COSMO BIO CO., LTD.
Inspiration for Life Science

TOYOBO

Can Get Signal™

Immunoreaction Enhancer Solution

Instruction Manual

(Code No.: NKB-101, NKB-101T, NKB-201, NKB-301)

Distributor

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Warning

All reagents contained in this kit are for use in research. Never use for diagnosis or other clinical purposes. When using this kit, follow general laboratory precautions and pay attention to safety.

1. Introduction

Can Get Signal™ Immunoreaction Enhancer Solution is a reactive reagent for use in immunoassays, and was developed to resolve the problems of low sensitivity and high background often encountered during procedures such as Western blotting and ELISA (enzyme-linked immunosorbent assay). It can be used in a range of immunoassays.

<Features of this product>

1. High signals and low background
   Can Get Signal™ enhances antigen-antibody reactions, yielding signals several times to several tens of times higher than those obtained with conventional methods. It has been verified that enhancement is particularly marked for relatively inactive antibodies. Compositions optimized for primary and secondary antibodies respectively minimize background and yield high S/N ratios.

2. Strong versatility
   Can Get Signal™ can be used for various assays involving antigen-antibody reactions, such as Western blotting, dot blotting and ELISA. Components do not affect the activity of enzymes used for labeling (peroxidase, alkaline phosphatase, etc.), allowing use in assays using any of these enzymes. It can also be used for detection of both fluorescence and coloration.

3. Ready to use
   Can Get Signal™ is supplied in a ready-to-use form, is at an appropriate concentration for use, and does not need to be diluted. Simply dilute antibodies with this product instead of conventional diluents to use. It does not lengthen the time required for the assay.

2. List of components

This product is composed of the following solutions:

	NKB-101	NKB-101T	NKB-201	NKB-301
Solution 1 for primary antibodies	250 ml	50 ml	250 ml	—
Solution 2 for secondary antibodies	250 ml	50 ml	—	250 ml

Store both solutions at 4 ℃, shielded from light.

3. How to use

・The antibody is diluted with this reagent to the concentration required by the assay. Usually, this reagent takes the place of conventional antibody diluents such as TBS (TBS-T), PBS (PBS-T) and diluting and blocking fluids. The assay method does not require modification when using this product.
・This product is composed of Solution 1, for diluting primary antibodies, and Solution 2, for diluting secondary antibodies, except for NKB-201, which is composed of Solution 1 alone, and NKB-301, which is composed of Solution 2 alone. In assays involving only one antibody (e.g., labeled primary antibodies), Solution 2 should be used. However, depending on the type of antibody Solution 1 will sometimes yield better results. For sandwich ELISA involving solid-phase antibody and antigen, Solution 1 may be used to dilute the antigen (cf. 6. ELISA).

<Precautions>
・This product may be used in combination with blocking agents such as casein, BSA, etc. However, since it contains substances with blocking activity, the concentration of blocking agents can become excessively high if used in combination.
・This product cannot be used for blocking reactions. It cannot be used for solidification of antigen or antibody during ELISA.

4. Western blotting

Western blotting is aimed at detecting proteins using specific antibodies after transferring proteins isolated by SDS polyacrylamide gel electrophoresis, etc. onto a nitrocellulose or PVDF membrane. Proteins thus detected are analyzed as to their molecular weight, concentration and level of expression. This method usually uses secondary antibodies such as anti-IgG antibody (enzyme-labeled antibody) attached to horseradish peroxidase (HRP) or alkaline phosphatase (AP).
An example of the protocol used for Western blotting is given below (semi-dry transfer method).

[1] Devices and tools

・Power supply cables
・Semi-dry transfer device
・Plastic container (large enough to accommodate gel or membrane)
・Agitator
・Luminescence imager (Toyobo FAS-1000, etc. when detecting chemiluminescence)
・X-ray film and film cassette (when detecting chemiluminescence with film)

[2] Reagents and consumables

(For articles marked * see 7. Preparing reagents)

- Primary antibody specific to the protein to be detected
- Secondary antibody (labeled with HRP, AP, etc.) specific to the primary antibody
- Blocking agent (skim milk, etc.)
- Pre-stained protein molecular weight marker
- PVDF membrane (Amersham Hybond-P, etc.)
- Filter paper
- Methanol
- Transfer buffer*
- TBS-T* or PBS-T*
- HRP-detecting reagent (Amersham ECL Plus, etc., when using HRP-labeled secondary antibody)
- AP-detecting reagent (Promega Western Blue, etc., when using AP-labeled secondary antibody)

[3] SDS-PAGE

The protein to be analyzed and the pre-stained protein molecular weight marker are subjected to electrophoresis. The pre-stained protein molecular weight marker is convenient for confirming the efficiency of protein transfer onto the PVDF membrane.

[4] Pre-wetting of PVDF membrane

Nitrocellulose and PVDF membranes are often used in Western blotting. As they are less likely to break and more efficient in transfer, PVDF membranes have been used more frequently than nitrocellulose membranes in recent years. However, since PVDF membranes are highly hydrophobic, they need wetting with methanol in advance. The pre-wetting method is as follows.

1. Cut PVDF membrane to the appropriate size for use.
   Wear gloves to avoid protein sticking to the hands.

2. Immerse membrane in methanol and agitate for one minute.

3. Immerse membrane in distilled water and agitate for 5 minutes.

4. Immerse membrane in transfer buffer and agitate for 10 minutes.

[5] Transfer onto PVDF membrane (semi-dry transfer)

1. Prepare eight sheets of filter paper, cut to a size slightly larger than the PVDF membrane.

2. Immerse filter papers in transfer buffer.

3. Stack in the following order from the cathode side: 4 sheets of filter paper → SDS-PAGE gel → PVDF membrane → 4 sheets of filter paper → anode
   Take care when determining the direction of the cathode and anode, since it can vary depending on the transfer device used. If gel and membrane are stacked in the reverse direction, protein can transfer to the filter paper. Care is also needed to prevent bubbles from entering the space between the gel and the PVDF membrane.

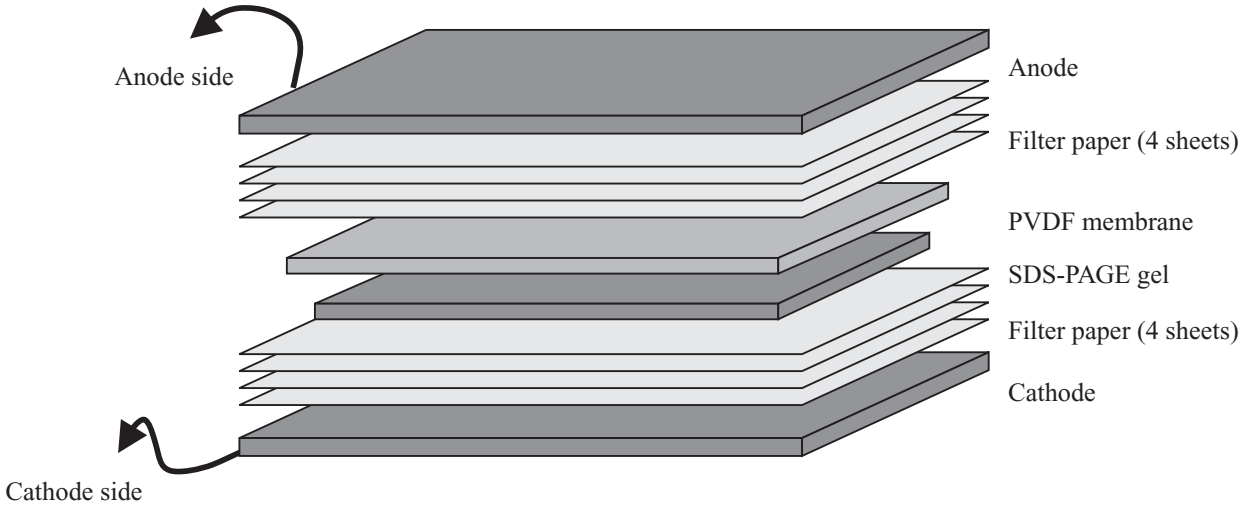

Fig. 1. Structure of the semi-dry transfer device

4. Apply electric current for one hour at a level roughly equivalent to the PVDF membrane area (cm^2) × 0.8 mA.
   Usually, if electricity is applied at this level, about 80% of protein is transferred onto the PVDF membrane. Transfer efficiency depends highly on the molecular weight of the protein and the gel concentration. If satisfactory results cannot be obtained, transfer conditions need to be optimized, referring to the transfer efficiency of the pre-stained protein marker.

5. Upon completion of transfer, put the PVDF membrane into a plastic container filled with TBS-T and agitate for 5 minutes to wash.
   PBS-T can be used instead of TBS-T as a cleansing buffer (the same can be said of the following steps). However, since the phosphoric acid contained in PBS-T can affect antibody-labeled AP activity we recommend TBS-T for AP detection.

[6] Blocking

1. Dissolve 1g of skim milk in 20 ml of TBS-T. Agitate well.
   Other blocking agents include cows milk-derived purified protein (casein), BSA (bovine serum albumin) and artificially synthesized macromolecules. However, some blocking agents involve cross-reactivity with the antibody used, possibly leading to increased nonspecific signals. Skim milk and casein are reported to be unsuitable for high specificity detection of phosphorylated proteins. Skim milk and casein are not suitable for detection using avidin-biotin systems. Use a blocking agent optimal to the antibody assay system.

2. Immerse PVDF membrane in skim milk solution and agitate for one hour at room temperature.

3. Rinse membrane twice with TBS-T.

4. Wash membrane with TBS-T (one 15-minute agitation session and 2 subsequent 5-minute agitation sessions).

[7] Exposure to the primary antibody

1. Dilute primary antibody with *Can Get Signal*™ Solution 1.
   The optimal dilution ratio for the primary antibody depends highly on the type of antibody, the concentration of the antigen, the sensitivity of the detection system, etc. It should be determined referring to the conditions recommended by the antibody supplier. If the optimal dilution ratio is unknown, it can be determined by a preliminary dot blotting experiment, etc. (see 5. Dot blotting).
   Solution 2 should be used when only one antibody is used but in some cases, Solution 1 may yield better results. In assays involving only one antibody, skip step [8] Exposure to the secondary antibody.

2. Immerse the PVDF membrane in dilute primary antibody solution and agitate for one hour at room temperature.

3. Wash the membrane with TBS-T (3 sessions of 10-minute agitation).

[8] Exposure to the secondary antibody

This step is omitted if using only one antibody

1. Dilute secondary antibody with *Can Get Signal*™ Solution 2.

The optimal dilution ratio for the secondary antibody is highly dependent on the type of antibody, the concentration of the antigen, the sensitivity of the detection system, etc. It should be determined refferring to the conditions recommended by the antibody supplier. If the optimal dilution ratio is unknown, it can be determined by a preliminary dot blotting experiment, etc. (see 5. Dot blotting).

2. Immerse the PVDF membrane in dilute secondary antibody solution and agitate for one hour at room temperature.

3. Wash the membrane with TBS-T (3 agitation sessions of 10-minutes).

[9] Detection (chemiluminescence detection using HRP-labeled antibody)

Detection can also use chemical coloration with HRP-labeled antibody.

1. Combine solutions A and B of ECL Plus (Amersham) in a ratio of 40:1.
   The volume of the mixture should be roughly equivalent to PVDF membrane area (cm^2) × 0.1 ml.

2. Place the PVDF membrane in a dry plastic container. Drop the ECL solution onto it. Leave the membrane to stand for 5 minutes at room temperature.

3. Remove the mixed ECL solution from the PVDF membrane. Place the membrane on a thin plastic plate (a plain black pad, etc. is recommended).

4. Take pictures with luminescence imager (Toyobo FAS-1000, etc.) or on X-ray film, etc.

[10] Detection (detection of chemical coloration, using AP-labeled antibody)

Detection can also use chemiluminescence with AP-labeled antibody

1. Put the PVDF membrane into a dry plastic container. Apply Western Blue (Promega). Leave the membrane to stand for about 15 minutes at room temperature or 37℃.
   The volume of Western Blue should be roughly equivalent to PVDF membrane area (cm^2) × 0.1 ml. Coloration can be detected macroscopically. The reaction should be stopped in accordance with the degree of coloration.

3. Remove Western Blue fluid remaining on the PVDF membrane. Take pictures and save with a camera, scanner, photocopying machine, gel imager (Toyobo FAS-III Series, etc.) etc.

5. Dot blotting

Dot blotting is aimed at quantifying proteins with high specificity using enzyme-labeled antibody, etc., after immobilization on a nitrocellulose or PVDF membrane without electrophoretic or other separation. (Dot blotting is sometimes used for quantification of DNA or RNA as well, but the term as used here refers only to the detection of protein).
Dot blotting is occasionally used as a preliminary assay to determine the concentration of antibodies used for Western blotting. It needs to be borne in mind that dot blotting cannot distinguish between specific and nonspecific reactions, it does not involve attenuation during transfer from gel to PVDF membrane, etc., and signals tend to be higher than with Western blotting.
The basic dot blotting method is similar to that for Western blotting. The method for determining antibody concentration by dot blotting will be presented below. Dot blotting uses reagents similar to those employed in Western blotting.

[1] Devices and tools

- Plastic container (large enough to accommodate the membrane; one container per sample to be tested)
- Agitator
- Luminescence imager (Toyobo FAS-1000, etc. when detecting chemiluminescence)
- X-ray film and cassette (when detecting chemiluminescence with film)

[2] Reagents and consumables

(For articles marked * see 7. Preparing reagents)

- Primary antibody specific to the protein to be detected
- Secondary antibody (labeled with HRP, AP, etc.) specific to the primary antibody
- Blocking agent (Difco skim milk, etc.)
- PVDF membrane (Amersham Hybond-P, etc.)
- Methanol
- TBS-T* or PBS-T*
- HRP detection reagents (Amersham ECL Plus, etc.; when using HRP-labeled secondary antibody)

[3] Pre-wetting of PVDF membrane

1. Cut PVDF membrane to the appropriate size for use.
   Wear gloves to avoid protein sticking to the hands.

2. Immerse membrane in methanol and agitate for one minute.

3. Immerse membrane in distilled water and agitate for 5 minutes.

[4] Dropping protein solution (spotting)

1. Dilute the protein solution appropriately with TBS or other diluent, and drop onto several points on the PVDF membrane. Dry the membrane.
   The volume of the solution dropped is about 2 μl. The dropped fluid sometimes assumes a semi-globular form on the membrane, but is absorbed in a few minutes. The membrane should be left standing for a while at room temperature.

[5] Blocking

1. Dissolve 1g of skim milk in 20 ml of TBS-T. Agitate well.
   See 4. Western blotting for the types of blocking agent used.

2. Immerse PVDF membrane in skim milk solution and agitate for one hour at room temperature.

3. Rinse membrane twice with TBS-T.

4. Wash membrane with TBS-T (one 15-minute agitation session and 2 subsequent 5-minute agitation sessions).

[6] Exposure to the primary antibody

1. Serially dilute primary antibody with *Can Get Signal*™ Solution 1.

The serial dilution ratios for the primary antibody should be about 1:100, 1:500, 1:1000 and 1:10000. These ratios should be changed as needed. The optimal dilution ratio for the secondary antibody may be determined by serially diluting the antibody at a fixed ratio with the primary antibody.
Solution 2 should be used when there is only one antibody but in some cases Solution 1 may yield better results). The step shown in [7] Exposure to the secondary antibody is skipped if there is only one antibody.

2. Cut PVDF membrane into pieces for individual spots, using scissors. Place membrane pieces in individual containers.
   A six-well plate for use in cell culturing is convenient for this purpose.

3. Add dilute primary antibody solution to each container, and agitate for one hour at room temperature.

4. Wash membrane pieces with TBS-T (3 agitation sessions of 10-minutes)
   If using a six-well plate for cell culturing, washing can be accelerated by applying cleansing fluid with a Komagome pipette or similar.

[7] Exposure to secondary antibody

This step is omitted if using only one antibody.

1. Dilute secondary antibody with *Can Get Signal*™ Solution 2.

The optimal dilution ratio for the secondary antibody is highly dependent on the type of antibody, the concentration of the antigen, the sensitivity of the detection system, etc. It should be determined referring to the conditions recommended by the antibody supplier. If the optimal dilution ratio is unknown, it can be determined by serial dilution at a fixed ratio with the primary antibody.

2. Immerse the PVDF membrane in dilute secondary antibody solution and agitate for one hour at room temperature.

3. Wash the membrane with TBS-T (3 agitation sessions of 10-minutes).

[8] Detection (chemiluminescence detection using HRP-labeled antibody)

Detection can also use chemical coloration with HRP-labeled antibody.

1. Combine solutions A and B of ECL Plus (Amersham) in a ratio of 40:1.
   The volume of the mixture should be roughly equivalent to PVDF membrane area (cm^2) × 0.1 ml.

2. Place the PVDF membrane in a dry plastic container. Drop the ECL solution onto it. Leave the membrane to stand for 5 minutes at room temperature.

3. Remove the mixed ECL solution from the PVDF membrane. Place the membrane on a thin plastic plate (a plain black pad, etc. is recommended).

4. Take pictures with luminescence imager (Toyobo FAS-1000, etc.) or on X-ray film, etc.

6. ELISA

ELISA (enzyme-linked immunosorbent assay) is aimed at quantifying antigen or antibody in a sample solution using enzyme-labeled antibody or antigen. It allows very sensitive detection and is used for quantifying minute amounts of antigen, etc.
There are various ELISA methods but generally the following are used: (1) direct method (antigen or antibody is transformed into a solid phase, and detection performed with enzyme-labeled antibody or antigen); (2) indirect method (antigen is transformed into a solid phase and detection performed using a primary antibody of the antigen and an enzyme-labeled secondary antibody of the primary antibody; and (3) sandwich method (the antibody to the antigen is transformed into a solid phase, and a sample solution containing the antigen added, followed by detection using a primary antibody (different from solid-phase antibody) of the antigen and an enzyme-labeled secondary antibody of the primary antibody.
An example sandwich method protocol is presented below. When measuring antigen or antibody concentrations, the assay should be performed on 3 or more wells for each sample under identical conditions, and the average of the three taken as the reading. In immunoassay, errors among different samples are usually very great, sometimes making it difficult to obtain accurate data if the number of assays for a given sample is small. For more accurate measurement of sample concentrations, we recommend preparing a calibration curve using data from serially diluted samples of known and unknown concentrations, and to calculate absolute and relative concentrations (compared to standard samples) from the calibration curve.

[1] Devices and tools

- ELISA plate reader
- Eight- or 12-member multichannel pipette (convenient if assaying many samples)

[2] Reagents and consumables

(For articles marked * see 7. Preparing reagents)

- Solid-phase antibody specific to the antigen to be detected
- Primary antibody specific to the antigen to be detected (If the solid-phase antibody is akin to the antigen determinant, antagonism can hamper successful reactions. It is therefore advisable to use solid-phase antibody and primary antibody derived from different animal species.)
- Secondary antibody (labeled with HRP, AP, etc.) specific to the primary antibody used
- Blocking agent (Difco skim milk, etc.)
- 96-well ELISA plate
- 96-well plate seal
- 50 mM carbonate buffer, pH 9.6*
- TBS or PBS*

・TBS-T* or PBS-T*
・HRP detecting reagent (BioFX TMB, etc, when using HRP-labeled antibody)
・1N sulfuric acid
1N sulfuric acid has strong corrosive action. Take full care when handling and disposing.

[3] Fixation of solid-phase antibody on plate

1. Pour solid-phase antibody (100 μl diluted with 50 mM carbonate buffer (pH 9.6) into a 96-well plate.
The dilution ratio for the solid-phase antibody should be 20-fold (10 μg/ml) or less. However, since detection sensitivity varies greatly depending on antibody type or antigen concentration, an optimal antibody concentration should be determined using the method shown below. When applying the antibody, care should be taken that fluid does not attach to the walls of the well.

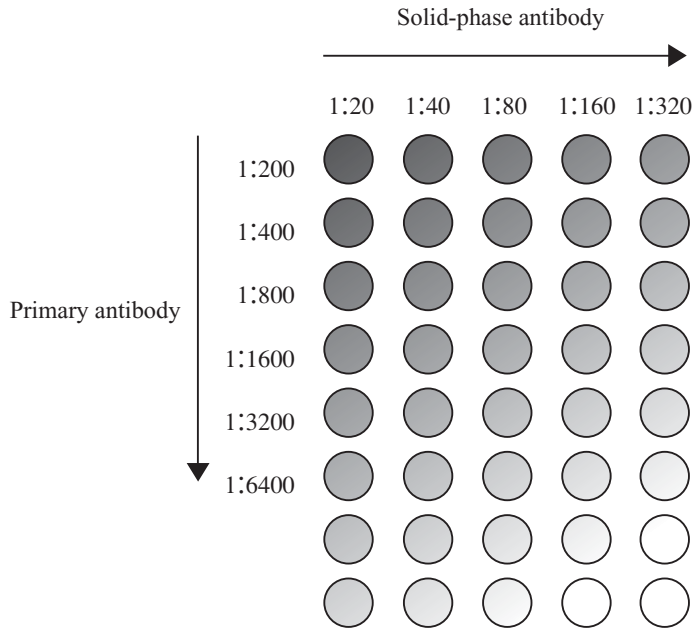

Fig. 2. Determining antibody concentration (titration)
When the concentration of the secondary antibody needs to be determined, serially dilute the secondary antibody using a fixed concentration of solid-phase antibody or by another method.

2. Apply a seal to the plate to prevent evaporation, and incubate for one hour at 37℃.
Solid-phase antibody can also be prepared by incubation for 16 hours at 4℃. The results will not differ greatly between the two methods. However, if the number of wells used for one-hour incubation at 37℃ is very large, it takes time to place the sample in all wells, and incubation time will differ greatly among different wells, possibly leading to unsatisfactory results. In such cases, incubation for 16 hours at 4℃ can minimize error.

3. Remove solid-phase antibody solution from 96-well plate by pipetting.
   Care is needed to prevent the fluid from attaching to the walls of the wells. After this step, care needs to be taken to prevent the wells from becoming dry. Otherwise, background signals may increase.

4. Pour 250 μl of PBS into each well. Put the plate upside down in a sink, and remove residual fluid completely. Repeat 3 times to wash the plate.

[4] Blocking

1. Dissolve skim milk in PBS to a concentration of 3%, and agitate the mixture well.
   Other suitable blocking agents include cow milk-derived purified protein (casein), BSA (bovine serum albumin) and artificially synthesized macromolecules. However, some blocking agents involve cross-reactivity with the antibody used, possibly leading to increased nonspecific signals. Skim milk and casein are reported to be unsuitable for high specificity detection of phosphorylated proteins. Skim milk and casein are not suitable for detection using avidin-biotin system. Use a blocking agent optimal to the antibody assay system.

2. Pour 250 μl of the skim milk solution into each well.

3. Apply a seal to the plate to prevent evaporation, and incubate for one hour at 37℃.
   Blocking can also be effected by incubation for 16 hours at 4℃. Following blocking, the plate can be used repeatedly for up to about one month if stored at 4℃ without removing the blocking agent. Even if solid-phase antibody is prepared using the same method, the features may vary from one occasion to the next. Therefore, if it is necessary to assay plates with equivalent features on multiple occasions, we recommend concomitant preparation of a large number of plates for solid-phase antibodies, filled with blocking agent, and stored so that they can be used in successive multiple assays.

4. The 96-well plate is put upside down in a sink, and the skim milk solution discarded.

5. Pour 250 μl of PBS into each well. Put the plate upside down in a sink, and remove residual fluid completely. Repeat 3 times to wash the plate.

[5] Exposure of antigen to primary antibody

1. Dilute antigen appropriately with *Can Get Signal*™ Solution 1.

2. Dilute primary antibody with *Can Get Signal*™ Solution 1.

The optimal dilution ratio for the primary antibody is highly dependent on the type of antibody, the concentration of the antigen, the sensitivity of the detection system, etc. It should be determined referring to the conditions recommended by the antibody supplier. If the optimal dilution ratio is unknown, it can be determined according to the methods for determination of antibody concentration mentioned above.

3. Pour 50 μl each of dilute antigen solution and dilute primary antibody solution into each well. Mix well.
   Another method is to apply the primary antibody after the plate exposed to the antigen alone is washed. Method selection can depend on decreases in background signal and detection sensitivity. However, the difference is often minimal so it is simpler to apply the antigen and primary antibody at the same time (first method).

4. Apply a seal to the plate to prevent evaporation, and incubate for one hour at 37℃.
   Exposure of the antigen to the primary antibody can also be performed with incubation for 16 hours at 4℃.

5. Remove the primary antibody solution remaining in the 96-well plate by pipetting.

6. Pour 250 μl of PBS-T into each well. Put the plate upside down in a sink and remove residual fluid completely. Repeat 3 times.

[6] Exposure to the secondary antibody

1. Dilute secondary antibody with *Can Get Signal*™ Solution 2.

The optimal dilution ratio for the secondary antibody is highly dependent on the type of antibody, the concentration of the antigen, the sensitivity of the detection system, etc. It should be determined referring to the conditions recommended by the antibody supplier. If the optimal dilution ratio is unknown, it can be determined according to the methods for determination of antibody concentration mentioned above.

2. Pour 100 μl of the dilute secondary antibody solution into each well.

3. Apply a seal to the plate to prevent evaporation, and incubate for one hour at 37℃.
   Exposure to secondary antibody can also be performed with incubation for 16 hours at 4℃.

4. Remove secondary antibody solution remaining in the 96-well plate by pipetting.

5. Pour 250 μl of PBS-T into each well. Put the plate upside down in a sink and remove residual fluid completely. Repeat 3 times to wash the plate.

[7] Detection (chemiluminescence detection using HRP-labeled antibody)

Detection can also use chemical coloration with AP-labeled antibody.

1. Pour 100 μl of TMB into each well.

2. Apply a seal to the plate to prevent evaporation, and incubate for 20 minutes at 37℃.
   The fluid gradually becomes vividly blue. Depending on detection sensitivity, coloring can take place immediately after the addition of TMB. In this case, check the degree of coloration continuously and carry out the next step, stopping the reaction, at the appropriate time.

3. Pour 100 μl of 1N sulfuric acid into each well to stop reactions.
   The fluid instantly changes color to yellow. If the reaction is not stopped, measurement with a plate reader is not possible. Stopping the reaction is therefore an indispensable step.

4. Measure absorbance at 495nm with an ELISA plate reader.
   Some types of plate reader are capable of mixing reaction systems automatically. When using a plate reader without this function, the reaction system needs to be mixed with a pipette, etc., before measurement. Large bubbles on the fluid surface can lead to large errors in measurement. Bubbles should be removed before measurement.

7. Preparing reagents

・1M Tris-Cl, pH 7.5 (500 ml)

Dissolve precisely 60.57 g of tris (hydroxymethyl) aminomethane in 400 ml of distilled water. Adjust pH to 7.5 with HCl. Since the pH of Tris buffer changes greatly depending on temperature, pH adjustment should be made while keeping the fluid temperature at 25℃.

After pH adjustment, add distilled water to the solution to make a total volume of 500 ml. If precise pH is essential, measure pH and adjust again. Autoclave the mixture if necessary and store at room temperature until use.

・TBS (500ml)

10 mM Tris-Cl, pH 7.5
100 mM NaCl (MW = 58.44)

Combine precisely 5 ml of 1M Tris-Cl (pH 7.5) and 2.9 g of NaCl with distilled water to make a total volume of 500 ml. Autoclave the mixture as necessary and store refrigerated or at room temperature until use. When stored at room temperature, the solution needs to be cooled immediately before use.

・TBS-T (500ml)

TBS
0.1% (v/v) Tween-20

Combine 500 ml of TBS with 500 μl of Tween-20. Store refrigerated until use.

・10x PBS(-) (10x PBS) (500ml)

43 mM $Na_2HPO_4•7H_2O$ (MW = 268.07)
14 mM KH_2PO_4 (MW = 136.09)
27 mM KCl (MW = 74.55)
1.37 M NaCl (MW = 58.44)

Combine precisely 5.75 g of $Na_2HPO_4 \cdot 7H_2O$, 1.0 g of KH_2PO_4, 40.0 g of NaCl and 1.0 g of KCl with distilled water to yield a 10x solution (500 ml). Autoclave the mixture as necessary and store refrigerated or at room temperature until use.

If 10x PBS is stored refrigerated, sedimentation can sometimes occur due to high salt concentrations. In such cases warm in a 37℃ water bath to dissolve sediment completely before use.

1 part 10x solution is diluted with 10 parts distilled or sterile distilled water to yield 1x solution. Store refrigerated or at room temperature for a while after preparation. If stored at room temperature, cool immediately before use.

・PBS-T (500 ml)

1x PBS(-)
0.1% (v/v) Tween-20

1x PBS(-) is combined with 500 ml of Tween-20 Mix well. Store refrigerated until use.

・50 mM Carbonate Buffer, pH 9.6 (500 ml)

15 mM Na_2CO_3 (MW = 105.99)
35 mM $NaHCO_3$ (MW = 84.01)

Combine precisely 0.79 g of Na_2CO_3 and 1.47 g of $NaHCO_3$ with distilled water to make a total volume of 500 ml. The pH does not need to be adjusted. Autoclave the mixture as necessary and store refrigerated or at room temperature until use. If stored at room temperature, cool immediately before use.

・Transfer Buffer (For Semi-Dry Transfer Use) (1 l)

192 mM Glycine (MW = 75.07)
25 mM Tris (MW = 121.14)
20% (v/v) Methanol

Combine precisely 14.4 g of glycine, 3.0 g of Tris (hydroxymethyl) aminomethane and 200 ml of methanol with distilled water to make a total volume of 1 l. Store at room temperature until use.

8. Trouble shooting

<Western blotting and dot blotting>

Trouble	Cause	Countermeasure
Weak signals	Low protein concentration after electrophoresis	Use samples of as high a concentration as possible in electrophoresis. Serial dilution of protein is useful in determining optimal concentration.
	Low antibody concentration	Determine optimal antibody concentration by dot blotting.
	Insufficient transfer to membrane	Elevate electric current or prolong transfer time. Usually, a higher gel concentration reduces transfer efficiency. Bear in mind that if a gradient gel is used, the difference in transfer efficiency between macromolecular and micromolecular regions is expanded. Efficiency may be improved by switching from semi-dry to wet transfer.
	Membrane transfer time and electric current either too long or large	If using a nitrocellulose membrane, excessive transfer can cause protein to permeate across the membrane to the opposite side. Reduce electric current or shorten time in such cases. Changing to a PVDF membrane may also help.
Colorless band center	Antibody concentration is too high	Depending on the detection reagent used, luminescence may be suppressed by excessive signals. Determine optimal antibody concentration by dot blotting.
Too many extra bands	Antibody concentration is too high	Excessive antibody can increase nonspecific signals. Determine optimal antibody level by dot blotting.
	Protein concentration is too high	Apply less concentrated protein in electrophoresis. Serial dilution is useful in determining optimal concentration.
	Insufficient blocking	Depending on the type of antigen and antibody involved, success or failure of blocking can depend greatly on the type and concentration of the blocking agent. Review the type and concentration of the blocking agent used.
	Insufficient washing	Increase frequency of washing.

<ELISA>

Trouble	Cause	Countermeasure
Weak signals	Antigen or antibody concentration is not high enough.	Review conditions of antigen and antibody concentrations (titration).
Color is too intense	Antigen or antibody concentration is too high.	Review conditions of antigen and antibody concentrations (titration).
	Duration of exposure is too long.	Shorten exposure time.
Background signals are high	Antigen or antibody concentration is too high.	Review conditions of antigen and antibody concentrations (titration).
	Insufficient blocking	Depending on the type of antigen and antibody involved, success or failure of blocking can depend greatly on the type and concentration of the blocking agent. Review the type and concentration of blocking agent used.
	Insufficient washing	Increase frequency of washing.
Great variance in reading	Problem with the ELISA plate	Protein adsorbing efficiency can vary greatly among different types of ELISA plate. Efficiency can also vary among different batches of the same type of ELISA plate. When more accurate measurement is needed, the ELISA plate should be selected carefully.

Manufacture

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