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Novex® Chromogenic Substrates

Cat. nos. WP20001, WP20004 **Store at 4°C**

Description

Novex® AP Chromogenic Substrate and Novex® HRP Chromogenic Substrate are ready-to-use, single component substrates for sensitive immunodetection of alkaline phosphatase (AP) or horse radish peroxidase (HRP) on western blots or dot blots. The sensitivity of the AP substrate is in the 100 pg range, while the HRP substrate is in the 1 ng range.*

Novex® AP Chromogenic Substrate consists of a ready-to-use solution of 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) which forms a black-purple precipitate upon reaction with alkaline phosphatase. Reducing components formed during alkaline phosphatase hydrolysis of phosphate groups in BCIP react rapidly with NBT to form a very insoluble, purple formazan. This product is not recommended for immunohistochemical techniques.

Novex® HRP Chromogenic Substrate consists of a ready-to-use, non-toxic solution of 3,3′,5,5′-tetramethylbenzidine (TMB) which forms a blue precipitate upon reaction with horse radish peroxidase.

* Sensitivity may vary depending upon the antigen and the antibody used for detection.

Products

Cat. no.	Description	Volume
WP20001	Novex® AP Chromogenic Substrate	250 ml
WP20004	Novex® HRP Chromogenic Substrate	250 ml

Storage Conditions

Store all reagents at 4°C. Avoid exposure to direct sunlight.

Safety Considerations

Substrate formulations contain dilute solutions of irritants. Wear gloves, safety glasses, and a lab coat when using Novex® Chromogenic Substrates.

Method

An example of a standard western blot or western dot blot detection procedure for nitrocellulose (NC) or polyvinylidene fluoride (PVDF) membranes is described below. The protocol can be modified based on initial results.

Materials Required

- Blotted membrane with antigen of interest
- Purified water — autoclaved or ultrafiltered to remove alkaline phosphatase activity (for AP detection)
- Clean containers for preparing solutions and incubating the membrane
- Clean forceps for manipulating blotted membrane
- Orbital shaker capable of rotating at 1 revolution/second
- Blocking buffer such as WesternBreeze® Blocker/Diluent (see page 4) or 5% non-fat dry milk in Tris Buffered Saline with Tween (0.05 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5)
- Wash buffer such as WesternBreeze® Wash Solution (see page 4)
- High affinity antigen-specific primary antibody diluted in blocking buffer at manufacturer recommended concentrations
- AP or HRP conjugated secondary antibody (see page 4)

General Guidelines

To obtain the best results with Novex® Chromogenic Substrates:

- Use enough solution to keep the membrane completely covered at all times.
- Use a single, clean dish for each blot. Containers should be large enough to accommodate the membrane and allow it to be fully immersed by solutions.
- Avoid touching the surface of the membrane. Wear clean gloves and handle the blot only with clean forceps.
- Use pure water, free from alkaline phosphatase activity for making solutions and washes. Fresh ultra-filtered water is preferred. Autoclave or ultra-filter stored water to remove alkaline phosphatase activity.
- Avoid cross-contamination of system solutions with the alkaline phosphatase substrate solution.

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General Guidelines, Continued

- Perform all washing, blocking, and incubation steps on an orbital shaker rotating at 1 revolution/second.
- Work quickly when changing solutions to ensure membranes remain wet. PVDF membranes dry quickly and must be re-wet with methanol and rinsed with water before proceeding if they become dry (see **Preparing Stored Membranes**).
- Add solutions to the trays slowly, at the membrane edge, to avoid bubble formation under the membrane. Decant from the same corner of the dish to ensure complete removal of previous solutions.
- Do not use sodium azide as a preservative for buffers when using an HRP detection system. Sodium azide inhibits HRP enzyme activity.

Suggestions for Antibody Usage

Antibody concentrations for chromogenic detection are higher than those used for chemiluminescent detection. For best result empirically determine the optimal concentration of antibody needed by dot blot. Antibody solutions that are too dilute result in weak or no signal, whereas overly concentrated solutions cause high background or non-specific binding. Dilute antibodies (*i.e.* primary antibodies or concentrated AP or HRP conjugated secondary antibodies) in blocking buffer (see **Materials Required**).
Note: Do not dilute secondary antibody if using Invitrogen 2° Antibody Solutions.

Preparing Stored Membranes

Membranes can be stored after transfer for subsequent immunodetection after being washed to remove gel and transfer buffer components. When starting with washed and dried membranes, perform the following steps:

Membrane	Procedure
NC	Wash the NC membrane with water for 5 minutes twice, then proceed directly to step 2 of the **Western Blot Procedure**.
PVDF	Re-wet the PVDF membrane in methanol and then wash with water for 5 minutes twice before proceeding to step 2 of the **Western Blot Procedure**.

When performing native-PAGE western blots, a drying step is recommended to improve protein binding to the membrane. To re-wet the membrane, follow the appropriate instructions from the table above.

Western Blot Procedure

1. After transfer, wash the membrane with water for 5 minutes twice, to remove any gel and transfer buffer components.
2. Place the membrane in blocking buffer at room temperature for 10–60 minutes with shaking (1 revolution/second), or if desired, overnight at 4°C without shaking. Decant blocking buffer.
3. Add diluted primary antibody solution. Incubate for 30–60 minutes with shaking. Decant solution.
4. Add an excess volume of wash buffer. Wash for 1 minute with shaking. Decant solution.
5. Add a volume of fresh wash buffer and wash with shaking for 5 minutes, then decant. Repeat this step up to 3 times.
6. Add secondary antibody solution. Incubate for 30 minutes. Decant solution.
7. Add an excess volume of wash buffer. Wash for 1 minute with shaking. Decant solution.
8. Add another volume of wash buffer and wash with shaking for 5 minutes, then decant. Repeat this step up to 3 times.
   Note: If using Novex® AP Chromogenic Substrate, **do not use phosphate buffer to wash.** Inorganic phosphate is a powerful inhibitor of AP.
9. Rinse the blot briefly with water for 2 minutes, then decant.
10. Add enough ready-to-use, Novex® Chromogenic Substrate to cover the blot. Incubate with shaking until the desired band intensity is achieved. Decant solution.
11. Stop reaction by rinsing briefly with water and decanting the wash. Rinse with water for 2 minutes. Repeat 2 minute water rinse twice.
    Note: If using Novex® HRP Chromogenic Substrate, **stop the reaction with reagent grade water, do not use tap water, buffer, or acid.** Buffer or tap water can cause fading, and acid turns the bands yellow.
12. Air dry the membrane and record an image of the blot. Store membrane protected from light to prevent band fading. Bands remain visible for years when protected from light.

Troubleshooting

Review the information below to troubleshoot your experiments. For additional troubleshooting, refer to the manual for the WesternBreeze® Chromogenic Western Blot Immunodetection Kit available at www.invitrogen.com.

Problem	Possible cause	Possible Solution
Weak or No Signal	Poor or incomplete transfer	Make sure transfer apparatus and membrane sandwiches are assembled correctly. Use appropriate transfer times. Repeat blot. After blotting, stain membrane to measure transfer efficiency.
	Protein of interest ran off the gel	Use positive control and/or molecular weight marker to match gel separation range to size of protein being blotted. After blotting, stain membrane to measure transfer efficiency.
	Membrane not completely wetted	Follow instructions for pre-wetting the membrane.
	Inactive or overly dilute primary antibody	Determine antibody activity by performing a dot blot. Increase antibody concentration as necessary.
	Sample improperly prepared or antigenicity compromised	SDS and reducing agents may interfere with some antibody/antigen affinities.
	Sample too dilute	Load a larger amount of protein onto the gel.
	Poor retention of proteins or protein weakly bound to membrane	Ensure that transfer buffer contains 10–20% methanol. Use membranes with appropriate binding capacity.
	Insufficient substrate incubation	Remove blot from substrate when signal-to-noise ratio is acceptable.
	Blots are too old	Protein may have degraded over time. Use freshly prepared blots.
High Background	Blot is overdeveloped	Follow recommended developing time or remove blot from substrate when signal-to-noise ratio is acceptable.
	Blocking time or washing time is too short	Perform each step for the specified amount of time.
	Primary and/or secondary antibody concentration too high	Determine optimal antibody concentration by performing a dot blot. Decrease antibody concentration as necessary.
	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. Shake or agitate during each step.
	Membrane, 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. Use forceps when handling membranes.
Non-Specific Binding	Insufficient removal of SDS or weakly bound proteins from membrane after blotting	Follow instructions for membrane preparation before immunodetection.
	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 protein standard manufacturer for homologies with primary antibody.
	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.
"Spotted" Membrane	Membrane blotting pads are dirty or contaminated	Soak pads with detergent and rinse thoroughly with purified water before use. Replace pads when they become worn or discolored.
	Blocking was uneven	The incubation dish must be sufficient 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	Make sure transfer apparatus and membrane sandwiches are assembled correctly. Use appropriate transfer times. Repeat blot.

Related Products

Product	Amount	Catalog no.
WesternBreeze® Blocker/Diluent (part A and B)	2 × 80 ml	WB7050
WesternBreeze® Wash Solution (16X)	2 × 100 ml	WB7003
2° Antibody Solution Alk-Phos. Conjugated (anti-mouse)	2 × 100 ml	WP20006
2° Antibody Solution Alk-Phos. Conjugated (anti-rabbit)	2 × 100 ml	WP20007
iBlot™ Gel Transfer Device	1 unit	IB1001
iBlot™ Transfer Stack, Regular (Nitrocellulose)	10 sets/box	IB3010-01
iBlot™ Transfer Stack, Mini (Nitrocellulose)	10 sets/box	IB3010-02
iBlot™ Transfer Stack PVDF, Regular	10 sets/box	IB4010-01
iBlot™ Transfer Stack PVDF, Mini	10 sets/box	IB4010-02
Nitrocellulose, 0.2 µm/Filter Paper Sandwiches	20 membranes	LC2000
Nitrocellulose, 0.45 µm/Filter Paper Sandwiches	20 membranes	LC2001
PVDF, 0.2 µm/Filter Paper Sandwiches	20 membranes	LC2002
Invitrolon™ PVDF/Filter Paper Sandwiches	20 membranes	LC2005
Novex® Reversible Membrane Protein Stain Kit	1 kit	IB7710
Sponge Pad for blotting	8 pads	EI9052
Primary antibody	---	visit www.invitrogen.com/antibodies

Product Qualification

The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available on our website at www.invitrogen.com/cofa, and is searchable by product lot number, which is printed on each box.

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