5 minutes using a shaker. Repeat.

4. Procedure

1. Dilute the working solution from the Streptavidin Biotin Complex Peroxidase Kit at a ratio of 1:10 with PBS Tween® 20 or TBS Tween® 20, and then apply the biotin-labeled secondary antibody.

2. Wash by immersing the membrane that was done Antigen-Antibody reaction in PBS Tween® 20 or TBS Tween® 20 and then agitate for 5 minutes using a shaker. Repeat the wash process three times.

3. Prepare the Chemi-Lumi One L working solution.

4. Grasp the membrane with tweezers and remove the excess PBS Tween® 20 or TBS Tween® 20 by touching the edge of membrane with a Kimwipe® laboratory wipe.

5. Place the membrane on a piece of plastic wrap with the transferred proteins side facing up.

*Please note:*
*Be careful to prevent air bubbles from forming between the membrane and plastic wrap.*

6. For peroxidase and luminol reactions, pour the Chemi-Lumi One L working solution on the membrane and allow to stand for 1 minute.

7. Grasp the membrane with tweezers and remove excess working solution by touching the edge of membrane to Kimwipe® laboratory wipe. Next, place the membrane onto a new large sheet of plastic wrap with the transferred proteins side facing down and wrap securely.

8. Place the wrapped membrane on the film cassette with the transferred proteins side facing up.

*(Please proceed to the dark room steps described below)*

9. Place the X-ray film on the wrapped membrane and close the film cassette. Then expose for 3 minutes.

10. Take out the X-ray film from the film cassette and immerse it in developer until the band image is visible.

11. Immerse the X-ray film in a stop solution, 0.3% acetic acid aqueous solution.

*Please note:*
*Please use 0.3% acetic acid aqueous solution for the stop solution. To prevent degradation, we recommend preparing a fresh stop solution prior to each use.*

12. Immerse the X-ray film in the fixer.

13. Thoroughly wash the X-ray film in running water to remove any remaining fixer. Then hang the X-Ray film to dry.

*Please Note:*
*When Blocking One is used as the blocking agent on detection with avidine-biotin reaction, the bovine-derived proteins in the Blocking One and avidine might cause cross-reactions. Therefore, pretest it in advance.*
Improvement the Specificity of Antigen-Antibody Reaction with Signal Enhancer HIKARI

Required Reagents

1. Procedure

1-1. For western blot, separate the proteins via electrophoresis and transfer them from the polyacrylamide gel onto a PVDF membrane or nitrocellulose membrane. For dot blot, spot the proteins directly onto the membrane without electrophoresis or transfer.

1-2. Block the membrane using Blocking One or Blocking One-P.

1-3. Dilute the primary antibody with HIKARI Solution A. If the primary antibody is linked an enzyme for detection, dilute it with HIKARI Solution B, and skip step 1-5. Optimize the dilution rate by referring to the recommendations of the antibody supplier. Immerse the membrane in the diluted antibody solution, and then agitate at room temperature for 1 hour.

1-4. Immerse the membrane in TBS Tween® 20 and then agitate for 5 minutes using a shaker. Repeat the wash process three times.

1-5. Dilute the secondary antibody with HIKARI Solution B. Optimize the dilution rate by referring to the recommendations of the antibody supplier.

1-6. Immerse the membrane in TBS Tween® 20 and then agitate for 10 minutes using a shaker. Repeat the wash process three times.

1-8. Continue with the appropriate detection procedure to detect the target protein.

Application

Example 1) Sensitivity and specificity improvement

For western blotting, the advantage obtained by diluting the primary and secondary antibody with HIKARI Solution instead of a conventional buffer like TBS Tween® 20 or PBS Tween® 20 is that the sensitivity and specificity of antibodies improve.

Detection enhancement of pJNK

Detection enhancement of Rac1
Use of Chemi-Lumi One Super or Chemi-Lumi One L and Signal Enhancer HIKARI for western blotting and ELISA can ensure both high detection sensitivity and low background.

**Example 2) High sensitivity detection with Signal Enhancer HIKARI and Chemi-Lumi One Super or Chemi-Lumi One L**

<table>
<thead>
<tr>
<th>Product</th>
<th>Membrane</th>
<th>Transfer</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemi-Lumi One L</td>
<td>PVDF membrane</td>
<td>15V, 50 minutes</td>
<td>mouse anti-β-actin (C4)</td>
<td>anti-mouse IgG (Goat)</td>
<td>LAS-3000, Expose time 3 minutes</td>
</tr>
<tr>
<td>Chemi-Lumi One L + HIKARI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product A, Competitor G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemi-Lumi One Super</td>
<td>PVDF membrane</td>
<td>15V, 50 minutes</td>
<td>mouse anti-β-actin (C4)</td>
<td>anti-mouse IgG (Goat)</td>
<td>LAS-3000, Expose time 3 minutes</td>
</tr>
<tr>
<td>Chemi-Lumi One Super + HIKARI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product B, Competitor G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sample:** HeLa cell extraction

Lane 1. 1667 ng/well
Lane 2. 333 ng/well
Lane 3. 67 ng/well
Lane 4. 13 ng/well
Lane 5. 3 ng/well

**Troubleshooting**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak signals</td>
<td>Low protein concentration after electrophoresis.</td>
<td>Begin by using samples with as a high concentration as possible during electrophoresis. Serial declining protein dilution testing is useful for determining optimal concentrations.</td>
</tr>
<tr>
<td>Low antibody concentration</td>
<td></td>
<td>Determine optimal antibody concentration by dot blotting.</td>
</tr>
<tr>
<td>Insufficient transfer to membrane</td>
<td></td>
<td>Increase electric current or transfer time. Usually the higher the gel concentration the lower the transfer efficiency. If a gradient gel is used, the difference in transfer efficiency between high and low molecular weight proteins is increased. Efficiency may be improved by switching to wet transfer from semi-dry.</td>
</tr>
<tr>
<td>Membrane transfer time too long and/or electric current too high</td>
<td>If using a nitrocellulose membrane, excessive transfer can cause proteins to permeate across the membrane to the opposite side. In such cases, reduce the electric current or shorten transfer times. Changing to a PVDF membrane may also help.</td>
<td></td>
</tr>
<tr>
<td>Colorless band center</td>
<td>Antibody concentration too high</td>
<td>Depending on the detection reagent used, chemiluminescence may be suppressed by excessive signals. Determine the optimal antibody concentration by dot blotting.</td>
</tr>
<tr>
<td>Too many extra bands</td>
<td>Antibody concentration too high</td>
<td>Excessive antibody concentrations can increase nonspecific signals. Determine optimal antibody concentration by dot blotting.</td>
</tr>
<tr>
<td></td>
<td>Protein concentration too high</td>
<td>Apply less concentrated protein during electrophoresis. Serial declining protein dilution testing is useful for determining optimal concentrations.</td>
</tr>
<tr>
<td>Insufficient blocking</td>
<td></td>
<td>Depending on the type of antigen and antibody, blocking success or failure can depend significantly on the type and concentration of the blocking agent used.</td>
</tr>
<tr>
<td>Insufficient washing</td>
<td></td>
<td>Increase washing frequency.</td>
</tr>
</tbody>
</table>
Comparison Data with Competitors

Chemi-Lumi One Super

Comparison Data 1
High Sensitivity for detection and low background of β-actin

A sensitivity and background comparison between Chemi-Lumi One Super and a competitor substrate was performed. To increase sensitivity, the prolongation of exposure time and the application of Signal Enhancer HIKARI treated. As can be seen in the photo below, the competitor’s substrate shows high background. The combination of Chemi-Lumi One Super and Blocking One (used as the blocking reagent) or Signal Enhancer HIKARI shows a lower background than seen on the competitor’s substrate.

Comparison Data 2
High sensitivity detection using Chemi-Lumi One Super

Chemi-Lumi One Super is much more sensitive than Competitor B Substrate.

Comparison Data 3
Reduced exposure time

Chemi-Lumi One Super can obtain the same sensitivity with a much shorter exposure time than competitor B.

<Condition>
Sample: HL-60 whole cell lysate
Lane 1. 0.5 μg
Lane 2. 0.25 μg
Lane 3. 0.125 μg
Blocking: Chemi-Lumi One Super (Product No. 02230-30), Blocking One (Product No. 03953-95), 30 minutes.
Competitor A: Blocking reagent, 60 minutes.
Primary Antibody: Mouse anti-β-actin (Santa Cruz: #sc-47778), 1:1000
Secondary Antibody: Goat anti-mouse IgG-HRP (Santa Cruz: #sc-2005), 1:10,000
Reaction time: Chemi-Lumi One Super (Product No. 02230-30), 1 minute
Competitor A: 5 minutes
Detector: LAS-3000 (Sensitivity: High, Expose time: 1 minute and 10 minutes.)
Comparison Data 4
Antibody amount reduction
Chemi-Lumi One enables customers to reduce the amount of valuable primary antibodies.

Comparison Data 5
Antigen amount reductions
Chemi-Lumi One enables customers to reduce the amount of antigens.

Chemi-Lumi One L
Longer light emission – strong light emission for more than 120 minutes
Highly sensitive – intense signal with low background
Fast – rapid blot substrate processing of blot

Comparison Data 6
Increased sensitivity and longer duration

Chemi-Lumi One L is more sensitive and provides longer signal duration than Competitor C.
Western blot stripping

Western blot stripping can…

1. detect the target protein using a few primary antibodies that recognize different epitopes on one transferred membrane.
2. detect a number of interested proteins on the same transferred membrane.

Therefore, this is an important method for researchers who are tasked with handling costly sample proteins in small amounts.

Please note:
When preparing the stripping solution by yourself, it is necessary to add 2-mercaptoethanol, which must be heated and has an unpleasant smell. However, WB Stripping Solution and WB Stripping Solution Strong do not contain 2-mercaptoethanol and can be incubated at room temperature.

Comparison of Each Method

<table>
<thead>
<tr>
<th>compares</th>
<th>Conventional Method</th>
<th>WB Stripping Solution &amp; WB Stripping Solution Strong</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-ME</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Warming</td>
<td>50°C</td>
<td>No</td>
</tr>
<tr>
<td>Treating time</td>
<td>30 minutes</td>
<td>Approx. 15 minutes</td>
</tr>
<tr>
<td>Suggested Membrane</td>
<td></td>
<td>PVDF membrane</td>
</tr>
</tbody>
</table>

Required Reagent

- Blocking One (Product No. 03953-95)
- Chemi-Lumi One L (Product No. 07880-70)
- WB Stripping Solution Strong (Product No. 05677-65)
- WB Stripping Solution (Product No. 05364-55)

Protocol

For blotting membrane (mini gel size), use 20 ml of this solution.

1. For WB Stripping Solution. Wash the chemiluminescence stain treated blotting membrane with PBS Tween® 20 or TBS-Tween for 5 minutes.
2. Pour enough room temperature stripping solution into a plastic tray to fully immerse the blotting membrane.
3. Dispose of the stripping solution and wash the membrane with PBS Tween® 20 or TBS Tween® 20 for 5 minutes.
4. Washed membranes can be used for a second antigen-antibody reaction.

Antibody Confirmation

Any antibodies remaining on the membrane after the stripping process that follows antigen-antibody reaction can cause false-positive signals.

1. Check whether any labeled antibody complex remains after stripping by repeating the chemiluminescence detection process.
2. Upon repeated reaction with a secondary antibody, check whether any non-labeled primary antibodies remains by performing chemiluminescence detection.
Application

Example 1)
Chemiluminescence detection with Chemi-Lumi One L

Example 2)
WB Stripping Solution (Data courtesy of Akaike Lab, Tokyo Institute of Technology)

Example 3) Difference between WB Stripping Solution and WB Stripping Solution Strong
Apply HPR-labeled anti-GST antibody to 5000 ng, 500 ng, 50 ng, or 5 ng (as desired) of c-Myc-GST antigen on a PVDF membrane, then remove the antibody by agitating gently for 10 minutes using one of the following stripping solutions.

After incubation the membrane with each above solution, detected the HRP-linked anti GST antibody that remain on the membrane with chamiluminescence kit. “e” shows it is detected the remaining antigens of membrane "d" with HRP-linked anti c-Myc antibody.

Usage Note

<table>
<thead>
<tr>
<th>Material required: Tray and tweezers</th>
<th>WB Stripping Solution</th>
<th>Plastic and metal equipment can be used.</th>
<th>WB Stripping Solution Strong</th>
<th>All equipment such as trays and tweezers expected to come into contact with this solution should be made of plastic. Use of metal equipment can compromise the performance of this solution.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Character</td>
<td>Acidic solution, pH2-3</td>
<td>Has reductive property</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safety</td>
<td>Wear protecting chemical-resistant clothes, gloves and safety goggles. If the product accidentally comes into contact with skin, wash the affected area thoroughly with water. Contact a physician if necessary.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Application</td>
<td>Use this product at room temperature. This solution should be stored at 4°C, but the surfactant may precipitate during long periods of cold storage. This may cause changes in the concentration. Therefore, wait until the precipitation has dissolved before using.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Alternately Use</td>
<td>This solution is not suitable for colorimetric stained membranes treated with agents such as TMB, DAB and 4-Chloronaphtol.</td>
<td></td>
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</tr>
<tr>
<td>Alternively Use</td>
<td>If WB stripping solution and WB stripping solution strong are mixed, a white precipitation will occur. When using both products in turn, the blotting membrane must be washed thoroughly 3-5 times with a proper buffer or deionized water. Additionally, please use separate trays for the each stripping solution.</td>
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