NeoPRO ECL substrates

NB-78-00001
NB-78-00002
NB-78-00003
NB-78-00004
NB-78-00005
NB-78-00006-20
NB-78-00006-100
NB-78-00007
NB-78-00008
NB-78-00009

Table of contents

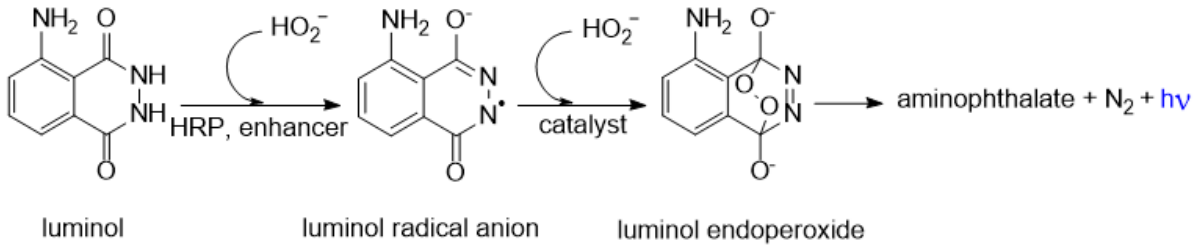
NeoPRO ECL substrates

#Cat: NB-78-00001	**Size:2x125mLkit**
#Cat: NB-78-00002	**Size:2x125mLkit**
#Cat: NB-78-00003	**Size:2x125mLkit**
#Cat: NB-78-00004	**Size:2x50mLkit**
#Cat: NB-78-00005	**Size:2x50mLkit**
#Cat: NB-78-00006-20	**Size:2x10mL**
#Cat: NB-78-00006-100	**Size:2x50mL**
#Cat: NB-78-00007	**Size:250mLkit**
#Cat: NB-78-00008	**Size:250mLkit**
#Cat: NB-78-00009	**Size:125mLkit**

NeoPRO and NeoPRO RTU are intended for research use only and shall not be used in any clinical procedures or for diagnostic purposes

1-Introduction

The peroxidase-catalyzed oxidation of luminol and its derivatives produces a weak flash of light at 425 nm. Incorporating 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 a further increase in light output is observed by adding a suitable acylation catalyst.



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

References:

1. Kricka, L.J. (2000) *Methods Enzymol.* 305, 370-390.
2. Heindl, D. and Josel, H.P. (1997) *Non-radioactive Analysis of Biomolecules*, 258-261. Springer, Berlin.
3. Marzocchi, E., Grilli. S., Della Ciana, L., Prodi, L., Roda, A. and Mirasoli, M., (2008) *Anal. Biochem*., 377, 189-194.
4. Vdovenko, M. M., Della Ciana, L., Sakharov, I. Yu., (2009) *Anal. Biochemistry*, 392, 54-58.
5. Vdovenko, M. M., Della Ciana, L., Sakharov, I. Yu., (2010) *Biotechnology Journal*, 5(8),886-90.
6. Vdovenko, M.M., Zubkov, A.V., Kuznetsova, G.I., Della Ciana, L., Kuzmina, N.S., Sakharov, I. Yu., (2010) *J Immunol Methods*, 362 (1-2), 127-130.

Storage/expiry
One year at room temperature (18-25°C).

NeoPRO product line
NeoPRO is a Neo Biotech product line of two-component chemiluminescent substrates for Western blotting. NeoPRO products offer different sensitivity levels for your Western blotting, allowing detections from picogram to femtogram.
Our proprietary technology enables the fine tuning of signal intensity to obtain the assay sensitivity and signal duration that are best suited to meet each experimental need.

NeoPRO	SUN	NOVA 2.0	ANTARES	ETA C ULTRA 2.0	SUPERNOVA	HYPERNOVA
Signal intensity	Standard	Medium	High	Very High	Ultra High	Extremely High
Signal duration	Medium	Medium	Extended	Extended	Short	Short
Protein abundance	High	High	Medium	Low	Ultra Low	Extremely Low

NeoPRO -RTU product line
NeoPRO -RTU is a Neo Biotech product line of one-component chemiluminescent substrates for Western blotting. NeoPRO -RTU products are available for different sensitivity levels to cover all detection needs, from picograms to low femtograms. Our premixed solution, which is based on proprietary technology, enables increased experimental consistency, avoiding pipetting errors and possible contaminations.

NeoPRO -RTU	BASIC	PLUS	EXTREME
Signal intensity	Medium	High	Ultra High
Signal duration	Short	Medium	Short
Protein abundance	High	Medium	Ultra Low

2- Components and other materials required

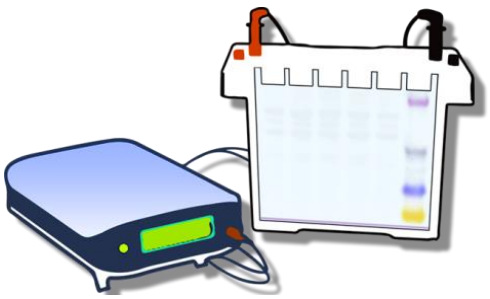
NeoPRO - Kit components
- **Solution A:** Luminol derivative/enhancer solution (amber bottle)
- **Solution B:** Peroxide solution (white bottle)

NeoPRO -RTU
- Luminol derivative/enhancer/peroxide solution (amber bottle)

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

3-Perform SDS-PAGE



I. Prepare fresh **Running Buffer.**
II. Load the gels being sure to keep a tight seal between the gel-cast and the gasket.
III. Pour the running buffer into the middle of the gels and <u>check for leaks.</u>
IV. Pour the rest of the running buffer into the bottom of the running tank.
V. Remove combs and use a pipette to clean away any unpolymerized acrylamide.
VI. Load a proper **prestained MW standard** in one lane.
VII. Load samples into the rest of the wells and fill any empty well with sample buffer.
VIII. <u>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.</u>

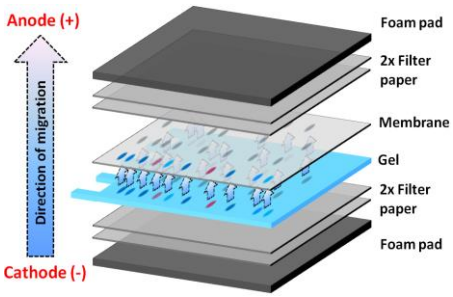
4-Prepare transfer membrane

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.

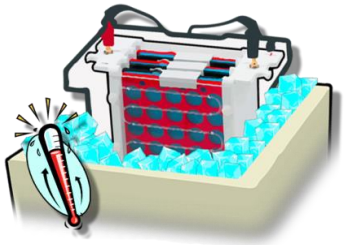
- **For proteins >15 kDa use membrane pore size 0.45 mm**
- **For proteins <15 kDa use membrane pore size 0.2 mm**

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.

5- Transfer to membrane



I. Wet four filter papers in **Transfer Buffer.**
II. 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.
III. 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.
IV. Place the gel and moisten its surface with **Transfer Buffer**.
V. 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.
VI. Place another two pre-wetted filter paper over the membrane and remove all air bubbles.
VII. Complete the assembly by placing the last foam pad and locking the top half of the transfer cassette.
VIII. Fill the transfer tank with **Transfer Buffer** and place the transfer cassette.
IX. Put a frozen cooling unit into the transfer tank and surround it with ice in a polystyrene box.



X. Run the transfer with the following settings:
Wet transfer: 80÷100 V for 30÷60min.
Semi-dry transfer: 15÷25 V for 20÷30min.
XI. When transfer is complete, remove the membrane and mark its orientation by cutting a corner.
XII. 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.

6-Membrane staining (optional)

I. Stain the membrane with protein side up using **Ponceau staining solution** for 5 minutes at RT to check transfer efficiency.
II. Rinse the membrane in distilled water until protein bands are distinct.
III. Scan the membrane if desired.
IV. Completely destain the membrane by immersing it for 10 min in a large volume of distilled water.
V. 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.

7- Blocking the membrane

I. Place the membrane with protein side up into a fresh tray with your choice of **Blocking Buffer.**
II. 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.
III. 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.

8- Antibody incubation

I. Dilute the primary antibody in fresh **TBS-T Buffer** to the suggested primary antibody dilution (see table below).

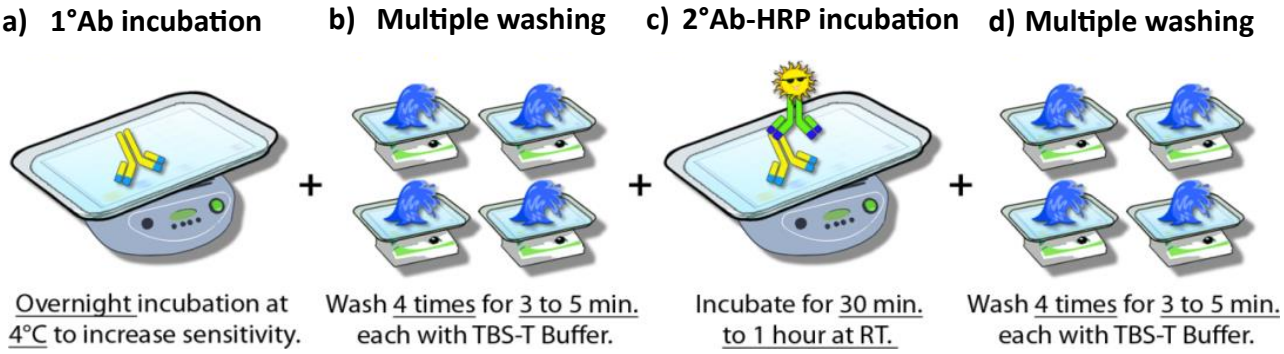
II. Incubate the membrane with protein side up in the primary antibody solution for 1 to 2 hours at RT. To increase sensitivity, try an overnight incubation at 4°C with agitation on a rocker. Make sure the membrane is completely covered with **TBS-T Buffer** with primary Ab.

III. 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.**

IV. Dilute the secondary Ab in fresh **TBS-T Buffer** to the suggested secondary antibody dilution (see table below).

V. 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.

VI. 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.

Product	Suggested antibody dilutions
NeoPRO Nano Western ECL Substrate Cod. **NB-78-00001** NeoPRO RTU Nano - Ready to Use Western ECL Substrate Cod. **NB-78-00007**	**Primary Ab** 1:100 - 1:5,000 **Secondary Ab** 1:1,000 - 1:15,000
NeoPRO Pico Western ECL SubstrateCod. **NB-78-00002**	**Primary Ab** 1:500 - 1:5,000 **Secondary Ab** 1:20,000 - 1:100,000
NeoPRO Pico PLUS Western ECL Substrate Cod. **NB-78-00003** NeoPRO RTU Pico - Ready to Use Western ECL Substrate Cod. **NB-78-00008**	**Primary Ab** 1:1000 - 1:15,000 **Secondary Ab** 1:25,000 - 1:150,000
NeoPRO Femto Western ECL Substrate Cod. **NB-78-00004**	**Primary Ab** 1:5000 - 1:50,000 **Secondary Ab** 1:50,000 - 1:250,000
NeoPRO Femto PLUS Western ECL Substrate Cod. **NB-78-00005** NeoPRO RTU Femto - Ready to Use Western ECL Substrate Cod. **NB-78-00009**	**Primary Ab** 1:5000 - 1:100,000 **Secondary Ab** 1:100,000 - 1:500,000
NeoPRO Atto Western ECL Substrate Cod. **NB-78-00006**	**Primary Ab** 1:10000 - 1:200,000 **Secondary Ab** 1:300,000 - 1:1,000,000

9- Chemiluminescent detection

NeoPRO:

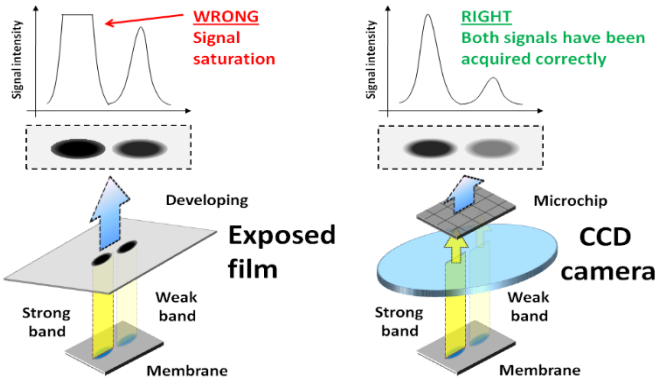
Prepare NeoPRO working solution **(NeoPRO WS)** by mixing properly each reagent in a 1:1 ratio. For best results, prepare WS immediately before use. Do not contaminate the solutions with the same pipette tips.

NeoPRO -RTU: the solution is ready to use.

I. Remove the membrane from its tray of **TBS-T Buffer**, rinse the membrane twice with TBS-T Buffer, and keep it in **TBS** until the incubation with **NeoPRO WS**.
II. Use 0.1 ml of **NeoPRO WS/ NeoPRO -RTU** per cm^2 of membrane.
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.
III. 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 quantifying samples relative to a control sample. By developing 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). Quantifying strong and weak signals with reliable results is possible on the same blot. Instead, potent signals get saturated in film, resulting in a wrong quantitation.

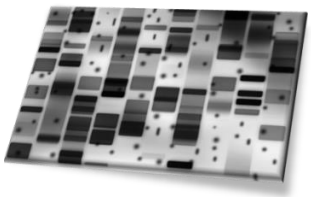


10- Troubleshooting

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 a blocking buffer if possible.
Insufficient washing. Increase both the volume, length and number of wash steps. Always use sufficient volumes to submerse the membrane.
The primary antibody is not specific to the protein of interest. Use monospecific or antigen-affinity purified antibodies. Always incubate your primary antibody at 4°C overnight, 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. The quality and age of primary and secondary antibodies may lead to background problems.
Poor handling of the membrane. Make sure to handle the membrane only with clean plastic tweezers and non-powdered gloves.
Contaminated buffer solutions. Check buffers for particulate or bacterial contaminants. Replace old buffers.

Irregular black spots



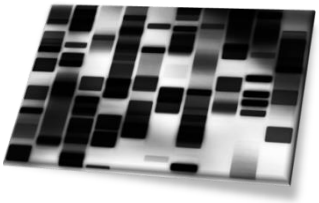
Air bubbles are trapped in the membrane. Remove air bubbles by gently rolling a clean pipette or a test tube during sandwich assembly.
Unevenly hydrated membrane. Make sure that the membrane is fully immersed during washes and antibody incubations.
Contaminated equipment. Protein or pieces of gel remaining on the unit may stick to the membrane. Antibodies can get trapped in the gel and then are washed out poorly, resulting in intense localized signals.

Aggregation of blocking agent. When the blocking agent is powder, stir it overnight at 4°C to ensure it is completely dissolved.
Interaction of the membrane with sample tray. Always use clean plastic trays to avoid any cross-reaction
Formation of aggregates in HRP-conjugate. Filter the secondary antibody solution through a 0.2 µm filter. Use fresh antibodies.

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 the 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 a minimum. Reduce NaCl in TBS-T Buffer (100mM÷350mM).
Non-fat dry milk may mask some antigens. Thus decrease blocking time and milk percentage in the Blocking Buffer or substitute with 5% BSA Blocking Buffer.
Sodium azide contamination. Ensure buffers do not contain sodium azide, which will quench the HRP signal.
Contaminated stock solutions. Do not contaminate the chemiluminescent substrate stock solutions using the same pipette tip. Use new reagents.

Non-specific bands

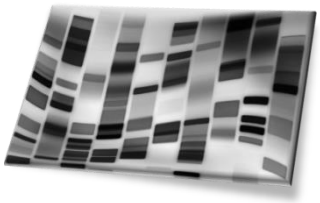


Aggregation of analyte. Increase the reducing agent to ensure a complete reduction of disulfide bonds.
SDS interference. The presence of SDS may result in the development of unspecific bands caused by antibodies binding to the charged SDS molecules associated with the proteins. Wash the membrane thoroughly after transfer with water.
High protein concentration. A commonly seen effect is the diffusion of protein bands. Reduce the amount of protein initially loaded.
The primary antibody is not specific to the protein of interest. Use monospecific or antigen-affinity purified antibodies. Always incubate your primary antibody at 4°C overnight, 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 proteins can cause high levels of localized signals. 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. First, try to dilute the secondary antibody further.

Uneven or jagged bands



Uneven gel run. Load all available wells. Empty wells can be loaded with sample buffer.

Voltage or current was 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).