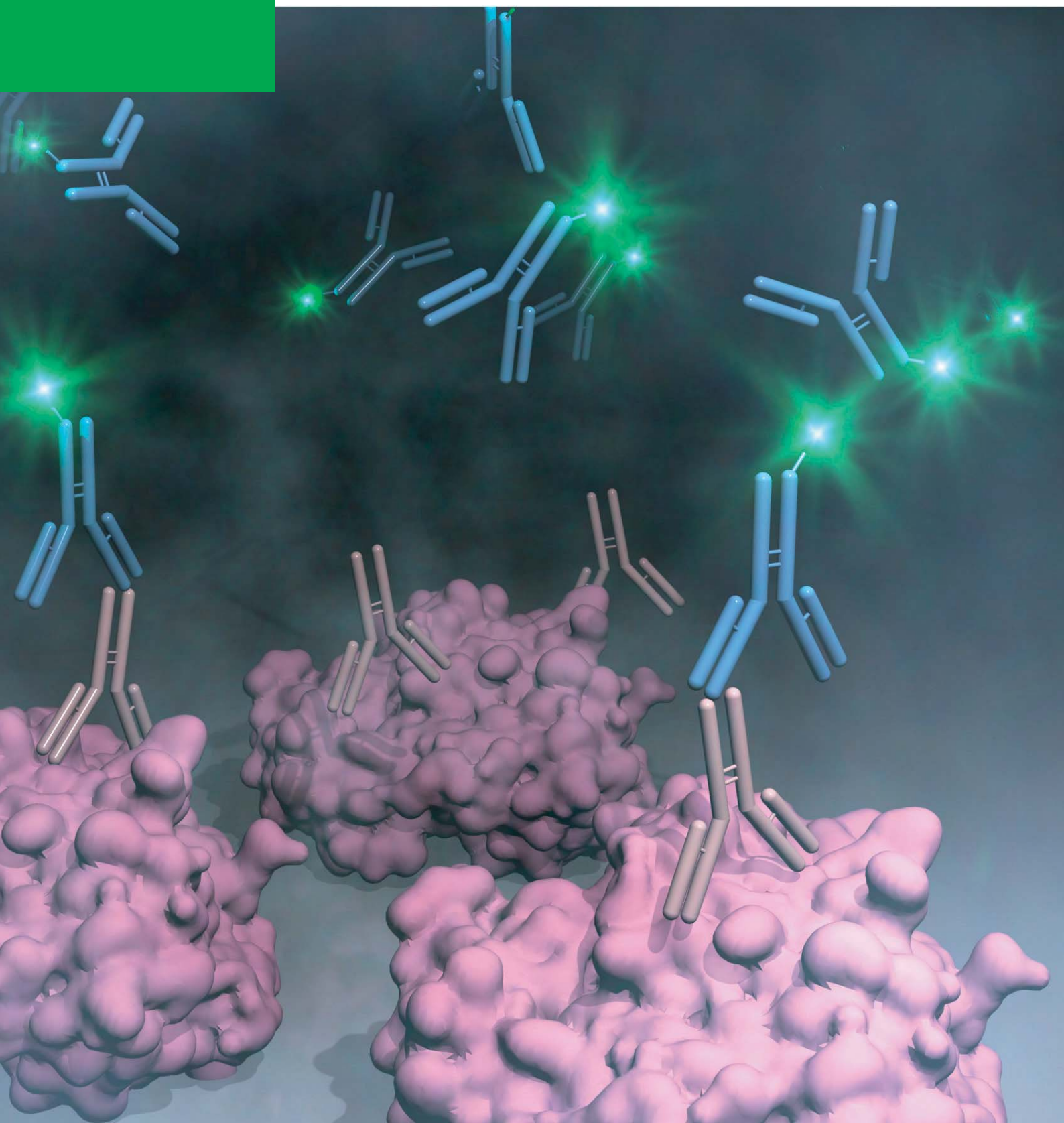
nacalai tesque
The quality for certainty.

Western Blot Protocol

Ver. 1.2



Contents

Introduction

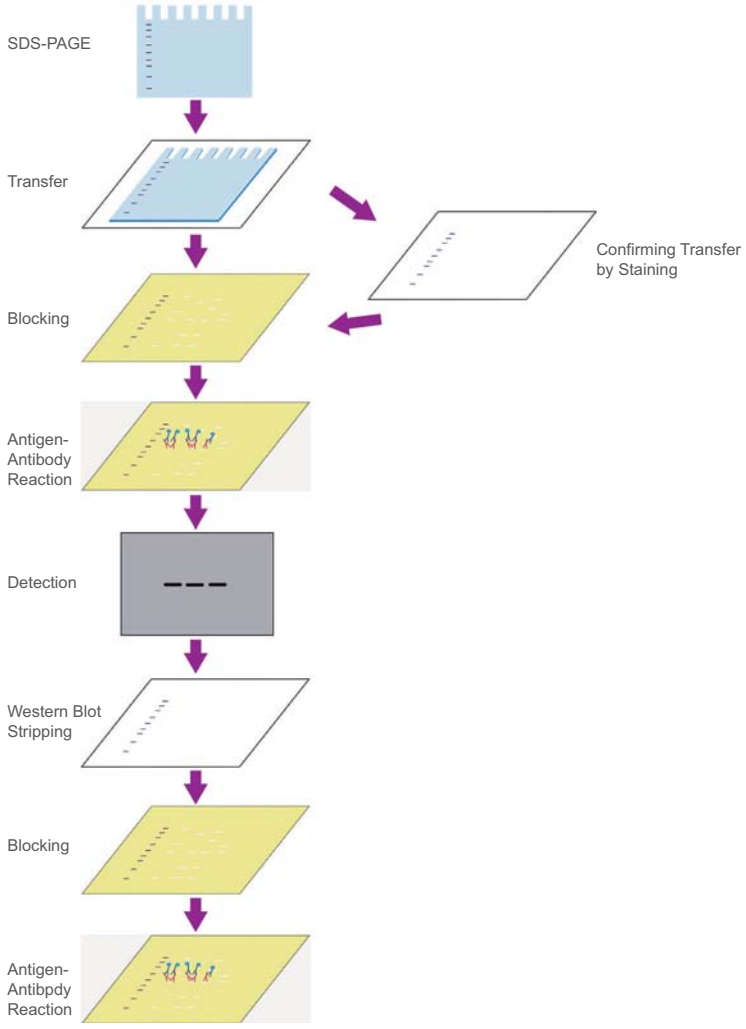
Western blotting is a protein detection method performed on nitrocellulose or PVDF membranes. After electrophoresis, proteins are transferred onto the membrane where they are reacted with specific antibodies. Proteins of interest are then identified using a detection reagent.
The blotting method itself 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

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

Authority: Baiozikken de shippaishinai! Kensyutsu to teiryo no kotsu. Naoya Moriyama p.41

Blotting Membranes Comparison

	PVDF (Polyvinylidene Difluoride) Membrane	Nitrocellulose Membrane
Property	Hydrophobic	Hydrophilic
Strength of membrane	Strong	Weak
Retaining amount of proteins	Approx. 250 µg / cm^2	Approx. 100 µg / cm^2
Cost	High	Low

Authority: Baiozikken de shippaishinai! Kensyutsu to teiryo 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

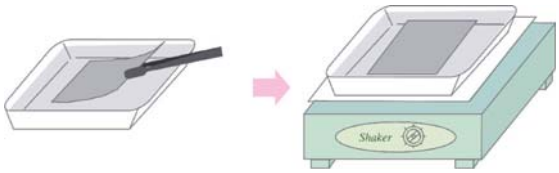
Required Reagents

Dispotray (for minigel staining)
(Product No. 06563-44)

Semi-dry Blotting Buffer Solution
for Western Blotting
(Product No. 30650-31)

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.



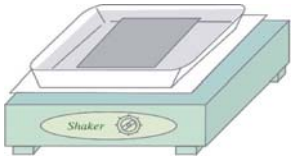
Please note:

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).

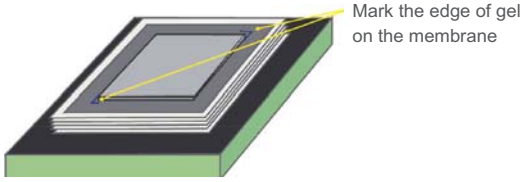
3-3. Immerse the filter papers for a second, one by one, in the Tray 3, and then use the wall of the tray (if necessary) to remove excess liquid. Place the filter papers in a row on the positive electrode plate, beginning from the edge. The six pieces of filter paper should overlap exactly.

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.

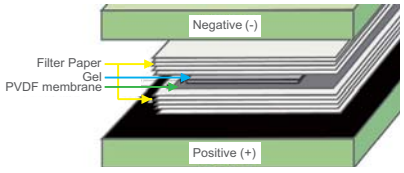
3-5. Place the polyacrylamide gel equilibrated according to the step 2, preparation of polyacrylamide gel, onto the PVDF membrane. Mark the edge of the gel on the membrane with a ballpoint pen in order to see the side of transferred protein and area easily. Then, stack the remaining six pieces of filter 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/cm^2 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 1.6 mA / cm^2. 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.

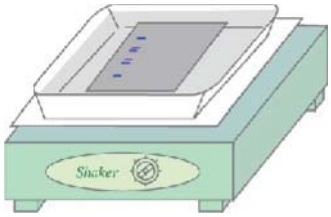
3-7. After step 3-6, disconnect the power supply from the blotting equipment, and then remove each filter paper, one by one, from the side of negative electrode plate. Next, immerse the PVDF membrane in PBS Tween® 20 or TBS Tween® 20. If it is necessary to stop this work temporarily, re-immerse the PVDF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

Please note:

When using an antibody for phosphorylated proteins deteciton, 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.



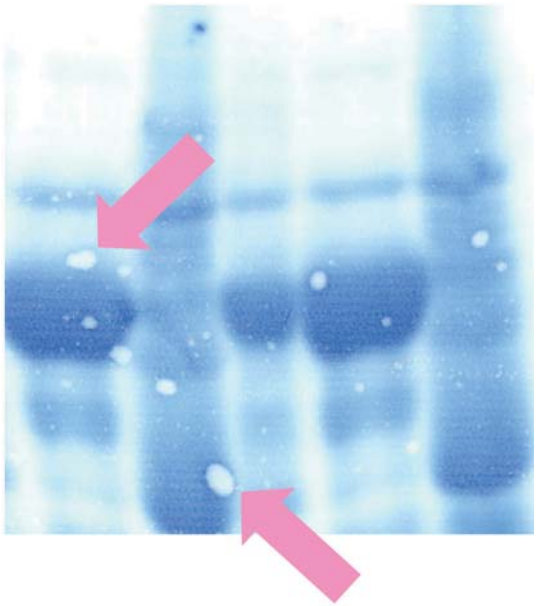
Please note:

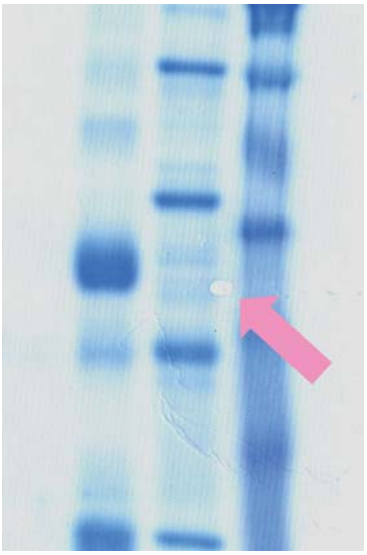
When confirming the transfer efficiency, we recommend the use of a pre-stained protein marker such as Protein Ladder One, Triple-color (Product No. 09547-74) and Pre-stained Protein Marker (Product No. 02525-35). Additionally, for confirming the transfer efficiency of whole proteins, we recommend staining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

Troubleshooting

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

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 efficeincy 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: Baiozikken 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

	CBB Stain One	Rapid Stain CBB Kit
Staining Solution	No methanol or acetic acid	Contains organic solvents
Staining Time	25 minutes, including two wash steps	1 minute
Background Destaining Step	Unnecessary	Necessary
Protein Band Destaining Step	5 minutes	5-10 minutes

Protocol for Membrane Staining with CBB Stain One

Required Reagents


CBB Stain One
(Ready To Use)
(Product No. 04543-51)

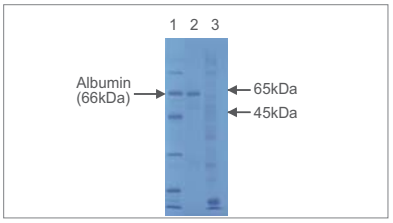

Rapid CBB Destain Kit
(Product No. 30046-74)


Protein Markers
(Product No. 29458-24)


Pre-stained
Protein Markers
(Product No. 02525-35)


Dispotray
(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.

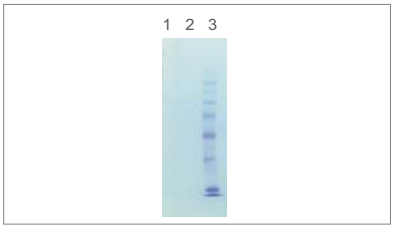
[Condition]

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

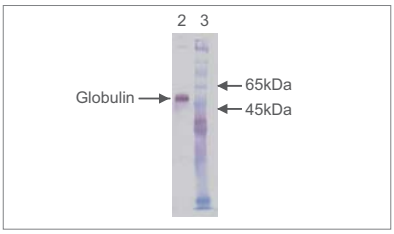
2. Destaining Stained Protein Bands for Western Blot Analysis



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.

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 Raid CBB Destaining has no affect on the antigen-antibody reaction.

Protocol for Membrane Staining with Rapid Stain CBB Kit

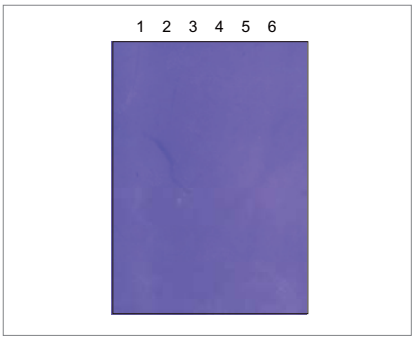
Required Reagents

Rapid Stain CBB Kit
(Product No. 30035-14)

Rapid CBB Destain Kit
(Product No. 30046-74)

Dispotray
(Product No. 06563-44)

1. Staining



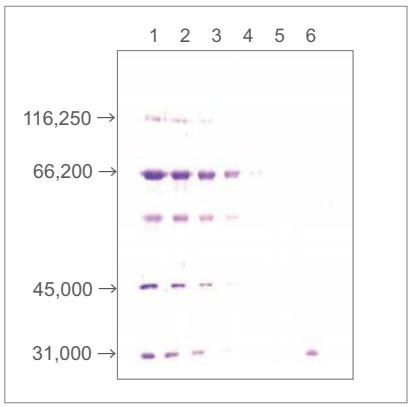
1-1. To prepare working solution, mix 30 ml of deionized or distilled water with 10 ml of solution A, then add 10 ml of solution B. This solution volume is suitable for one 10 cm x 10 cm piece of membrane. Prepare this staining solution fresh for each use.

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

Destain the CBB dyes from the membrane to confirm each protein band.



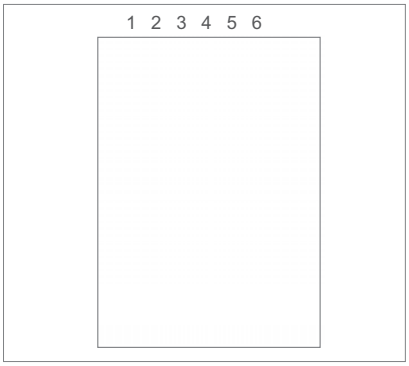
2-1. Pour 20 ml of solution A, 10 ml of solution B (included in the Rapid CBB Destain Kit) and 30 ml of deionized water into a disposable tray. This solution volume is suitable for one 10 cm x 10 cm piece of membrane.

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 transfering 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

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

Protocol for Blocking One

Required Reagents

Blocking One
(Product No.03953-95)

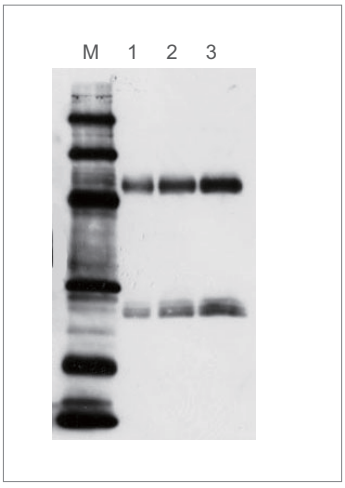
Dispotray
(Product No. 06563-44)

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

Detetion 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)

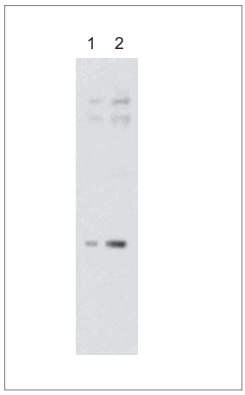
Protocol

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



<Condition>

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

FAQ

Question	Solution
Is Blocking One or Blocking One-P suitable for use when diluting the primary and secondary Abs?	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.

Please note:

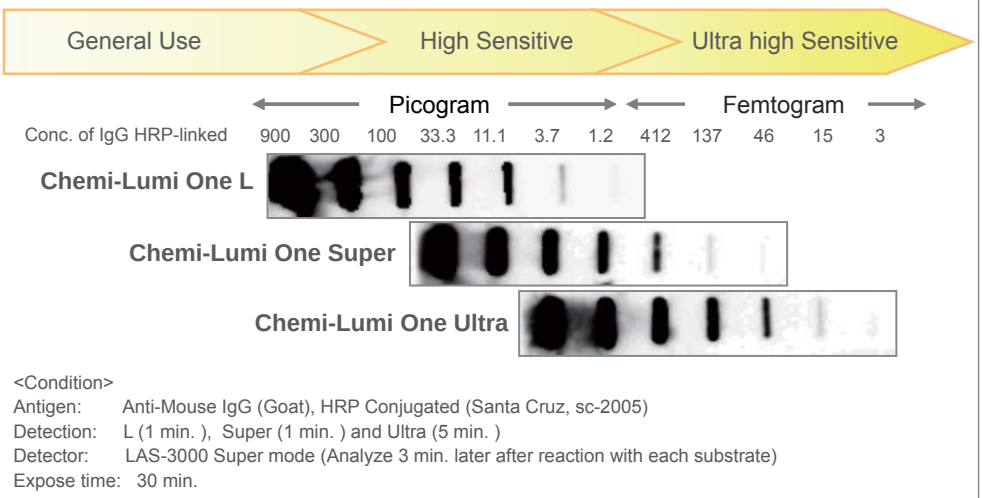
Blocking One-P should not be used to dilute alkaline phosphatase-linked antibodies.

Detection

Chemiluminescence Detection on Western Blot

We offer three types of chemiluminescence detection kit as follows.

Selection Guide

Product Name	Chemi-Lumi One L	Chemi-Lumi One Super	Chemi-Lumi One Ultra
Product No.	07880	02230	11644
Usage	General Use	High Sensitivity	Ultrahigh Sensitivity
Lower Detection Limit	**Low-picogram**	**Mid-femtogram**	**Low-femtogram**
Required Working Solution	Approx. 0.125 ml / cm^2	Approx. 0.1 ml / cm^2	Approx. 0.1 ml / cm^2
Suggested Antibody Dilution	Primary: 1:1,000-1:5,000 Secondary: 1:20,000-1:100,000	Primary: 1:1,000-1:20,000 Secondary: 1:20,000-1:200,000	Primary: 1:5,000-1:100,000 Secondary: 1:100,000-1:500,000
Reaction Period	1 min.	1 min.	5 min.
Comparable to	ECL SuperSignal Pico	ECL Prime SuperSignal Dura	ECL Select SuperSignal Femto
Sensitivity			

<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.)

Chemi-Lumi One Super Protocol

Required Reagents

Chemi-Lumi One Super
(Product No. 02230-30)

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-Limi One L = 1 : 1,000 - 1 : 5,000
Chemi-Limi 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.

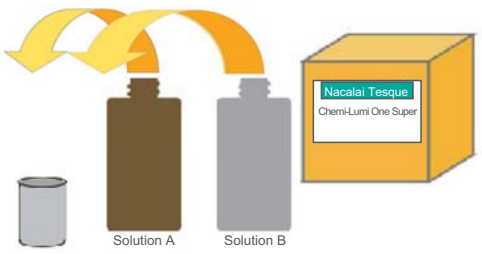
Dilution ratio of secondary antibody for each product
Chemi-Limi One L = 1 : 20,000 - 1 : 100,000
Chemi-Limi One Ultra = 1 : 100,000 - 1 : 500,000

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.

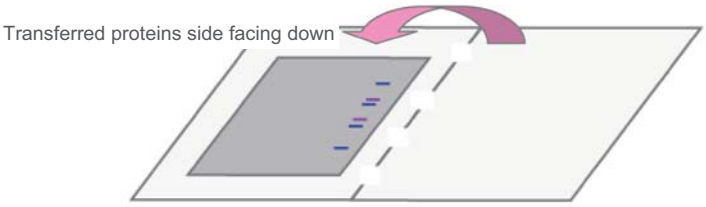
Required working solution and reaction time for each product
Chemi-Limi One L = required working solution : approximately 0.125 ml/cm^2, reaction time : 1 min.
Chemi-Limi One Ultra = required working solution : approximately 0.1 ml/cm^2, reaction time : 5 min.

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.



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.

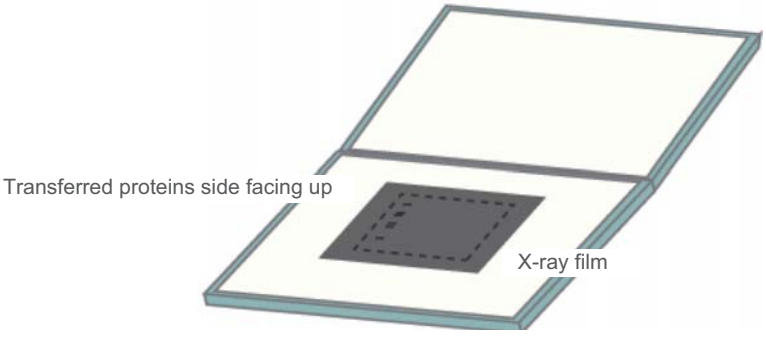
(Please proceed to the darkroom steps described below)

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:

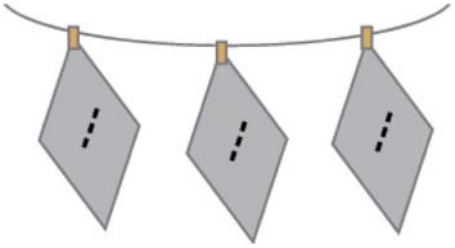
When it is necessary to use a 0.3% acetic acid aqueous solution, we recommend preparing a fresh stop solution each time to prevent solution degradation.

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

Please note:

Always be sure to follow the package directions of fixer solution.

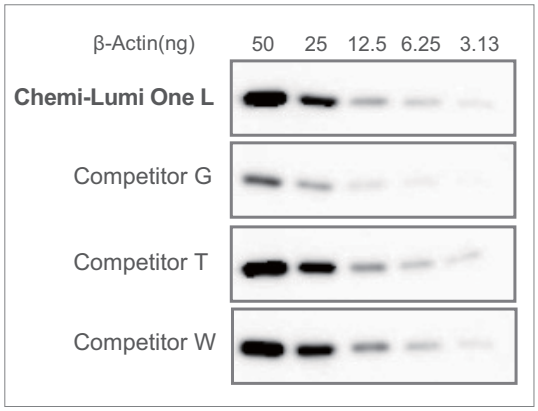
2-11. Wash the X-ray film with running water to remove fixer, then hang X-ray to dry.



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.

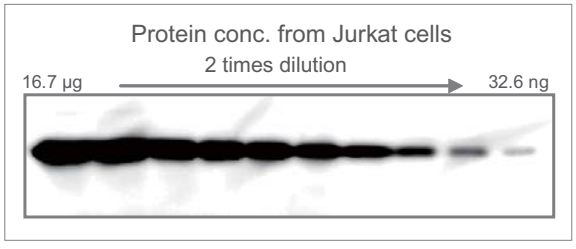


<Condition>

Gel:	10% PAGE gel
Wash:	0.1% t-TBS(1x), pH7.4
Blocking:	Blocking One
1st Ab:	Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruze sc-47778), 1:1,000
2nd Ab:	Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruze sc-2005), 1:20,000
Detection period:	Chemi-Lumi One L, 1 min. Competitor G, T, W 1min.
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.



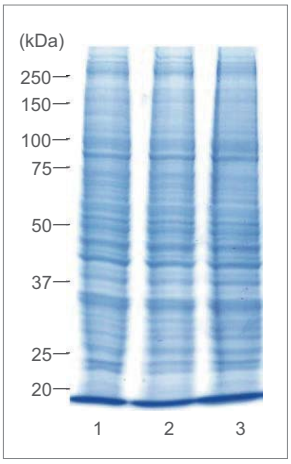
<Condition>

Gel:	10% PAGE gel
Wash:	0.1% t-TBS(x1), pH7.4
Blocking:	Blocking One
1st Ab:	Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruze sc-47778), 1:1,000
2nd Ab:	Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruze sc-2005), 1:20,000
Detection period:	Chemi-Lumi One L, 1 min.
Detector:	LAS-3000 High mode
Expose time:	30 min.

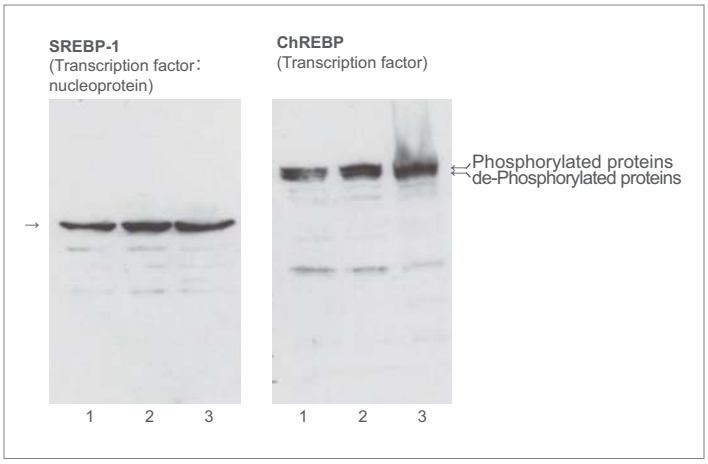
3. [Chemi-Lumi One L] Detection of transcription factor of SREBP-1 and ChREBP

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

CBB Stain
(Stained with CBB Stain One)



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

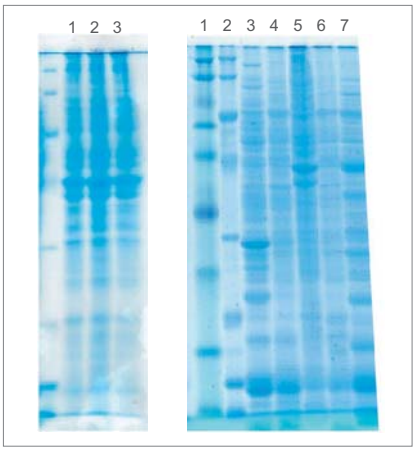


Lane 1: RIPA (w/ SDS), 2: RIPA (w/o SDS), 3:B Company (w/ SDS)
Data courtesy of associate professor Tatsuya Moriyama, Faculty of Agriculture, Kinki University

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

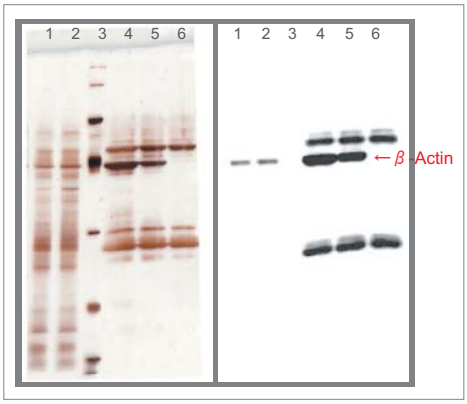


Left
Sample : Mouse stomach
Lane1 : RIPA (w/o SDS, Nacalai)
2 : RIPA (w/ SDS, Nacalai)
3 : RIPA (w/ SDS, A company)
Detection : CBB Stain One

Right
Lane1 : Pre-stained Protein Markers(Broad Range) (#02525-35)
2 : Protein Markers(10x) (#29458-24)
3 : Mouse Liver (RIPA w/ SDS, Nacalai)
4 : Mouse Kidney
5 : Mouse Stomach
6 : Mouse Brain
7 : Mouse Heart
Detection : CBB Stain One

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

Figure 2 : Gel staining and western blotting after immunoprecipitation



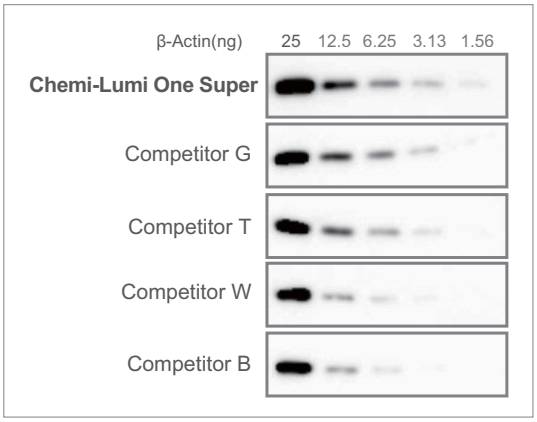
Sample : Jurkat Cell
Primary Antibody : Anti-β-Actin Mouse Monoclonal Antibody (Santa Cruz, sc-47778)
Secondary Antibody : Anti-Mouse IgG (Goat), HRP Conjugated (Santa Cruz, sc-2005)
Left : Stained with Sil-Best Stain One
Right : Detected with Chemi-Lumi One L
Lane1 : Protein extracted solution with 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-antigen.

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

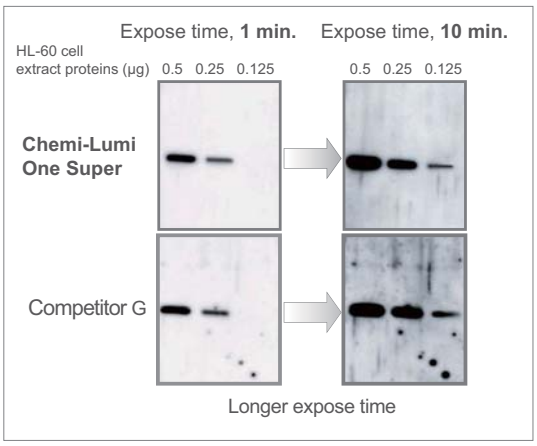
Chemi-Lumi One Super is the most sensitive of competitors under the above consition, and its expore time is only 1 min., while others are required 5 min.



<Condition>
Gel: 10% PAGE gel
Wash: 0.1% t-TBS, pH7.4
Blocking: Blocking One
1st Ab: Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruze sc-47778), 1:2,000
2nd Ab: Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruze sc-2005), 1:30,000
Detection period: Chemi-Lumi One Super, 1 min.
Competitor G, T, W, B 5 min.
Detector: LAS-3000 High mode
Expose time: 5 min.

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

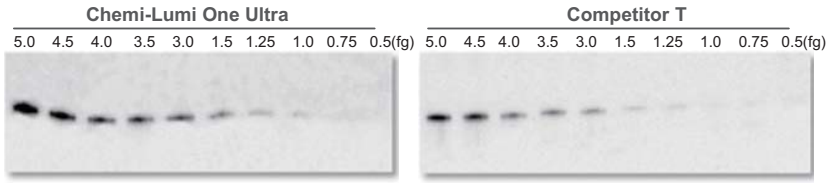
Chemi-Lumi One Super performs under lower background, so users enable expose time to be longer for achieving more sensitive analysis.



<Condition>
Gel: 10% PAGE gel
Wash: 0.05% t-TBS, pH7.4
Blocking: Blocking One
1st Ab: Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruze sc-47778), 1:1,000
2nd Ab: Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruze sc-2005), 1:10,000
Detection period: Chemi-Lumi One Super, 1 min.
Competitor G, 5 min.
Detector: LAS-3000 High mode
Expose time: 1 min. and 10 min.

7. [Chemi-Lumi One Ultra] Comparison data of sensitivity with competitors 1

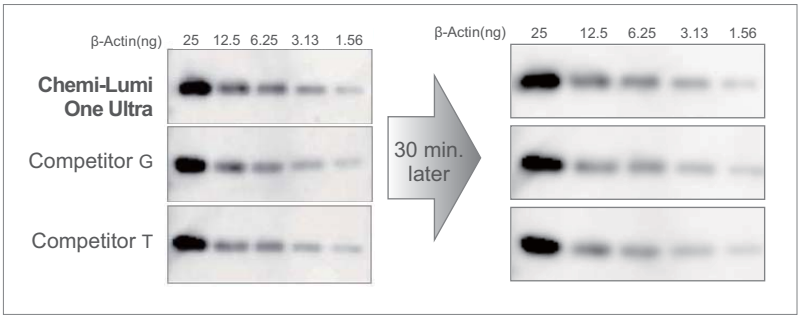
Chemi-Lumi One Ultra is an extremely sensitive chemiluminescent substrate. Western blot of identical transferrin samples. The membranes were incubated with substrate that was prepared according to the manufacturers' instructions. The membranes were exposed to film for 2 minutes.



Primary antibody : Rabbit anti-transferrin, 1: 30,000
Secondary antibody: Goat anti-rabbit IgG-HRP, 1: 200,000

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

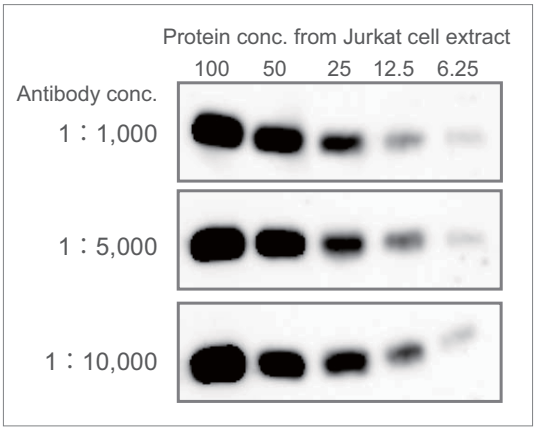
Chemi-Lumi One Ultra shows equal to or higher sensitivity compared to competitors'.



<Condition>
Gel: 10% PAGE gel
Wash: 0.1% t-TBS(x1), pH7.4
Blocking: Blocking One
1st Ab:
Anti-β-Actin Mouse Monoclonal Antibody, (Santa Cruze sc-47778), 1:300
2nd Ab:
Anti-Mouse IgG (Goat), HRP Conjugated, (Santa Cruze sc-2005), 1:1,000
Detection period: 5 min.
Detector: LAS-3000 High mode
Expose time: 3 min.

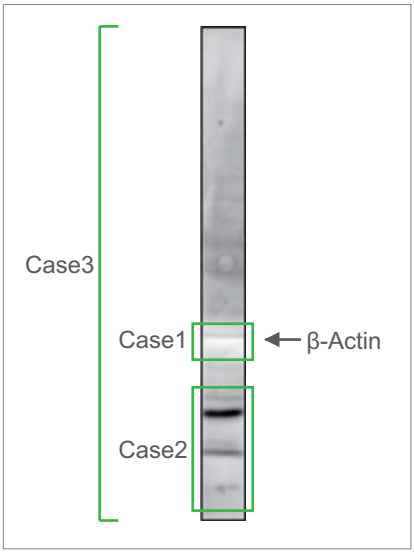
9. [Chemi-Lumi One Ultra] Comparison data of Abs concentration

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



<Condition>
Gel: 10% PAGE gel
Wash: 0.1% t-TBS(1x), pH7.4
Blocking: Blocking One
1st Ab: Anti- PCNA Mouse Monoclonal Antibody, (Santa Cruze sc-56c)
2nd Ab: Anti-Mouse IgG (Goat) HRP, (Santa Cruze sc-2005), 1:100,000
Detection period: 5 min.
Detector: LAS-3000 High mode
Expose time: 1:1,000, 3 min.
1:5,000, 5 min.
1:10,000, 8 min.

Troubleshooting



<Condition>
Sample: HL-60 cells extract (6.4 µg)
Membrane: PVDF membrane
Blocking: Blocking One (Product No. 03953-95) for 15 minutes at room temperature
Primary Abs: Anti- β -Actin for 1 hour at room temperature (1:1,000 Santa Cruz)
Secondary Abs: Anti-Mouse IgG HRP conjugated for 1 hour at room temperature (1:2,000 Santa Cruz)
Detection: Chemi-Lumi One L (Product No. 07880-70)
Detector: LAS-3000

Case	Problem	Cause	Solution
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.

Colorimetric Detection 1-1 : Peroxidase Stain Kit

Required Reagents

Peroxidase Stain Kit
(Product No. 26652-70)

Dispotray
(Product No.06563-44)

Components

Regent Name	Main Composition	Volume	Storage
Staining Stock Solution	Naphthol / Benzidine derivative solution	10 ml x 1 btl	Freezer
Buffer Solution	Phosphate-Citrate Buffer Solution, Hydrogen Peroxide	200 ml x 1 btl	Refrigerator

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.

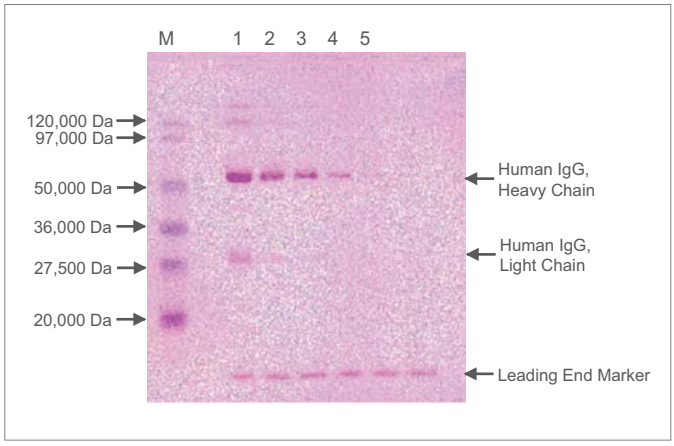
2-4. After an image appears, remove the membrane from the plastic tray and wash it in running water for more than 10 minutes to ensure the staining reaction has stopped.

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



<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
Primary Antibody:	POD-linked Goat Anti- Human IgG
Electrophresis:	12.5% SDS-PAGE (35 mA, 40 minutes)
Membrane:	PVDF Membrane
Staing Time:	60 minutes

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

Reagent Name	Main composition	Volume	Cap Color
DAB Stock Solution	Diaminobenzidine Solution	10 ml x 1 btl	Red
Buffer Solution	Imidazole Buffer Solution	10 ml x 1 btl	Yellow
Substrate Reagent	Hydrogen Peroxide Solution	10 ml x 1 btl	Purple

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.

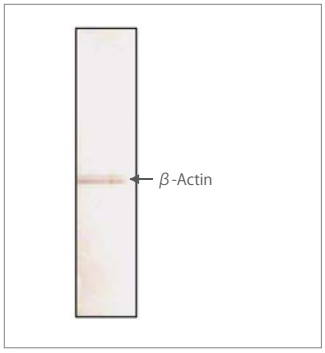
2-4. After the image appears, remove the membrane from the plastic tray and wash it in running water for more than 10 minutes to ensure the staining action has stopped.

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.

Requierd Reagents


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


Metal Enhancer
for DAB Stain
(Product No. 07388-24)


Dispotray
(Product No. 06563-44)

Components

Reagent Name	Main composition	Volume	Cap Color
DAB Stock Solution	Diaminobenzidine Solution	10 ml x 1 btl	Red
Buffer Solution	Imidazole Buffer Solution	10 ml x 1 btl	Yellow
Substrate Reagent	Hydrogen Peroxide Solution	10 ml x 1 btl	Purple

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 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:

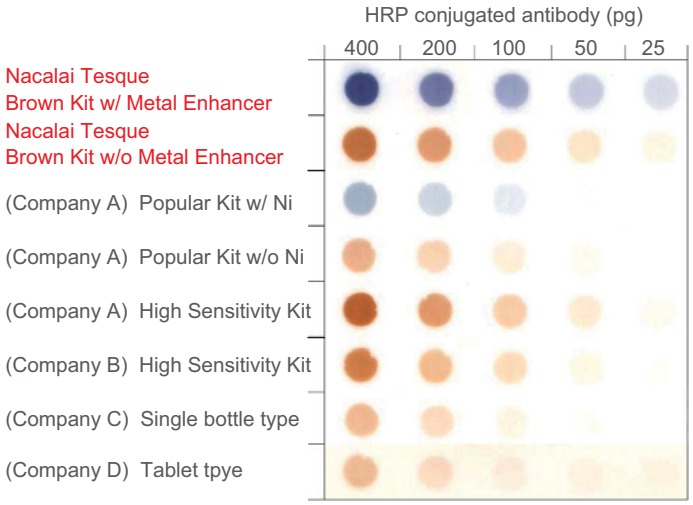
Due to its high detection sensitivity, high background may occur. Accordingly, optimize the conditions such as reaction time and antibody concentrations beforehand. Use of a disposable plastic tray is recommended as dyes might be absorbed into stainless steel trays.

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



- 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

BCIP-NBT Solution Kit
for Alkaline Phosphatase Stain
(Product No. 03937-60)

Dispotray
(Product No. 06563-44)

Components

Reagent Name	Main composition	Volume	Storage
Staining Stock Solution	5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitrotetrazolium blue chloride	2 ml x 1 btl	Freezer
Buffer Solution	Tris-HCl Buffer	200 ml x 1 btl	Refrigerator

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.*

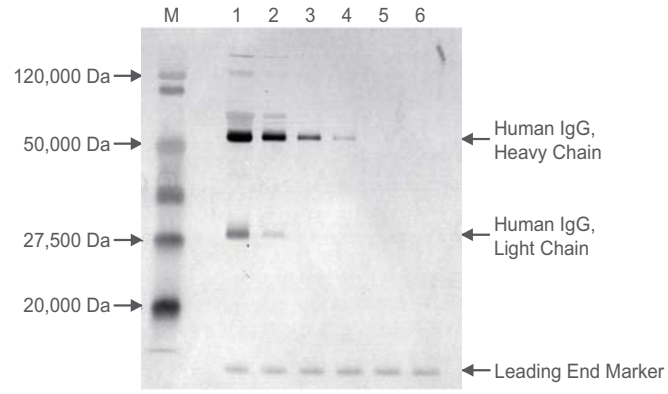
3. Pour the working solution into a clean plastic tray, and immerse the membrane into the solution. Agitate the tray with a shaker at room temperature.
4. After an image appears, remove the membrane from the plastic tray, and wash it with running water for more than 10 minutes to ensure staining reaction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



<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 rabit anti-goat IgG
Staining Time:	30 minutes
Electrophresis:	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

Reagent of Dispenser Name	Main composition or Purpose of Use	Volume	Package
Solution A	Streptavidin Solution	2 ml x 1 btl	Blue
Solution B	Biotinylated Proxidase Solution	2 ml x 1 btl	Green
Empty Bottle C	Bottle for Mixing Solution A and Solution B	for 10 ml x 1 btl	Purple

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

Reagent Name	Main composition	Volume	Packge
Solution A	Luminol Solution	250 ml x 1 btl	Brown Plastic Bottle
Solution B	Peroxide Solution	250 ml x 1 btl	White Plastic Bottle

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 cm^2 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 largre 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% acedic 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

Signal Enhancer HIKARI for Western Blotting and ELISA (Product No. 02270-81)

Chemi-Lumi One Super (Product No. 02230-30)

Chemi-Lumi One L (Product No. 07880-70)

Blocking One (Product No.03953-95)

Blocking One-P (Product No. 05999-84)

Dispotray (Product No. 06563-44)

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 the diluted antibody solution and agitate at room temperature for 1 hour.

1-7. Immerse the membrane in TBS Tween® 20 and then agitate for 10 minutes using a shaker. Repeat the wash process three times.

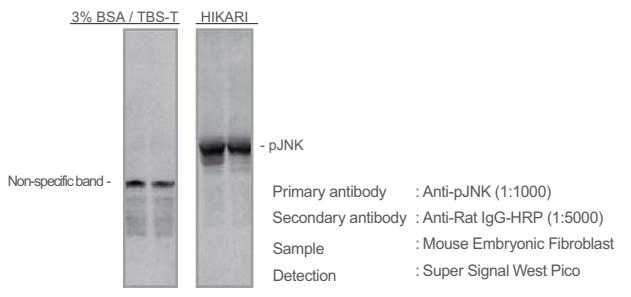
1-8. Continue with the appropriate detection procedure to detect the target protein.

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

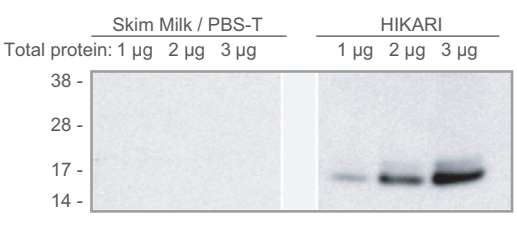


Primary antibody	: Anti-pJNK (1:1000)
Secondary antibody	: Anti-Rat IgG-HRP (1:5000)
Sample	: Mouse Embryonic Fibroblast
Detection	: Super Signal West Pico

Treatment with Signal Enhancer HIKARI resulted in clear signal enhancement and background suppression compared with the conventional method using TBS-T.

Data courtesy of Dr. S. Matsuzawa, Signal Transduction, NCI Cancer Center, Burnham Institute for Medical Research

Detection enhancement of Rac1



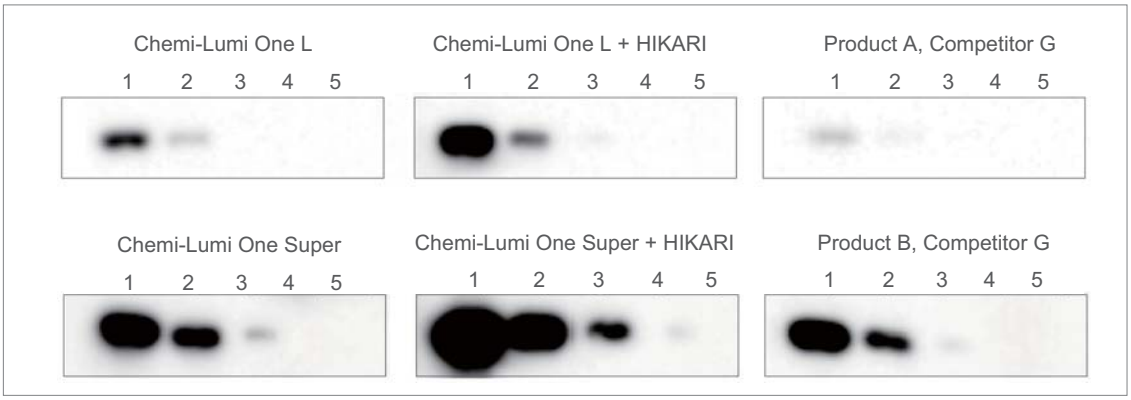
Primary antibody	: Anti-Rac1 (1:1000)
Secondary antibody	: Anti-Mouse IgG-HRP (1:2500)
Sample	: Rat Cortical Primary Neuron
Detection	: Chemiluminescence

Treatment with Signal Enhancer HIKARI resulted in clear signal, whereas dilution with conventional PBS-T resulted in no signal.

Data courtesy of Dr. K. Ishizuka, Depertment of Psychiatry, Johns Hopkins University School of Medicine

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

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.



Sample:	HeLa cell extraction	Membrene:	PVDF membrene
	Lane 1. 1667 ng/well	Transfer:	15V, 50 minutes
	Lane 2. 333 ng/well	Primary antibody:	mouse anti-β-actin (C4) (Santa Cruz: #sc-47778)
	Lane 3. 67 ng/well	Secondary antibody:	anti-mouse IgG (Goat) (Santa Cruz: #sc-2005)
	Lane 4. 13 ng/well	Detection:	LAS-3000, Expose time 3 minutes
	Lane 5. 3 ng/well		

Troubleshooting

Problem	Possible Cause	Solution
Weak signals	Low protein concentration after electrophoresis.	Begin by using samples with as a high concentration as possible during electrophoresis. Serial declining protein dilution testing is useful for determining optimal concentrations.
	Low antibody concentration	Determine optimal antibody concentration by dot blotting.
	Insufficient transfer to membrane	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 swiching to wet transfer from semi-dry.
	Membrane transfer time too long and/ or electric current too high	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.
Colorless band center	Antibody concentration too high	Depending on the detection reagent used, chemiluminescence may be suppressed by excessive signals. Determine the optimal antibody concentration by dot blotting.
Too many extra bands	Antibody concentration too high	Excessive antibody concentrations can increase nonspecific signals. Determine optimal antibody concentration by dot blotting.
	Protein concentration too high	Apply less concentrated protein during electrophoresis. Serial declining protein dilution testing is useful for determining optimal concentrations.
	Insufficient blocking	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.
	Insufficient washing	Increase washing frequency.

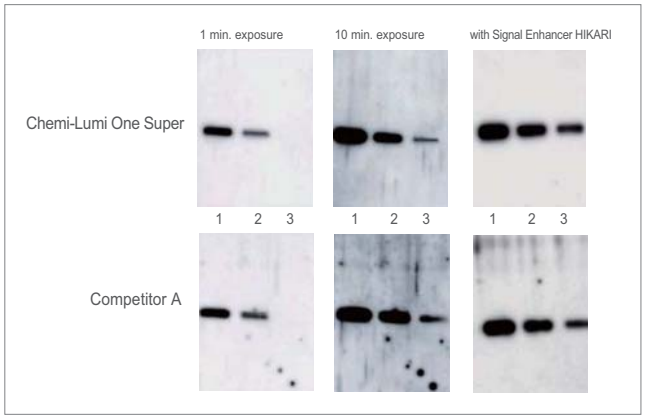
Comparison Data with Competitors

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.



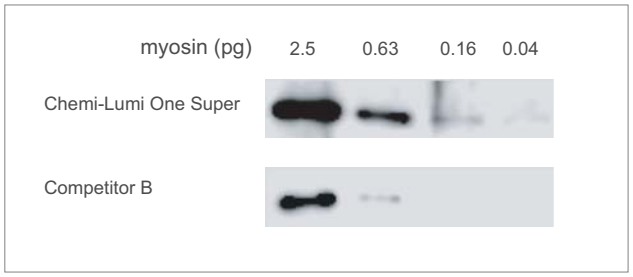
<Condition>

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 2

High sensitivity detection using Chemi-Lumi One Super

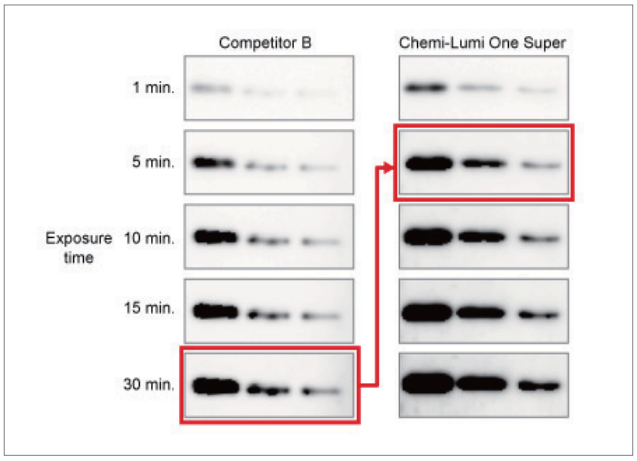
Chemi-Lumi One Super is much more sensitive than Competitor B Substrate.



Comparison Data 3

Reduced exposure time

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

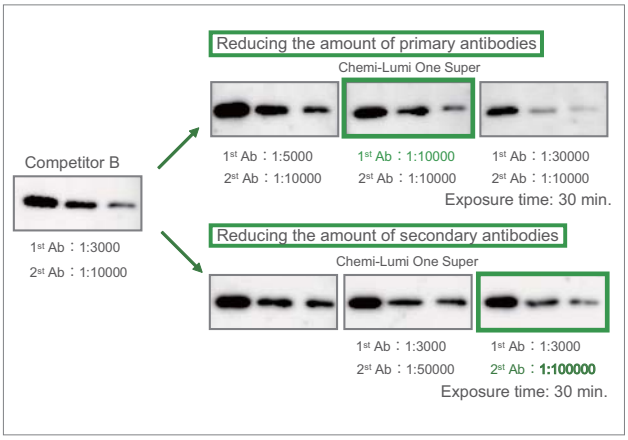


30 minutes exposure Primary Ab 1:3,000 Secondary Ab 1:10,000

Comparison Data 4

Antibody amount reduction

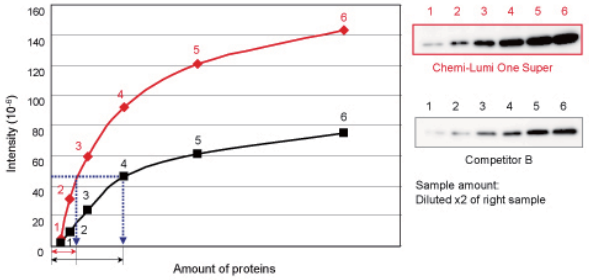
Chemi-Lumi One enables customers to reduce the amount of valuable primary antibodies.



Comparison Data 5

Antigen amount reductions

Chemi-Lumi One enables customers to reduce the amount antigens.



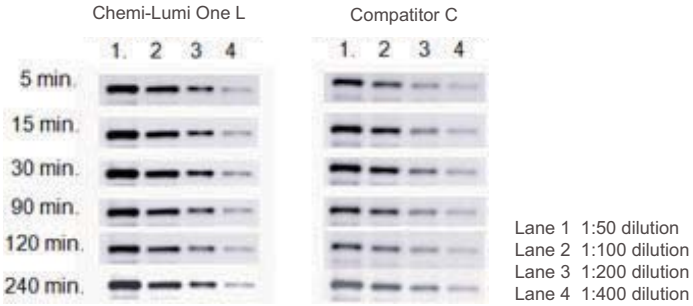
30 minutes exposure Primary Ab 1:3,000 Secondary Ab 1:10,000

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

	Conventional Method	WB Stripping Solution & WB Stripping Solution Strong
2-ME	Yes	No
Warming	50℃	No
Treating time	30 minutes	Approx. 15 minutes
Suggested Membrane	PVDF membrane	

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.
   For WB Stripping Solution Strong. Wash the chemiluminescence stain treated blotting membrane with deionized water for 1 minute.
2. Pour enough room temperature stripping solution into a plastic tray to fully immerse the blotting membrane.
3. Agitate gently for 5-15 minutes with a shaker.
4. Dispose of the stripping solution and wash the membrane with PBS Tween® 20 or TBS Tween® 20 for 5 minutes.
5. Washed membranes can be used for a second antigen-antibody reaction.

Antibody Confirmation

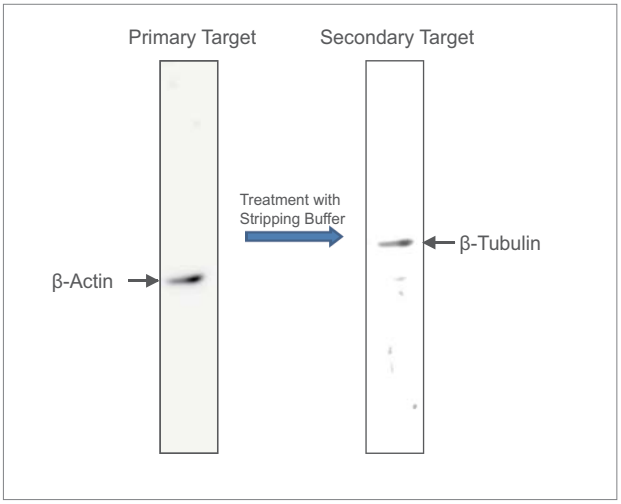
Any antibodies remaining on the membrane after the stripping process that follows antigen-antibody reaction can cause false-positive signals.

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

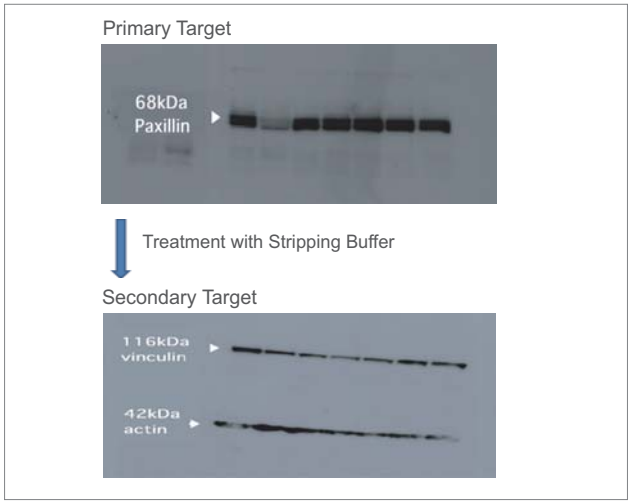
Example 1)

Chemiluminescence detection with Chemi-Lumi One L



Example 2)

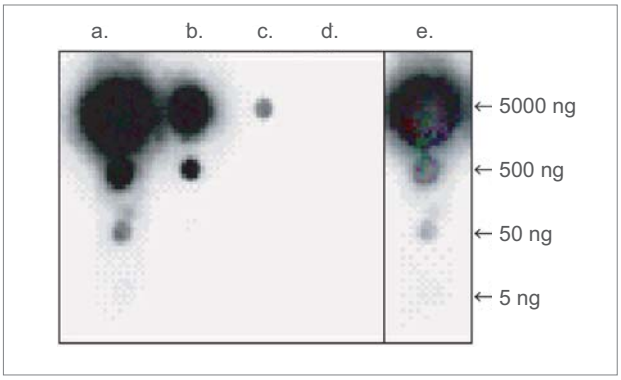
WB Stripping Solution (Data courtesy of Akaike Lab, Tokyo Institute of Technology)



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.



a: 0.05%(v/v) t-TBS
b: 2%(w/v) SDS, 100mM 2-Meraptoethanol, 62.5 mm Tris-HCl (pH 6.7)
c: WB Stripping Solution
d: WB Stripping Solution Strong

Usage Note

	WB Stripping Solution	WB Stripping Solution Strong
Material required: Tray and tweezers	Plastic and metal equipment can be used.	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.
Character	Acidic solution, pH2-3	Has reductive property
Safety	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.	
Usage	Use this product at room temperature. This solution should be stored at 4ºC, 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.	
Application	This solution is not suitable for colorimetric stained membranes treated with agents such as TMB, DAB and 4-Chloronaphtol.	
Alternately Use	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.	

For research use only, not intended for diagnostic or drug use.

NACALAI TESQUE, INC.

Nijo Karasuma, Nakagyo-ku, Kyoto 604-0855 JAPAN
TEL : +81-(0)75-251-1730
FAX : +81-(0)75-251-1763
Website : www.nacalai.com
E-mail : info.intl@nacalai.com