LumiGO / LumiPRO
ECL Reagent

LumiGO and LumiPRO

Instruction Manual

Catalogue Numbers

ECLONE

ECLULTRA

Contents

Packing List

Kit components

SKU	Solution A	Solution B
ECLONE	Luminol/enhancer solution (amber bottle) 125 ml	Peroxide solution (white bottle) 125 ml
ECLULTRA	Luminol/enhancer solution (amber bottle) 50 ml	Peroxide solution (white bottle) 50 ml

Packing List Checked by: _____

Date: _____

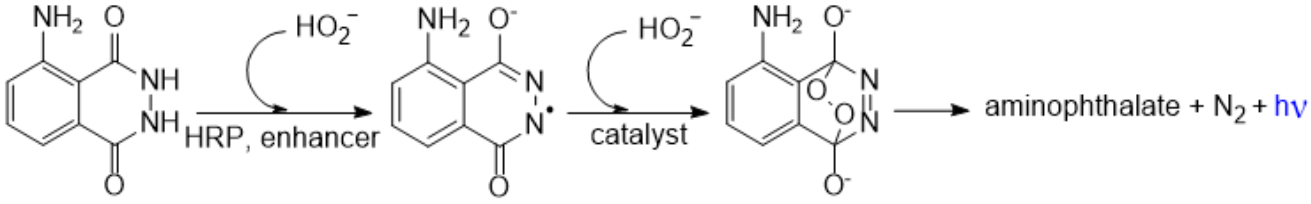
The packing lists should be referred to as soon as the units are received to ensure that all components have been included. The unit should be checked for damage when received.

Cleaver Scientific is liable for all missing or damaged parts within 7 days after customers have received this Kit. Please contact Cleaver Scientific immediately regarding this issue. If no response within such period is received from the customer, Cleaver Scientific will no longer be liable for replacement/damaged parts.

Please contact your supplier if there are any problems or missing items.

Introduction

The peroxidase-catalysed oxidation of luminol produces a weak flash of light at 425 nm. The incorporation of an electron transfer mediator into the buffer forces the flash signal into a glow and greatly improves the analytical characteristics of the reaction in terms of increased signal intensity and duration[1-2]. Recent works[3-6] have shown that, by addition of a suitable acylation catalyst, a further large increase in light output is observed.



luminol | luminol radical anion | luminol endoperoxide

ECL detection reagents are non-isotopic, luminol-based chemiluminescence substrate, designed for the chemiluminescent detection of immobilized proteins and immobilized nucleic acids conjugated with horseradish peroxidase (HRP). ECL is intended for research use only, and shall not be used in any clinical procedures, or for diagnostic purposes.

Storage/expiry

One year at room temperature (25° C).

Cleaver product line

	LumiGO	LumiPRO
Signal intensity	Medium	Extreme
Signal duration	Long	Short
Protein quantity	High abundance	Very-low abundance

Other required solutions

Solution	Preparation	
Running Buffer	**For 1L of 10x Running Buffer (stock):** • 30.3 g TRIS (250mM) • 144.0 g Glycine (1.9M) • 10.0 g SDS (1% w/v) • Dilute to 1L with distilled water	**For 1L of Running Buffer:** • 100mL of **10x Transfer Buffer** • Dilute to 1L with distilled water
Transfer Buffer	**For 1L of 10x Transfer Buffer (stock):** • 30.3 g TRIS (250mM) • 144.0 g Glycine (1.9M) • Dilute to 1L with distilled water	**For 1L of Transfer Buffer:** • 100mL of **10x Transfer Buffer** • 200mL of methanol • Dilute to 1L with distilled water
TBS-T Buffer	**For 1L of 10× TBS Buffer (stock):** • 24.23 g TRIS-HCl (20mM) • 80.06 g NaCl (136mM) • Dilute to 800mL with distilled water • Add NaOH 1M until pH is about 7.6 • Dilute to 1L with distilled water	**For 1L of TBS-T Buffer:** • 100 ml of **10× TBS Buffer** • While stirring add 1 mL Tween-20 • Dilute to 1L with distilled water
Blocking Buffer	**With 5% non-fat dried milk:** • 5 g Non-fat dried milk • Dissolve in 100 ml **1× TBS-T Buffer**	**With 5% BSA:** • 5 g BSA (Cohn fraction V) • Dissolve in 100 ml **1× TBS-T Buffer**
Ponceau staining solution	**For 100mL of 10x Ponceau staining solution (stock):** • Dissolve 0.5 g Ponceau S in 1.0 ml glacial acetic acid • Dilute to 100 ml with distilled water • Wrap bottle with foil to protect solution from light	**For 1L of Ponceau staining solution:** • 100 ml of **10x Ponceau S staining solution** • Dilute to 100 ml with distilled water

Cleaver Compatible Imaging Devices

- ChemiLITE
- chemiPRO
- chemiPROXS 6/9
- chemiPROXL 6/9

Protocols

Perform SDS-PAGE

1. Prepare fresh Running Buffer.
2. Load the gels being sure to keep a tight seal between the gel-cast and the gasket.
3. Pour the running buffer into the middle of the gels and check for leaks.
4. Pour the rest of the running buffer into the bottom of the running tank.
5. Remove combs and use a pipette to clean away any unpolymerized acrylamide.
6. Load a proper pre-stained MW standard in one lane.
7. Load samples into the rest of the wells and fill any empty well with sample buffer.
8. Run at 90-130 V constant voltage until the dye front reaches the bottom of the gel. If the current is too high band smiling and smearing (diffuse band) are commonly seen effects.

Prepare transfer membrane

If using nitrocellulose membrane place into distilled water slowly, with one edge at a 45° angle. If inserted too quickly into the water, air gets trapped and protein will not transfer onto these areas. Once wet, equilibrate the membrane in Transfer Buffer for 15 min.

If using PVDF membrane activate it with methanol for 30 seconds. Rinse with distilled water and equilibrate in Transfer Buffer for 15 min.

For proteins >15 kDa use membrane pore size 0.45 □m

For proteins <15 kDa use membrane pore size 0.2 □m

NOTE: Low molecular weight proteins (<15kDa) are sometimes transferred through nitrocellulose membranes, therefore may be not visible on the blot. PVDF membrane has higher protein binding capacity than nitrocellulose membrane and is recommended for best detection sensitivity.

Transfer to membrane

1. Wet four 3M filter papers in Transfer Buffer.
2. Assemble the transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with Transfer Buffer so that the cassette is covered.
3. Place the first foam pad onto the black side of the transfer cassette then place two pre-wetted filter papers on the top of it.
4. Place the gel and moisten its surface with Transfer Buffer.
5. Place pre-wetted membrane directly on the top side of the gel, then gently remove all air bubbles. The proteins will transfer as soon as the gel is placed on the membrane, its repositioning can generate a smeared image.
6. Place another two pre-wetted filter paper over the membrane and remove all air bubbles.
7. Complete the assembly by placing the last foam pad and locking the top half of the transfer cassette.
8. Fill the transfer tank with Transfer Buffer and place the transfer cassette.
9. Put a frozen cooling unit into the transfer tank and surround it with ice in a polystyrene box.
10. Run the transfer with the following settings:
11. Wet transfer: 80-100 V for 30-60min.
12. Semi-dry transfer: 15-25 V for 20-30min.
13. When transfer is complete, remove the membrane and mark its orientation by cutting a corner.
14. Wash the membrane twice with distilled water.

Constant voltage or current during transfer?

The buffer composition changes as salts are eluted from the gels, resulting in an increase in current and a drop in resistance. A transfer using constant current leads to decrease in voltage as well as resistance (I=V/R). Therefore, the use of constant voltage provides the best driving force during transfer. However, when current reaches over 500mA in constant voltage setting cooling the gel is crucial for preventing joule heating in the tank.

Membrane staining (optional)

1. Stain the membrane with protein side up using Ponceau staining solution for 5 minutes at RT to check transfer efficiency.
2. Rinse the membrane in distilled water until protein bands are distinct.
3. Scan the membrane if desired.
4. Completely destain the membrane by immersing it for 10 min in a large volume of distilled water.
5. Re-activate PVDF membrane with methanol then wash in TBS-T Buffer.

NOTE: The background staining tends to be high with some dyes while Ponceau staining solution gives a very clean pattern. Re-activate PVDF membrane after staining. The LOD for Ponceau staining solution is 250 ng of protein.

Blocking the membrane

1. Place the membrane with protein side up into a fresh tray with your choice of Blocking Buffer.
2. Incubate the membrane in Blocking Buffer for 30-60 minutes with gentle agitation on a rocker/shaker. A maximum blocking time of 2 hours at RT should not be exceeded. Blocking for too long can result in antigen masking and loss of protein.
3. Rinse the membrane twice with TBS-T Buffer.

NOTE: Add 3% non-fat dry milk in TBS-T Buffer when dilute Ab to reduce non-specific bindings. Milk contains many proteins which bind to the membrane. So, after transfer, proteins contained in the milk bind to the membrane and fill a lot of potential non-specific sites. After this, when you incubate with your antibody, it binds to the antigen and has less possibilities of non-specific binding. If you are working with anti-phosphoproteins or with biotinylated antibodies the adding of milk is not appropriate. Use 5% BSA instead.

Antibody incubation

1. Dilute the primary antibody in fresh TBS-T Buffer to the suggested primary antibody dilution (see table below).
2. Incubate the membrane with protein side up in the primary antibody solution for 1 to 2 hours at RT. To increase sensitivity, try an overnight incubation at 4°C with agitation on a rocker. Make sure the membrane is completely covered with TBS-T Buffer with primary Ab.
3. Wash the membrane with protein side up 4 times for 3 to 5 minutes each with TBS-T Buffer with gentle agitation on a shaker. After each washing place the membrane on a new clean tray with fresh TBS-T buffer.
4. Dilute the secondary Ab in fresh TBS-T Buffer to the suggested secondary antibody dilution (see table below).
5. Incubate the membrane with protein side up for 30 minutes to 1 hour at RT. Increasing the incubation time of the secondary antibody usually leads to higher background.
6. Wash the membrane with protein side up 4 times for 3 to 5 minutes each with TBS-T Buffer with gentle agitation on a shaker. After each washing place the membrane on a new clean tray with fresh TBS-T buffer.

IMPORTANT: Optimal Ab dilutions may vary between different applications and depend on quality and affinity for the target protein. It is crucial to optimize both primary and secondary Ab dilutions for best results with high signal and low background. Optimal Ab dilutions can be determined by Dot-Blot assay.

Suggested Ab dilution for LumiGO

Primary Ab	from 1:500 to 1:5,000
Secondary Ab	from 1:20,000 to 1:100,000

Suggested Ab dilutions for LumiPRO

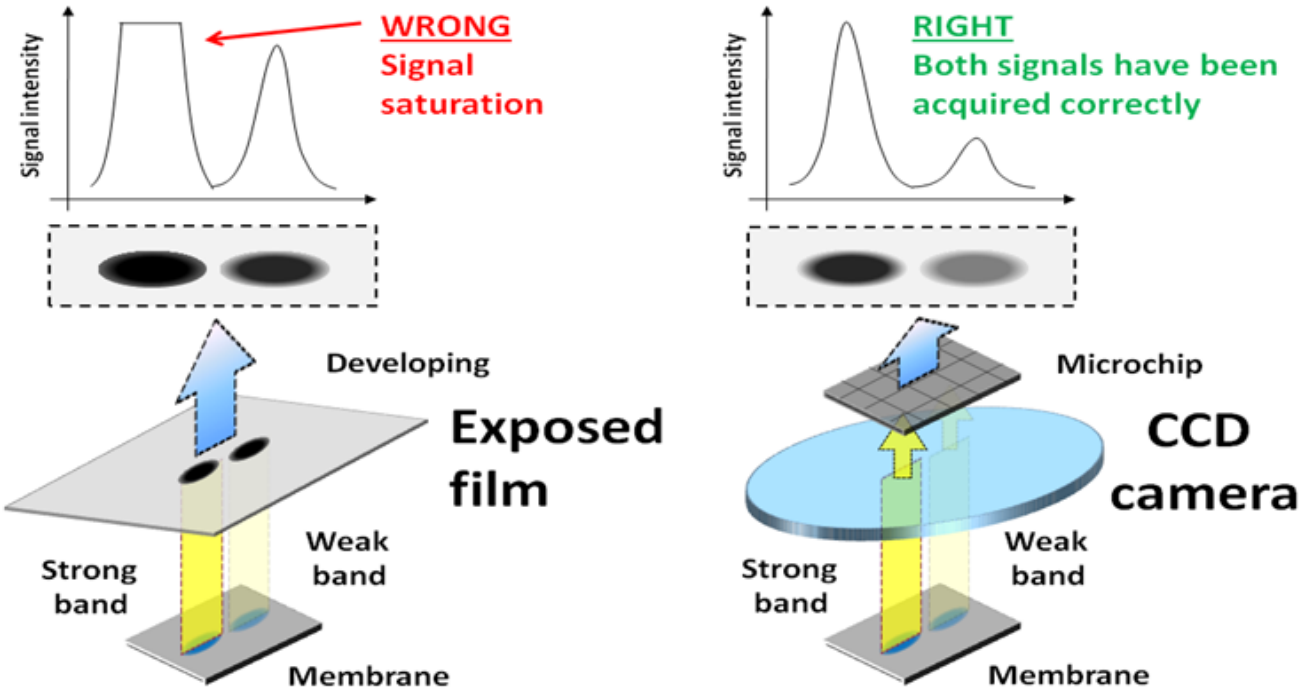
Primary Ab	from 1:5,000 to 1:100,000
Secondary Ab	from 1:100,000 to 1:500,000

Chemiluminescent detection

1. For reproducible performance allow the detection solutions to equilibrate to RT before using.
2. Prepare ECL detection working solution by mixing properly each reagent in a 1:1 ratio. For best results prepare the substrate immediately before use. Do not contaminate the solutions with the same pipette tips.
3. Remove the membrane from its tray of TBS-T Buffer and rinse the membrane twice with TBS-T Buffer and keep it in TBS until the incubation with ECL detection solution
4. Use 0.1 ml of LumiGO or LumiPRO per cm2 of membrane.
5. Allow the excess buffer to run off from a corner. Do not let the membrane dry out. Just pipette the volume required directly onto the membrane with protein side up and incubate for 1,5 min ensuring that the entire surface is covered.
6. Acquire the signal with autoradiography film or imaging devices. For an unknown signal, try to expose 15 s, 30 s, 1 min and 5 min to start with.

Autoradiography film vs. imaging devices

Nowadays, Western Blotting is used either for absolute quantification (in combination with a calibration curve of the recombinant protein of known concentration) or for quantification of samples relative to a control sample. Through the development of new technologies most imagers offer a wide dynamic range (3-5 orders of magnitude) generating a high-quality image compared with the limited linear dynamic range of film (1.5 orders of magnitude). This means that is possible to quantify both strong and weak signals on the same blot with reliable results. Instead, on film strong signals get saturated resulting in a wrong quantitation.



Troubleshooting

High membrane background

- High concentration of Ab. Further dilute primary and secondary Ab. Follow suggested Ab dilutions.
- Inefficient blocking. Increase Tween-20 in TBS-T Buffer (0.1%-0.5% v/v). Use 5% non-fat dried milk as blocking buffer if possible.
- Insufficient washing. Increase both the volume, length and number of wash steps. Always use sufficient volumes to submerse the membrane.
- Primary antibody is not specific for the protein of interest. Use monospecific or antigen affinity purified antibodies. Always incubate your primary antibody at 4°C overnight and not at room temperature. Reduce NaCl in TBS-T Buffer (100mM-350mM). Use monospecific or antigen affinity purified Ab.
- Non-specific binding of secondary antibody. Confirm the secondary is specific by omitting the primary and running a secondary only blot. If bands develop choose an alternative secondary antibody.
- Incompatible blocking agent. Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin systems. Substitute with 5% BSA.
- Poor quality of antibodies. Quality and age of primary and secondary antibody may lead to background problems.
- Poor handling of membrane. Be sure to handle the membrane only with clean plastic tweezers and non-powdered gloves.
- Contaminated buffer solutions. Check buffers for particulate or bacterial contaminate. Replace old buffers.

Irregular black spots

- Air bubble trapped in membrane. Remove air bubbles by gently rolling a clean pipette or a test-tube during sandwich assembling.
- Unevenly hydrated membrane. Make sure that the membrane is fully immersed during washes and antibody incubations.

- Contaminated equipment. Protein or pieces of gel remaining on the unit may stick to the membrane. Antibody can get trapped in the gel, and then are washed out poorly, resulting in intense localized signal.
- Aggregation of blocking agent. When blocking agent is powder stir it over night at 4°C to make sure it is completely dissolved.
- Interaction of the membrane with sample tray. Always use clean plastic trays to avoid any type of cross-reaction.
- Formation of aggregates in HRP-conjugate. Filter secondary antibody solution through a 0.2 □m filter. Use fresh antibody.

No bands or weak bands

- Excessive signal generated. The enzyme in the system depleted the substrate and caused the signal to fade quickly. Further dilute secondary Ab.
- Inefficient transfer. Ensure that there is good contact between membrane and gel during sandwich assembling. High MW protein may require more time for transfer. Reduce voltage or time of transfer for low molecular weight proteins (< 10 kDa).
- Antibodies may have lost activity. Perform a Dot Blot. Follow manufacturer's recommended storage and avoid freeze/thaw cycles.
- Incorrect secondary antibody used. Confirm host species/Ig type of primary Ab.
- Low protein-antibody binding. Reduce the number of washes to minimum. Reduce NaCl in TBS-T Buffer (100mM-350mM).
- Non-fat dry milk may mask some antigens. Decrease blocking time. Decrease milk percentage in Blocking Buffer or substitute with 5% BSA Blocking Buffer.
- Sodium azide contamination. Make sure buffers do not contain sodium azide as this will quench HRP signal.
- Contaminated stock solutions. Do not contaminate the chemiluminescent substrate stock solutions using the same pipette tip. Use new reagents.

Non-specific bands

- Aggregation of analyte. Increase amount of reducing agent to ensure complete reducing of disulphide bonds.
- SDS interference. The presence of SDS may result in the development of unspecific bands caused by antibodies binding to the charged SDS molecules associated with the proteins. Wash thoroughly the membrane after transfer with water.
- High protein concentration. A commonly seem effect is the diffusion of protein bands. Reduce the amount of protein initially loaded.
- Primary antibody is not specific for the protein of interest. Use monospecific or antigen affinity purified antibodies. Always incubate your primary antibody at 4°C overnight and not at room temperature. Reduce NaCl in TBS-T Buffer (100mM-350mM). Use monospecific or antigen affinity purified Ab.
- Non-specific binding of secondary antibody. Confirm the secondary is specific by omitting the primary and running a secondary only blot. If bands develop choose an alternative secondary antibody.

White bands or "ghost bands"

- Excessive signal generated. Excessive antibodies or loaded protein can cause high levels of localized signal. This results in rapid consumption of substrate at this point. Since there is no light production after the completion of this reaction, white bands are the result. Try first to further dilute secondary antibody.

Uneven or jagged bands

- Uneven gel runs. Load all available wells. Empty wells can be loaded with sample buffer.
- Voltage or current were too high during electrophoresis. Reduce voltage or current during electrophoresis.
- Effects of high salt in samples. Reduce NaCl concentration in TBS-T Buffer (100mM-350mM).

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