invitrogen™

Novex® Chemiluminescent Substrates

Cat. no. WP20002, WP20003, WP20005 **Store at 4°C**

Description

Novex® AP Chemiluminescent Substrate and Novex® ECL Chemiluminescent Substrate are non-radioactive substrates for chemiluminescence-based immunodetection of alkaline phosphatase (AP) or horse radish peroxidase (HRP) on western blots or dot blots. Novex® Chemiluminescent Substrates provide detection sensitivities superior to that of precipitating chromogenic substrates. Low picogram levels of detection can be achieved using either X-ray film or imaging equipment.

Novex® AP Chemiluminescent Substrate consists of a ready-to-use solution of CDP-Star®, a dioxetane-based substrate for detection of alkaline phosphatase. The Novex® AP Chemiluminescent Substrate Enhancer consists of a 20X solution of Nitro-Block-II™ that is mixed with the AP Chemiluminescent Substrate when probing blots on nitrocellulose (NC) membranes. **Do not** use the enhancer with polyvinylidene fluoride (PVDF) membranes, as it can produce high background. Light emission for this substrate builds rapidly in the first 20 minutes after membrane incubation and reaches peak intensity after 45–60 minutes. Light emission has a maximal wavelength at 461–466 nm, and continues for several hours and in some cases, for days.

Novex® ECL Chemiluminescent Substrate Reagent Kit is a two-part reagent consisting of a luminol and an enhancer for the detection of horse radish peroxidase. Reagent A and Reagent B are mixed in equal volumes before application to the blot. Light emission for Novex® ECL Chemiluminescent Substrate is most intense from 5–30 minutes after membrane incubation and decreases slowly with time over the course of several hours.

Products

Cat. no.	Description	Volume
WP20002	Novex® AP Chemiluminescent Substrate	100 ml
WP20003	Novex® AP Chemiluminescent Substrate Enhancer (20X)	5 ml
WP20005	Novex® ECL Chemiluminescent Substrate Reagent Kit	
	HRP Chemiluminescent Substrate, Reagent A	125 ml
	HRP Chemiluminescent Substrate, Reagent B	125 ml

Storage Conditions

Store all reagents at 4°C.

Safety Considerations

Avoid skin contact. Novex® HRP Chemiluminescent Substrate contains irritants and components that can be toxic when exposed to the skin. Wear gloves, safety glasses, and a lab coat when using Novex® Chemiluminescent Substrates.

Method

An example of a standard western blot or western dot blot detection procedure for NC or 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 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® Chemiluminescent Substrates:

- 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.

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General Guidelines, Continued

- Use enough solution to keep the membrane completely covered at all times.
- 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 preparing 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.
- Perform all washing, blocking, and incubation steps on a rotary shaker platform rotating at 1 revolution/second.
- Work quickly when changing solutions to ensure membranes remain wet. PVDF membranes dry quickly and if they do, must be re-wet with methanol and rinsed with water (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 milk in blocking or diluent buffers when using avidin/biotin detection systems. Endogenous biotin in milk can cause high background.
- Do not use sodium azide as a preservative for buffers when using an HRP detection system. Sodium azide inhibits HRP enzyme activity.
- Do not expose the substrate working solutions to intense light. Short term exposure to laboratory light is not harmful to the substrates.

Suggestions for Antibody Usage

Antibody concentrations for chemiluminescent detection are lower than those used for chromogenic 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/sec), 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 diluted 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.
9. Rinse the blot briefly with water for 2 minutes, then decant.
10. Mix the chemiluminescent substrate working solution as described below (use 0.06 ml/cm^2 membrane surface area). For best results, use the working solutions immediately after mixing.

Substrate	Membrane	Substrate Working Solution
HRP	PVDF or NC	Mix equal parts HRP Chemiluminescent Substrate Reagent A and HRP Chemiluminescent Substrate Reagent B.
AP	PVDF	Use Novex® AP Chemiluminescent Substrate alone.
AP	NC	Make a 1:20 dilution of Novex® AP Chemiluminescent Enhancer in Novex® AP Chemiluminescent Substrate (0.125 ml enhancer + 2.375 ml substrate for a 2.5 ml working solution).

Western Blot Procedure, Continued

11. Lay the blot on a clean piece of plastic sheet (such as clear copier transparency material), antigen side up. **Do not** allow the membrane to dry out.
12. Pipet the chemiluminescent substrate working solution onto the blot surface, taking care to cover the entire blot.
13. Allow the reaction to develop as follows:
    - 1 minute for HRP Chemiluminescent Substrate
    - 5 minutes for AP Chemiluminescent Substrate
14. Absorb excess substrate by gently blotting with clean absorbent material (such as filter paper or tissue) or by tilting the plastic sheet so excess substrate drains off onto the absorbent material, but **do not** let the membrane dry out.
15. Overlay another clean piece of plastic on top of the blot to prevent drying during imaging. Gently lowering the top plastic from one side of the blot to the other will prevent bubble formation on top of the blot. If excess substrate squeezes out from between the plastic sheets take care to blot it away. If X-ray film imaging is used, no liquid must contact the film. Plastic wrap can be used as an alternative to plastic sheets.
16. Place the protected blot in a film cassette or imaging system and expose for 1 second to several minutes depending on the signal produced and image desired.

Troubleshooting

Review the information below to troubleshoot your experiments. For additional troubleshooting, refer to the manual for the WesternBreeze® Chemiluminescent 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 exposure time	Re-expose film for a longer period of time.
	Blots are too old	Protein may have degraded over time. Use freshly prepared blots.
High Background	Film overexposed or became wet during exposure	Decrease exposure time or allow signal to further decay. Prevent leakage of solutions by encasing membrane in transparency film and blotting excess substrate from edges before exposure.
	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.

Troubleshooting Continued

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 http://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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