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Instruction Manual

WesternBreeze®

Chromogenic Western Blot Immunodetection Kit

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Important Information

Introduction

The WesternBreeze® Chromogenic Immunodetection Kit contains complete, optimized, ready-to-use or ready-to-dilute reagents for sensitive immunodetection of western blots or dot blots. The kit is used to detect primary antibodies immobilized on nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes.

Contents

The components included in the WesternBreeze® Chromogenic Immunodetection Kit are listed below. Sufficient reagents are supplied to detect 20 mini-blots.

Item	Description	Amount
Blocker/Diluent (Part A)	Concentrated buffered saline solution containing detergent	80 ml
Blocker/Diluent (Part B)	Concentrated Hammersten casein solution	80 ml
Antibody Wash (16X)	Concentrated buffered saline solution containing detergent	2 x 100 ml
Chromogenic Substrate	Ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase.	100 ml
Secondary Antibody Solution	Ready-to-use solution of alkaline phosphatase-conjugated, affinity purified, anti-species IgG (anti-mouse and anti-rabbit kits: goat conjugate, anti-goat kits: rabbit conjugate)	2 x 100 ml
Incubation Dishes	100 mm x 100 mm x 15 mm plastic dishes with lids (Fisher No. O8-757-11A)	2

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Important Information, Continued

Storage

All solutions are proprietary formulations and contain chlorobutanol as a preservative; once diluted, they should be used that day. Store all solutions at 2-8°C.

Safety Issues

The WesternBreeze® Chromogenic Immunodetection Kit, contains a solution of 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT) which is a possible carcinogen and dilute solutions of irritating chemicals. We recommend wearing gloves, safety glasses, and a lab coat when using the WesternBreeze® Chromogenic Immunodetection Kit.

Product Qualification

Invitrogen qualifies the WesternBreeze® Chromogenic Immunodetection Kit as follows:

10 -100 pg human IgG in NuPAGE® LDS sample buffer is electrophoresed on a 4-12% NuPAGE® Novex Bis-Tris Gel using NuPAGE® MES SDS Running Buffer. After electrophoresis, proteins are transferred onto a nitrocellulose membrane using NuPAGE® Transfer Buffer. Immunodetection is performed as described in this manual using anti-human IgG primary antibody. The amount of antigen as shown in the table below must be detected within 40 minutes of color development.

Kit	Amount
Rabbit	10 pg
Mouse	100 pg
Goat	100 pg

General Guidelines

General Guidelines

To obtain the best results with WesternBreeze® Chemiluminescent Immunodetection Kit:

- Use a single, clean dish for each blot.
- Avoid touching the working surface of the membrane, even with gloves.
- Use pure water, free from alkaline phosphatase activity. Stored water should be autoclaved or ultra-filtered to remove alkaline phosphatase activity. Freshly ultra-filtered water is preferred.
- Avoid cross-contamination of system solutions especially with the alkaline phosphatase substrate solution.
- Perform all washing, blocking, and incubating steps on a rotary shaker platform rotating at 1 revolution/second.
- Work quickly when changing solutions as PVDF membranes dry quickly. If the membrane dries, re-wet the membrane with methanol and rinse with water before proceeding (see page 4).
- Add solutions to the trays slowly, at the membrane edge, to avoid bubbles forming under the membrane. Decant from the same corner of the dish to ensure complete removal of previous solutions.

Note

Since the amount of reagents used for the detection of PVDF and nitrocellulose membranes is different, it is normal for some reagents to be in excess in the kit after detecting 20 mini-blots.

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General Guidelines, Continued

Materials Needed

- Blotted membranes containing applied antigen or primary antibody samples
- Primary antibody to detect applied antigen, if appropriate
- Purified water autoclaved, sterile, or ultra filtered to remove alkaline phosphatase activity from all solutions used in the procedure
- Clean flasks for preparing solutions
- Forceps for manipulating blotted membranes.
- Orbital shaker platform

Preparing the Membrane

For western blots or western dot blots, from SDS-PAGE gels to NC or PVDF membranes, wash the membranes twice for 5 minutes with 20 ml of pure water to remove gel and transfer buffer components and some weakly bound proteins. Proceed to immunodetection (see page 7).

If you are using water-washed and dried NC membranes, proceed to immunodetection (see page 7). For water-washed and dried PVDF membranes, re-wet the membrane in methanol followed by two 20 ml water washes for 5 minutes. Proceed to immunodetection (see page 7).

For native-PAGE western blots or western dot blots, a drying step is recommended to improve protein binding to the membrane. Wash the dried NC membranes twice with 20 ml water for 5 minutes before proceeding to immunodetection (see page 7). Re-wet the dried PVDF membranes in methanol, followed by two water washes of 20 ml for 5 minutes before proceeding to immunodetection (see page 7).

Preparing Solutions

Nitrocellulose Membranes

For western blots from Novex® or other mini gels, prepare the solutions for nitrocellulose membranes as described in the table below for ~ 60 cm^2 membrane.

Note: The Blocking Solution is used for blocking and as a Primary Antibody Diluent for NC membranes.

Solution	For NC Membrane	
Blocking Solution	Ultra filtered Water	14 ml
	Blocker/Diluent (Part A)	4 ml
	Blocker/Diluent (Part B)	2 ml
	Total Volume	20 ml
Primary Antibody Diluent	Dilute your primary antibody according to the manufacturers recommendations into 10 ml of NC Blocking Solution (see above). Typically, commercial primary antibody preparations are diluted 1:1000 to 1:5000 to a concentration of about 1 to 0.2 µg/ml	
Antibody Wash	Ultra filtered Water	150 ml
	Antibody Wash Solution (16X)	10 ml
	Total Volume	160 ml

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Preparing Solutions, Continued

PVDF Membranes

For western blots from Novex® or other mini gels, prepare the solutions for PVDF membranes as described in the table below for ~ 60 cm^2 membrane.

Solution	For PVDF Membrane	
Blocking Solution	Ultra filtered Water	5 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	3 ml
	Total Volume	10 ml
Primary Antibody Diluent	Ultra filtered Water	7 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	1 ml
	Total Volume	10 ml
	Dilute your primary antibody into this diluent according to the manufacturer's recommendations. Typically, commercial primary antibody preparations are diluted 1:1000 to 1:5000 to a concentration of about 1 to 0.2 µg/ml.	
Antibody Wash	Ultra filtered Water	150 ml
	Antibody Wash Solution (16X)	10 ml
	Total Volume	160 ml

WesternBreeze® Chromogenic Immunodetection Protocol

Protocol for Small Membranes (60 cm^2)

1. Place the membrane in 10 ml of the appropriate Blocking Solution in a covered, plastic dish provided in the kit. Incubate for 30 minutes on a rotary shaker set at 1 revolution/sec. Decant the Blocking Solution.
2. Rinse the membrane with 20 ml of water for 5 minutes, then decant. Repeat once.
3. Incubate the membrane with 10 ml of Primary Antibody Solution for 1 hour, then decant.
4. Wash the membrane for 5 minutes with 20 ml of prepared Antibody Wash, then decant. Repeat 3 times.
5. Incubate the membrane in 10 ml of Secondary Antibody Solution for 30 minutes, then decant.
6. Wash the membrane for 5 minutes with 20 ml of Antibody Wash, then decant. Repeat 3 times.
7. Rinse the membrane with 20 ml of water for 2 minutes, then decant. Repeat twice.
8. Incubate the membrane in 5 ml of Chromogenic Substrate until purple bands develop on the membrane. Development is complete in 1 to 60 minutes.
9. Rinse the membrane with 20 ml of water for 2 minutes. Repeat twice.
10. Dry the membrane on a clean piece of filter paper to open air, by a stream of slightly warm air, or under an infrared lamp.

Protocol for Large Membranes (200 cm^2)

To blot standard size gels (~200 cm^2), scale up the required solution volumes by 3.3X. Use a tray that closely matches the dimensions of the membrane for most efficient use of the solutions.

Troubleshooting

Introduction

Review the information provided below to troubleshoot your experiments.

High Background

- **Membrane not completely wetted**

  Follow instructions for pre-wetting the membrane. Use an incubation dish which is small enough to allow thorough coverage of membrane to prevent drying out. Shake or agitate during each step.

- **Membrane is contaminated**

  Use only clean, new membranes. Wear clean gloves at all times and use forceps when handling membranes.

- **Blocking solution is incorrect**

  Nitrocellulose and PVDF require different blocking conditions; make sure the appropriate solution volumes were used for preparation.

- **Blocking time or washing time is too short**

  Make sure that each step is performed for the specified amount of time. Strictly adhere to all wash times.

- **Solutions or incubation tray is contaminated**

  Use clean glassware and purified water to prepare solutions. Replace or clean the tray thoroughly with a glassware-cleaning detergent. Rinse thoroughly with purified water. Wear clean gloves at all times.

- **Blot is overdeveloped**

  Follow recommended developing time or remove blot from substrate when signal-to-noise ratio is acceptable.

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Troubleshooting, Continued

High Background, continued

- **Concentrated Primary antibody used**

  Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.

Non-Specific Binding

- **Membrane contaminated by fingerprints or keratin proteins**

  Wear clean gloves at all times and use forceps when handling membranes. Always handle membranes around the edges.

- **Primary antibody too concentrated**

  Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.

- **Insufficient removal of SDS/weakly bound proteins from membrane after blotting**

  Follow instructions for membrane preparation before immunodetection (see page 4).

- **Short blocking time or long washing time**

  Make sure that each step is performed for the specified amount of time.

- **Affinity of the primary antibody for the protein standards**

  Check with the protein standard manufacturer for homologies with primary antibody.

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Weak or No Signal

- **Poor or incomplete transfer**

  Refer to Western Blotting instructions (IM-9051) and repeat blot. After blotting, stain membrane to measure transfer efficiency. Use positive control and/or molecular weight marker.

- **Membrane not completely wet**

  Follow instructions for pre-wetting the membrane. The incubation dish must be small enough to allow thorough coverage of membrane to prevent drying out. Shake or agitate during each step.

- **Primary antibody concentration too low**

  Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.

- **Inactive primary antibody**

  Determine activity by performing a dot-blot.

- **Low Affinity of primary antibody to antigen**

  Obtain a higher affinity primary antibody.

- **Contaminated secondary antibody solution**

  Wear gloves at all times and keep bottles tightly capped when not in use. Use only purified water when preparing reagents.

- **Protein of interest ran off the gel**

  Match gel separation range to size of protein being transferred.

- **Poor retention of proteins**

  Match gel separation range to size of protein being transferred. Use a molecular weight marker with relevant size proteins. Larger proteins require more transfer time, smaller proteins less. Use membrane with the appropriate binding capacity (see Western Blotting instructions).

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Weak or No Signal, continued

- **Sample improperly prepared; antigenicity weakened, or destroyed**

  SDS and reducing agents may interfere with some antibody/antigen affinities.

- **Sample too dilute**

  Load a higher concentration or amount of protein onto the gel.

- **Protein weakly bound to membrane**

  Ensure that transfer buffer contains 10-20% methanol.

- **Incorrect blocking solution**

  Nitrocellulose and PVDF require different blocking conditions; make sure the appropriate solution volumes were used for preparation.

- **Insufficient substrate incubation**

  Perform each step for the specified amount of time or remove blot from substrate when signal-to-noise ratio is acceptable.

- **Substrate is contaminated**

  Wear gloves at all times and keep bottles tightly capped when not in use.

- **Blots are too old**

  Protein may have broken down over time. Use freshly prepared blots.

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Troubleshooting, Continued

"Spotted" Membrane	• **Poor or incomplete transfer** Refer to Western Blotting instructions (IM-9051) and repeat blot. • **Membrane pads are dirty or contaminated** Soak with detergent and rinse thoroughly with purified water before use. Replace pads when they become worn or discolored. • **Membrane not completely wetted** Follow instructions for pre-wetting the membrane. The incubation dish must be small enough to allow thorough coverage of membrane to prevent drying out. Shake or agitate during each step. • **Membrane is contaminated by fingerprints or keratin proteins** Wear clean gloves at all times and use forceps when handling membranes. Always handle membranes around the edges. • **Uneven blocking** The incubation dish must be small enough to allow thorough coverage of membrane. Shake or agitate during each step.
Large, Scattered Signal	• **Protein is overloaded** Reduce load or dilute concentration of sample. • **Poor or incomplete transfer** Refer to Western Blotting instructions (IM-9051) and repeat blot. • **Primary antibody is too concentrated** Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.

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