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Instruction manual Can Get Signal 0810

F0991K

Can Get Signal ®

Immunoreaction Enhancer Solution

NKB-101T	50 mlx2
NKB-101	250 mlx2
NKB-201	250 ml
NKB-301	250 ml

Store at 4°C

Contents

CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precaution and utilize safety while using this kit.

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FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

[1] Introduction

Description

Can Get Signal® is a solution containing an accelerator for antigen-antibody reactions. This reagent improves sensitivity, specificity, and signal-to-noise ration (S/N) for western blotting, dot blotting, enzyme-linked immunosorbent assay (ELISA), etc.

Features

- Can Get Signal® enhances immunoassay signals up to several dozen times by keeping low background signals.
- This reagent can be used for various assays such as western blotting, dot blotting, and ELISA.
- This reagent can also be used in combination with secondary antibodies labeled with peroxidase or alkaline phosphatase etc.
- The reagents consist of solutions 1 and 2. These solutions correspond to the reactions of the primary and secondary antibodies, respectively. The solutions can be used directly without dilution (ready-to-use type). In sandwich ELISA, solution 1 can be used in the reaction between the captured antibodies and antigens.

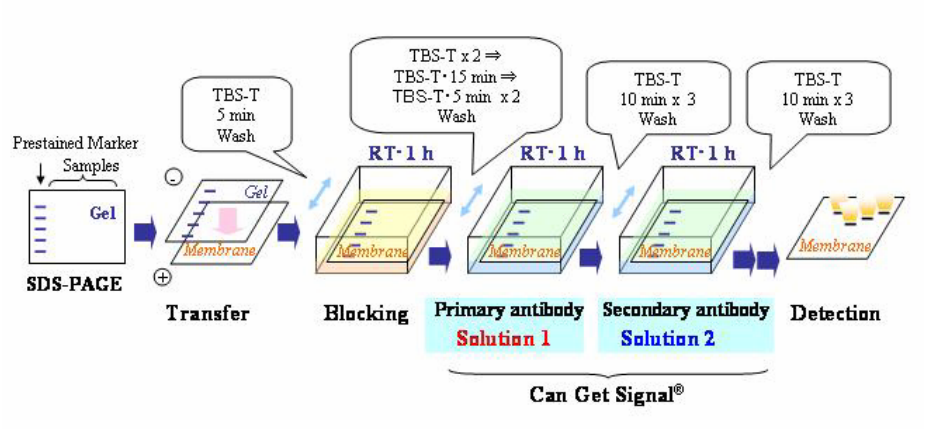

Fig. 1. Flow chart of western blotting with Can Get Signal®

Notes

Can Get Signal® cannot be used as a blocking reagent. The blocking and detection steps must be performed using conventional methods.

[2] Components

This kit includes the following components. All reagents should be stored at 4 °C, and protected from light.

Reagent Name	Code no.			
	NKB-101T	NKB-101	NKB-201	NKB-301
Solution 1 for primary antibody	50 ml	250 ml	250 ml	-
Solution 2 for secondary antibody	50 ml	250 ml	-	250 ml

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[3] Western blotting

During western blotting, proteins that have been separated on polyacrylamide gels are transferred to membranes such as nitrocellulose or polyvinylidene fluoride (PVDF) membrane for detection with specific antibodies. The membrane is subsequently stained with antibodies labeled with horseradish peroxidase (HRP) or alkaline phosphatase (AP). The following procedures are a standard protocol for western blotting using Can Get Signal®.

1. Materials required

(1) Equipments

- Power supply
- Eletroblotting apparatus (semi-dry or wet type)
- Shaker

(2) Reagents and consumables

- Blocking reagent
- Molecular weight markers (prestained)
- PVDF membrane (*e.g.*, Hybond-P)
- Whatman 3MM filter paper
- Methanol
- Transfer buffer*
- TBS-T or PBS-T*
- Detection solution (*e.g.*, ECL Plus)
- (X-ray film)

* see [6] Reagents

2. Protocol

(1) Electrophoresis

After loading the treated samples in the narrow wells of a polyacrylamide gel, submerge the gel in the migration buffer, and leave to spread for an appropriate time. The use of prestained molecular weight markers will enable the determination of protein size, and monitor the progress of electrophoresis and the subsequent transfer step.

(2) Transfer

Transfer can be performed under semi-dry or wet conditions. Semi-dry transfer is generally faster but wet transfer allows better reproducibility and the transfer of large proteins (> 100 kDa). The following protocol is for semi-dry blotting using PVDF membranes. PVDF membranes should be prewetted with methanol because the membrane has a hydrophobic surface.

(A) Precut a PVDF membrane to the size of the gel.

(B) Soak the membrane in methanol for 1 min while shaking.

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(C) Transfer the membrane to distilled water, and shake for 5 min.

(D) Transfer the membrane to the transfer buffer and shake for 10 min.

(E) Precut 8 pieces of Whatman 3MM filter paper of a slightly larger size than the gel.

(F) Saturate the filter paper with transfer buffer.

(G) **Optional** Presoak the gel in transfer buffer for 5 min.

(H) On the bottom electrode (cathode), place in the following order: 4 sheets of filter paper → polyacrylamide → PVDF membrane → 4 sheets of filter paper.

Notes
Bubbles should be removed after each step.

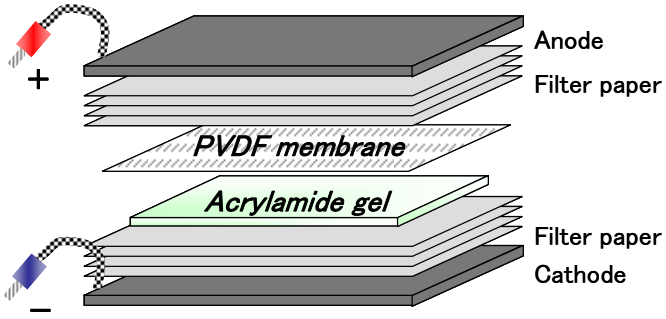

Fig. 2. Semi-dry blotting

(I) Perform transfer at the following current condition for 1 h.

Optimal current (mA) = membrane area (cm^2) x 0.8

Notes
Transfer efficiency depends on the molecular sizes of the proteins or the acrylamide gel concentration. Therefore, experimental conditions should be optimized using a prestained molecular weight marker as an indicator of transfer.

(J) Following transfer, the membrane should be washed with TBS-T for 5 min while shaking.

Notes
TBS-T can be replaced by PBS-T as well as the following steps. However, phosphate ions may inhibit AP activity, so TBS-T is recommended for the detection of AP.

(3) Blocking

Blocking of membranes decreases non-specific background signals from primary and/or secondary antibodies on membranes. The following is a standard protocol for blocking.

(A) Block the membrane in an appropriate blocking solution for 1 h.

Notes

-Two types of blocking solution are traditionally used: non-fat milk or BSA (Cohn fraction V). However, non-fat milk is not recommended for the study of phosphorylated proteins. Milk contains casein, which is a phosphorylated protein, causing high background.

-To prepare a 5% non-fat milk/TBS-T or BSA/TBS-T solution, weight 1 g per 20 ml of TBS-T.

(B) Remove the blocking solution and rinse the membrane twice in TBS-T.

(C) Wash the membrane once in TBS-T for 15 min while shaking.

(D) Wash the membrane twice in TBS-T for 5 min per wash while shaking.

(4) Primary antibody reaction

When performing direct detection using labeled antibodies, this step must be omitted.

(A) Dilute the primary antibody in Can Get Signal® Solution 1.

Notes

- The optimal concentrations of the antibodies used in this method tend to be lower than those used in conventional methods. Therefore, the optimization of the antibody concentrations should be investigated on the basis of previously suggested concentrations to lower the concentration range.

- Pretest by dot blotting is effective to determine the appropriate dilution rate of the primary antibodies.

(B) Incubate the membrane in the primary antibody solution at room temperature for 1 h while shaking.

Notes

-This reaction can be performed at 4 °C overnight.

(C) Wash the membrane in TBS-T 3 times for 10 min per wash while shaking.

(5) Secondary antibody reaction

(A) Dilute labeled (secondary) antibodies in Can Get Signal® Solution 2.

Notes

- The optimal concentrations of the antibodies used in this method tend to be lower than those used in conventional methods. Therefore, the optimization of the antibody concentration should be performed based on previously suggested concentrations to lower the concentration range.

- Pretest by dot blotting is effective to determine the appropriate dilution rate of the primary antibodies.

(B) Incubate the membrane in the secondary antibody solution at room temperature for 1 h while shaking.

(C) Wash the membrane in TBS-T 3 times for 10 min per wash while shaking.

(6) Detection

For the detection of HRP-conjugated antibodies, various kinds of detection systems can be used. The following is a standard protocol for detection using the ECL Plus Kit (GE healthcare). Details of the experimental conditions should be determined according to the instruction manual of this kit.

(A) Place the washed membrane, protein-side up, on a clean plastic sheet, on a flat surface.

(B) Prepare the ECL Plus substrate (40:1 mixture of components A and B)

(C) Apply the ECL Plus substrate at 100 µl / cm^2 of membrane, so that the membrane is completely covered.

(D) Incubate the membrane at room temperature for 5 min.

(E) Carefully pick up the membrane with clean forceps. Briefly drain the excess reagents, and place on a clean black plastic coaster.

(F) Expose the membrane to an X-ray film or using an imaging apparatus.

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[4] Dot blotting

Dot blotting is a technique for the detection of proteins, similar to western blotting. In the dot blotting procedure, the protein samples are not separated electrophoretically but are spotted directly onto the membrane. Dot blotting can be used as a pretest of western blotting to determine optimal antibody concentrations. However, dot blotting tends to exhibit higher sensitivity than western blotting.

1. Materials required

(1) Equipments
- (Imaging apparatus)

(2) Reagents and consumables

- Blocking reagent
- PVDF membrane (*e.g.*, Hybond-P)
- Methanol
- TBS-T or PBS-T*
- Detection solution (*e.g.*, ECL Plus)
- (X-ray film)

* see [6] Reagents

2. Protocol

(1) Spotting

(A) Precut a PVDF membrane to an appropriate size.

(B) Soak the membrane in methanol for 1 min while shaking.

(C) Transfer the membrane to distilled water, and shake for 5 min.

(D) Using a narrow-mouthed pipet tip, spot 2-µl aliquots of diluted samples onto the membrane.

(E) Let the membrane dry.

(2) Blocking

Blocking membranes decreases non-specific background signals from the primary and/or secondary antibodies on membranes. The following is a standard protocol for blocking.

(A) Block the membrane in an appropriate blocking solution for 1 h.

Notes
- Two types of blocking solution are traditionally used: non-fat milk or BSA (Cohn fraction V). However, non-fat milk is not recommended for the study of phosphorylated proteins. Because milk contains casein, which is a phosphorylated protein, high background signals may develop.

-To prepare a 5% non-fat milk / TBS-T or BSA / TBS-T solution, weight 5 g per 100 ml of TBS-T.

(B) Remove the blocking solution, and rinse the membrane twice in TBS-T.

(C) Wash the membrane once for 15 min in TBS-T while shaking.

(D) Wash the membrane twice for 5 min per wash in TBS-T while shaking.

(3) Primary antibody reaction

When direct detection using labeled antibodies is performed, this step must be omitted.

(A) Dilute the primary antibody in Can Get Signal® Solution 1.

Notes
- For the optimization of the concentrations of the primary antibodies, the dilution ranges of the primary antibodies should be set at 1:100-1:10,000.

(B) Incubate the membrane in the primary antibody solution at room temperature for 1 h while shaking.

Notes
- This reaction can be performed at 4 °C overnight.

(C) Wash the membrane in TBS-T 3 times for 10 min per wash while shaking.

(4) Secondary antibody reaction

(A) Dilute labeled (secondary) antibodies in Can Get Signal® Solution 2.

(B) Incubate the membrane in the secondary antibody solution at room temperature for 1 h while shaking.

(C) Wash the membrane in TBS-T 3 times for 10 min per wash while shaking.

(5) Detection

For the detection step, refer to the western blotting protocol.

[5] ELISA

Sandwich Enzyme-linked Immunosorbent Assay (ELISA) is an effective technique to measure the amount of antigens using capture and detection antibodies. In this system, the antigen must contain at least 2 epitopes capable of binding to antibodies. Either a monoclonal or polyclonal antibody can be used as the capture and detection antibodies in the sandwich ELISA system. A polyclonal antibody is often used as the capture antibody to capture as many antigens as possible.

The following is a standard protocol for sandwich ELISA using Can Get Signal® reagent. In this system, each data should be obtained as an average of at least 3 wells, because this assay system often results in analytical errors. Making an analytical curve using standard samples is also useful for determining the amount of antigens with accuracy.

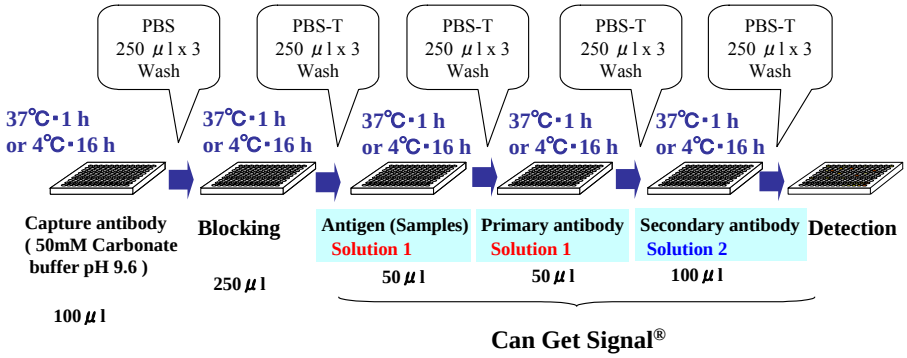

Fig. 3. Flow chart of sandwich ELISA using Can Get Signal®

Notes
Can Get Signal® cannot be used as a blocking reagent. Blocking and detection steps must be performed using conventional methods.

1. Materials required

(1) Equipments

- Plate reader
- Multipipetter (8 or 12 nozzle type)

(2) Reagents and consumables

- 50 mM Carbonate buffer (pH 9.6)
- Blocking reagent
- PBS or TBS*
- PBS-T or TBS-T*
- Detection solution (e.g., TMB)
- 1 N H_2SO_4
-96-well microplate
-Adhesive plastic sheet

* see [6] Reagents

2. Protocol

(1) Coating with the capture antibody

(A) Coat the wells of a 96-well microplate with 100 µl capture antibody solution at a concentration of ≤ 10 µg/ml in 50 mM carbonate buffer (pH 9.6).

Notes

- The optimal concentrations of the capture and primary antibodies can be determined according to the titration method (Fig. 4).

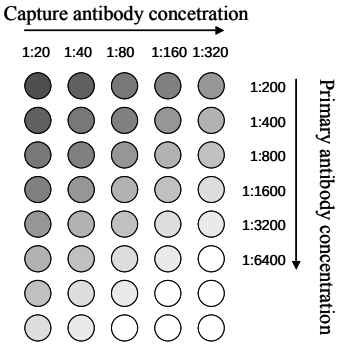

Fig.4 Titration method

(B) Cover the plate with an adhesive plastic sheet, and incubate at 37 °C for 1 h.

Notes

- This step can be performed at 4 °C for 16 h.

(C) Remove the coating solution carefully.

Notes

- The plate should not be allowed to get dried after this step to avoid background signal.

(D) Wash the plate 3 times with 250 µl PBS.

(2) Blocking

(A) Block the coated wells by adding 250 µl blocking solution.

Notes

- Two types of blocking solution are traditionally used: non-fat milk or BSA (Cohn fraction V). However, non-fat milk is not recommended for the study of phosphorylated proteins. Because milk contains casein, which is a phosphorylated-protein, it results in high background signals.

- To prepare a 5% non-fat milk / PBS or BSA / PBS solution, weight 5 g per 100 ml of PBS.

(B) Cover the plate with an adhesive plastic sheet and incubate at 37 °C for 1 hr.

(C) Remove the blocking solution.

(D) Wash the plate 3 times with 250 µl PBS.

(3) Antigen / Primary antibody reactions

(A) Dilute all samples (antigen) in Can Get Signal® Solution 1.

(B) Dilute the primary antibodies in Can Get Signal® Solution 1.

Notes

- The optimal concentrations of the capture and primary antibodies can be determined according to the titration method (Fig. 4).

- The antigen and primary antibody reactions can be preformed independently.

(C) Add 50 µl of the appropriately diluted sample to each well

(D) Add 50 µl of the diluted primary antibody to each well.

(E) Cover the plate with an adhesive plastic sheet, and incubate at 37 °C for 1h.

(F) Remove the solution carefully.

(G) Wash the plate 3 times with 250 µl PBS-T.

(4) Secondary antibody reaction

(A) Dilute the labeled (secondary) antibodies in Can Get Signal® Solution 2.

Notes

- The optimal concentrations of the antibodies used in this method tend to be lower than those used in conventional methods. Therefore, the optimization of the antibody concentration should be performed based on previously suggested concentrations to lower the concentration range.

(B) Add 100 µl of diluted antibody to each plate.

(C) Cover the plate with an adhesive plastic sheet, and incubate at 37 °C for 1h.

(D) Remove the solution carefully.

(E) Wash the plate 3 times using 250 µl PBS-T.

(5) Detection

Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are widely used labeling enzymes employed in ELISA. The following is a standard protocol of ELISA using HRP-conjugated antibodies.

(A) Add 100 µl 3,3',5,5'-tetramethyl benzidine (TMB) solution to each well.

(B) Cover the plate with an adhesive plastic sheet, and incubate at 37 °C for 20 min.

(C) Add 100 µl 1 N H_2SO_4 solution to each well.

(D) Read the optical density at 450 nm.

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[6] Reagents

1. 1 M Tris-HCl, pH 7.5 (500 ml)

60.57 g Tris
Dissolve in 400 ml distilled water
Adjust pH to 7.5 with HCl at 25 °C
Adjust volume to 500 ml
Autoclave

2. TBS (500 ml)

5 ml 1 M Tris-HCl, pH 7.5
2.9 g NaCl
Adjust volume to 500 ml

3. TBS-T (500 ml)

500 ml TBS
0.5 ml Tween-20

4. 10 x PBS(-) (10 x PBS) (500 ml)

5.75 g $Na_2HP0_4 \cdot 7H_20$
1 g KH_2PO_4
40 g NaCl
1 g KCl
Adjust volume to 500 ml

5. PBS-T (500 ml)

500 ml 1 x PBS(-)
0.5 ml Tween-20

6. 50 mM Carbonate buffer, pH 9.6 (500 ml)

0.79 g Na_2CO_3
1.47 g $NaHCO_3$
Adjust volume to 500 ml

7. Transfer buffer (1 l)

14.4 g Glycine
3 g Tris
200 ml methanol
Adjust volume to 1 l

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[7] Troubleshooting

Symptom	Cause	Solution
Western blotting, Dot blotting		
No bands or faint bands observed	Insufficient proteins	Increase the amount of total proteins loaded on the gel.
	Insufficient antibodies	Increase antibody concentrations. Pretest by dot blotting is effective to determine the optimal antibody concentrations.
	Poor or incomplete transfer	- Optimize transfer time. High molecular weight proteins may require more time to transfer. - Transfer efficiency depends on the gel concentration. Be careful of the difference in efficiency when using a gradient gel. - Alter the transfer method from semi-dry to wet.
	Over transfer	Reduce voltage or time of transfer, especially for low molecular weight proteins (< 10 kDa).
White bands (ECL system)	Excessive signal generated	Reduce antibody or protein concentrations. Excessive signals result in the rapid, complete consumption of substrates.
Extra bands	Excessive antibody	Decrease the antibody concentration.
	Excessive protein	Decrease the amount of protein.
	Insufficient blocking	Use different types of blocking reagents.
	Insufficient washing	Increase the number of washing steps.
High background	Casein in milk	Casein is a phosphorylated protein. When using anti-phospho antibodies, blocking solutions which do not contain casein should be used.
ELISA		
Faint signals	Insufficient antigens or antibodies	Identify the optimal concentrations of the antigen and antibody by titration.
Excessive signals	Excessive antigens or antibodies	Identify the optimal concentrations of the antigen and antibody by titration.
	Excessive reaction time	Decrease the reaction time.
High background	Excessive antigens or antibodies	Identify the optimal concentrations of the antigen and antibody by titration.
	Insufficient blocking	Change the blocking reagent.
	Insufficient washing	Increase the number of washing steps.
Data spread	Insufficient quality of the microplate	The absorption ability of proteins on the well surface depends on the quality of the microplate. Use different qualities of the microplate.

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