Clarity™ Western ECL Substrate
Instruction Manual

Catalog #	Description
170-5060	Clarity Western ECL Substrate, 200 ml
170-5061	Clarity Western ECL Substrate, 500 ml

BIO-RAD

Table of Contents

Introduction

The Bio-Rad Clarity™ western ECL substrate is compatible with any HRP-conjugate secondary detection reagent and ideal for both digital and film-based imaging. The Clarity substrate provides excellent sensitivity with an extremely long signal duration that allows re-imaging without loss of signal. In addition, Clarity substrate is formulated to exhibit very low background levels that yield exceptionally clear images. The combination of bright, long signal, and low background makes Clarity substrate the perfect choice for most blotting applications.

Quick Start Protocol

1. After immunodetection, keep the membrane moist in wash buffer as you prepare the substrate mixture.
2. Mix substrate kit components in a 1:1 ratio. Prepare 0.1 ml of solution/cm^2 of membrane.
   - For a mini-sized membrane (7 x 8.5 cm), 7 ml of solution is sufficient
   - For a midi-sized membrane (8.5 x 13.5 cm), 12 ml of solution is sufficient
3. Place the membrane protein side up on a clear surface.
   - Add substrate to the blot and incubate for 5 min
4. Image the membrane with a digital imager or by exposing to X-ray film.

Storage Conditions

Kit components are stable for at least one year at room temperature. Freezing will not adversely affect kit performance but do not expose to multiple freeze/thaw cycles.

Background on Chemiluminescence

Chemiluminescence is a chemical reaction that produces light and has become a common detection method for western blotting because of its high sensitivity. With chemiluminescent western blots, a secondary antibody is conjugated to the enzyme horseradish peroxidase (HRP). Once the secondary reagent is bound to the target protein on the membrane, the membrane is incubated with a solution containing the chemiluminescent substrate (Fig. 1). In the presence of peroxide, the HRP enzymes catalyzes the oxidation of luminol, which then generates light (Fig. 2). An enhancer is included in the substrate solution to increase the longevity and intensity of the emitted light. Depending on the substrate and enhancer formulation, the half-life of the light-generating reaction can range from a few minutes to over an hour. This light resulting from this reaction can be detected with either film or a digital imaging system. When combined with a Bio-Rad digital imager such as the ChemiDoc™ MP, the Bio-Rad Clarity substrate produces clear digital images that can be directly used for analysis or publication.

Antibody Incubations

A typical immunodetection experiment system utilizes two sets of antibodies.

- The primary antibody, which is directed against the target protein (Antigen)
- The secondary reagent, in this case an antibody that recognizes and binds to the primary antibody; it is conjugated to an enzyme such as HRP, which will convert the substrate into light, which is then detected by an imager of film

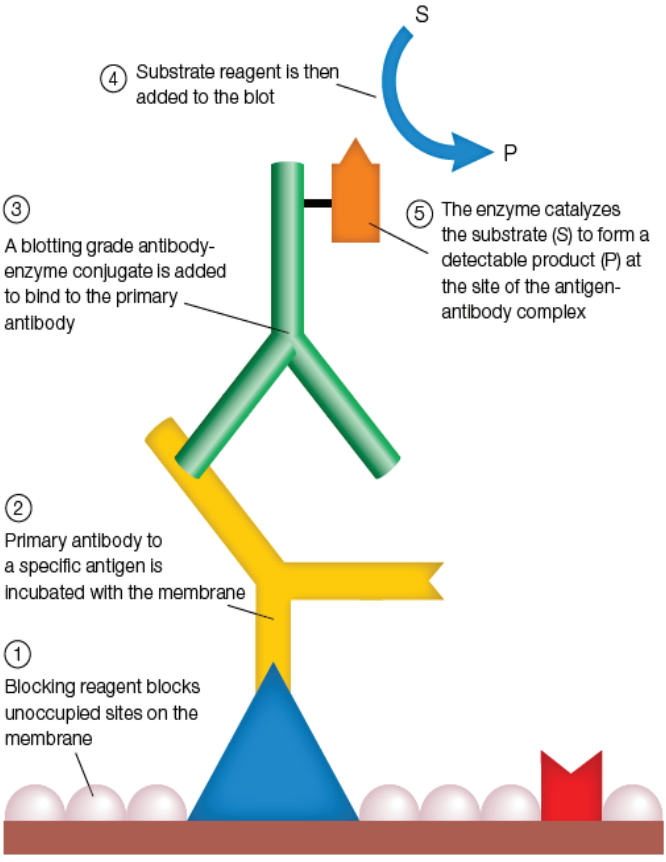

Fig. 1. Specific enzymatic detection of membrane-bound antigens.

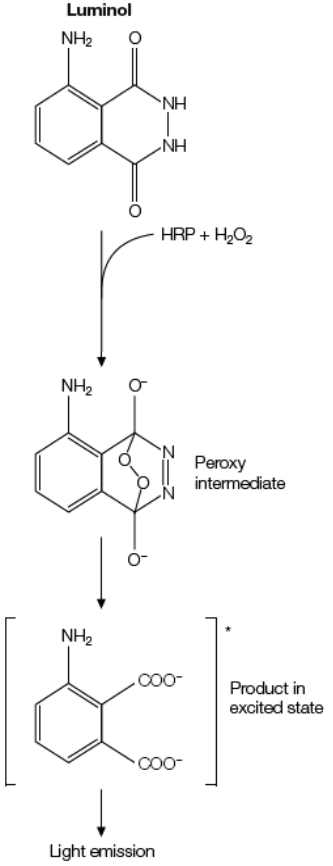

Fig. 2
Chemiluminescence detection by HRP.
The secondary antibody is linked to an enzyme, which catalyzes a reaction leading to light emission. Luminol oxidized by HRP in the presence of a peroxide leads to the formulation of a 3-aminophthalate dianion and the release of light.

Detailed Assay Procedure

Example Western Protocol

Materials

- PVDF, LF PVDF, or nitrocellulose membrane with transferred proteins
- Blocking buffer (Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) with 0.05% Tween-20 and 1–6% of a blocking reagent, typically BSA, gelatin, casein, or nonfat dry milk
- Wash buffer (TBS or PBS with 0.05% Tween-20)
- Primary antibody, diluted in blocking buffer
- HRP-conjugated secondary reagent, such as goat anti-rabbit or goat anti-mouse conjugated HRP, diluted in wash buffer

Immunodetection

1. Wash buffer volumes should be at least 20 ml for mini blots and 100 ml for midi blots. Block and antibody solution volumes should be at least 10 ml for mini blots and 25 ml for midi blots.
2. Equilibrate membrane with transferred proteins in wash buffer for 3 minutes. Dried PVDF and LF PVDF membranes should be briefly re-wet in methanol prior to equilibration in wash buffer.
3. Incubate the membrane, protein side up, in blocking buffer for 1 hr with continuous agitation.

4. Incubate the membrane in diluted primary antibody solution for 1 hr with continuous agitation.
    - Incubation in primary antibody may be carried out overnight at 4°C
5. Wash the blot in wash buffer 5 times for 5 min each with continuous agitation.
6. Incubate the blot in diluted secondary antibody solution for 1 hr with continuous agitation.
7. Wash the blot in wash buffer 6 times for 5 min each with continuous agitation.

Chemiluminescent Development

1. Keep the membrane moist in wash buffer as you prepare the substrate mixture.
    - Do not allow the membrane to dry out during the subsequent steps
2. Mix substrate kit components in a 1:1 ratio. Prepare 0.1 ml of solution/cm^2 of membrane.
    - For a mini-sized membrane (7 x 8.5 cm), 7 ml of solution is sufficient
    - For a midi-sized membrane (8.5 x 13.5 cm), 12 ml of solution is sufficient
3. Place the membrane, protein side up, on a clear surface.
    - Add substrate to the blot and incubate for 5 min
    - Ensure the surface of the blot is completely covered with substrate with no air bubbles

4. Remove the membrane from the substrate solution and drain off excess.
5. Place membrane in a plastic sheet protector or in plastic wrap to prevent the membrane from drying.
6. Image the membrane with a digital imager or by exposing to X-ray film.
    - If switching to Bio-Rad Clarity from another chemi substrate, optimal exposure times may be different. Refer to the **Substrate Transition Guide** below when switching substrates

Transition Guide. To transition to Clarity, use the following guidelines to adjust exposure times.

Current Substrate	Exposure Using Clarity
GE ECL/Pierce ECL	Decrease 20-fold*
SuperSignal West Pico	Decrease 5-fold*
Bio-Rad® Immun-Star™ HRP	Decrease 5-fold*
Pierce ECL Plus	Equal
Bio-Rad® Immun-Star™ WesternC™	Equal
SuperSignal West Dura	Equal
ECL Prime	Increase 2-fold
SuperSignal West Femto	Increase 2-fold
ECL Select	Increase 2-fold

*Or decrease antibody dilution by 2- to 5-fold.

Troubleshooting

Problem	Cause	Solution
High background	Blocking was incomplete	• Increase the concentration of blocking agent or increase blocking duration • Match the blocker to the membrane. For example, gelatin may give poor results on PVDF membranes
	Washing was insufficient	• Increase the number, duration, or stringency of the washes
	The primary or secondary antibody was too concentrated	• Decrease antibody concentrations • Perform a dot-blot experiment to optimize the working concentrations
Areas of no signal within a band (a donut appearance)	Localized substrate depletion	• Bands on the blot with high protein amounts will lead to excessive local concentrations of peroxidase conjugate. This may lead to localized substrate depletion. Use lower concentrations of primary and secondary antibody • Load less sample

Troubleshooting, continued

Problem	Cause	Solution
No reaction or weak signal	Proteins may be washed from the membrane during assays	• Reduce the number or stringency of washes
	Antigen binding to the membrane was insufficient	• Decrease antibody concentrations • Stain the gel after transfer or use prestained standards to assess transfer efficiency • Some total protein stains (such as amido black and colloidal gold) interfere with antibody recognition of the antigen. Do not use a total protein stain, or use a different stain • Optimize the blocking reagent. Some blocking reagents (such as nonfat dry milk) provide high stringency at the expense of sensitivity. Others (such as BSA) offer comparatively high sensitivity at the expense of higher background or nonspecific binding

Troubleshooting, continued

Problem	Cause	Solution
	Poor antibody binding to the antigen	• Detergents may affect the binding of some antibodies. Eliminate or reduce their amount from the assay • Increase the antibody incubation times
	Insuffecient reagent volume	• Use additional volumes of blocking, antibody, and wash solutions
	The enzyme conjugate was inactive	• Test the reagent for activity • Horseradish peroxidase is most active at optimal pH. Ensure excess wash buffer is removed from the membrane before application of substrate • Sodium azide is a potent inhibitor of horseradish peroxidase. Eliminate from antibody stocks
Blank spots in areas of membrane that should have signal	The membrane was allowed to dry during handling	• Ensure that no air bubbles were present during assembly of transfer stack • Ensure that warm membranes are not allowed to dry after transfer

Ordering Information

Catalog	Product
170-5060	**Clarity™ Western ECL Substrate**, 200 ml size contains Clarity western peroxide reagent, 100 ml, and Clarity western luminol/enhancer reagent,100 ml
170-5061	**Clarity Western ECL Substrate**, 500 ml size contains Clarity western peroxide reagent, 250 ml, and Clarity western luminol/enhancer reagent, 250 ml

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723*
Australia *61 2 9914 2800* ***Austria*** *01 877 89 01*
Belgium *09 385 55 11* ***Brazil*** *55 11 5044 5699*
Canada *905 364 3435* ***China*** *86 21 6169 8500*
Czech Republic *420 241 430 532* ***Denmark*** *44 52 10 00*
Finland *09 804 22 00* ***France*** *01 47 95 69 65*
Germany *089 31 884 0* ***Greece*** *30 210 9532 220*
Hong Kong *852 2789 3300* ***Hungary*** *36 1 459 6100*
India *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091*
Japan *03 6361 7000* ***Korea*** *82 2 3473 4460*
Mexico *52 555 488 7670* ***The Netherlands*** *0318 540666*
New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30*
Poland *48 22 331 99 99* ***Portugal*** *351 21 472 7700*
Russia *7 495 721 14 04* ***Singapore*** *65 6415 3188*
South Africa *27 861 246 723* ***Spain*** *34 91 590 5200*
Sweden *08 555 12700* ***Switzerland*** *061 717 95 55*
Taiwan *886 2 2578 7189* ***Thailand*** *800 88 22 88*
United Kingdom *020 8328 2000*

10026389 Rev A US/EG *0712 Sig 1211*