CYANAGEN
Reagents for Genomics and Proteomics

Westar EtaC

Cod. XLS100

Enhanced Western HRP Substrate

Suggested Ab Dilutions:

Primary: 1/1,000 ÷ 1/15,000

Secondary: 1/25,000 ÷ 1/150,000

Package Contents:

- 1 Luminol-Enhancer Solution – Amber Bottle
- 1 Peroxide Solution – Clear Bottle

Quantity:	Sufficient For:
50mL kit	500 cm^2 of membrane
100mL kit	1,000 cm^2 of membrane
500mL kit	5,000 cm^2 of membrane

Storage/Expiry:

Upon receipt stored at 4°C
Stock solutions stability: One year

Detailed Operative Procedure:

1) Sample Preparation:

i. Add **2x Laemmli Buffer (see Table 1)** to your samples in a 1:1 ratio. The optimal concentration is 1-5 mg/ml. It is best if this concentration is determined empirically by a total protein assay (e.g. **BCA assay Cod.PRTD1,1000**).
ii. Heat the protein samples for 5 minutes at 95°C.
iii. All samples should be mixed by vortexing before and after the heating step.
iv. Let the samples cool to RT before loading the gel.

2) Gel Casting:

Prepare **suggested SDS gel** according to **Table 2÷3.**

3) Perform SDS-PAGE:

i. Prepare fresh **1× Running Buffer (see Table 4).**
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 check for leaks.
v. 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.
viii. Fill any empty well with **2x Laemmli Buffer.**
ix. Run at 90÷130 V constant voltage until the dye front reaches the bottom of the gel.

4) Prepare Transfer Membrane:

- If using nitrocellulose membrane place it into Milli-Q water slowly, with one edge at a 45° angle. Once wet, equilibrate the membrane in **1×Transfer Buffer (see Table 5)** for 15 min.
- If using PVDF membrane activate it with methanol for 30 seconds. Rinse with Milli-Q water and equilibrate in **1×Transfer Buffer** for 15 min.

5) Transfer To Membrane:

i. Wet four filter papers in **1×Transfer Buffer.**
ii. Assemble the transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with **1×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 **1×Transfer Buffer**.
v. Place pre-wetted membrane directly on the top side of the gel, then gently remove all air bubbles.
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 **1×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:
  a. **Wet transfer:** 80÷100 V for 30÷60min.
  b. **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 Milli-Q water.

6) Membrane Staining (optional):

i. Stain the membrane with **Ponceau S Staining Solution (see Table 6)** for 5 minutes at RT to check efficiency of transfer and visualize protein bands.
ii. Rinse the membrane in Milli-Q water until protein bands are distinct. Scan the membrane if desired.
iii. Completely destain the membrane by immersing it for 10 min in a large volume of Milli-Q water.
iv. If using a PVDF membrane, re-activate it with methanol then wash in **TBS-T Buffer (see Table 8)**.

7) Blocking The Membrane:

i. Place the membrane into a fresh tray with your choice of **Blocking Buffer (see Table 7).**
ii. Incubate the membrane in **Blocking Buffer** for 30÷60 minutes with gentle agitation on a rocker/shaker.
iii. 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.
iv. Rinse the membrane twice with **TBS-T Buffer.**

8) Antibody Incubation:

i. Dilute the primary antibody in fresh **TBS-T Buffer** to the **suggested primary antibody dilution.**
ii. Incubate the membrane 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 4 times for 3 to 5 minutes each with **TBS-T Buffer** with gentle agitation on a shaker. Use at least 25 ml of **TBS-T Buffer** for each washing.
iv. Dilute the secondary Ab in fresh **TBS-T Buffer** to the **suggested secondary antibody dilution**.
v. Incubate the membrane 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 4 times for 3 to 5 minutes each with **TBS-T Buffer** with gentle agitation on a shaker. Use at least 25 ml of **TBS-T Buffer** for each washing.

9) Chemiluminescent Detection:

i. For reproducible performance allow the detection solutions to equilibrate to RT before using.
ii. Prepare **Westar Working Solution (Westar WS)** by mixing properly each reagent in a 1:1 ratio. For best results prepare WS immediately before use.
iii. Remove the membrane from its tray of **TBS-T Buffer** and allow the excess buffer to run off from a corner. Do not let the membrane dry out.
iv. Use 0.1 ml of **Westar WS** per cm^2 of membrane.
v. Just pipet the volume required directly onto the membrane and incubate for 1 min (protein side up).
vi. Agitate gently and ensure the entire surface is covered with **Westar WS.**
vii. Allow the **Westar WS** to run off from a corner.

a. Autoradiography Film Detection:

i. After incubating the membrane with **Westar WS** always cover it with plastic wrap to avoid direct contact with the film.
ii. Make sure excess substrate is removed from the membrane and the plastic film.
iii. Place the protected membrane into a clean cassette with the protein side facing up.
iv. Carefully place film on top of the protected membrane. Roll out any air bubbles but liquid must not come into contact with the film.
v. For an unknown signal, try to expose 15 s, 30 s, 1 min and 5 min to start with.

b. Digital Image Detection:

i. After incubating the membrane with **Westar WS** place it onto a plastic wrap with the protein side facing up.
ii. Make sure excess substrate is removed from the membrane.
iii. For an unknown signal, try to expose 15 s, 30 s, 1 min and 5 min to start with.

Compatible Instrumentation:

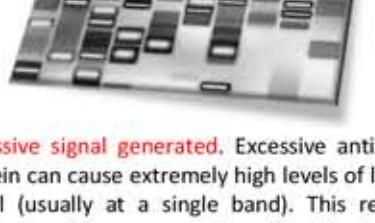
- ImageQuant™LAS-4000/Mini (GE Healthcare)
- DIAS-II (SERVA)
- ChemiDocXRS/VersaDoc (Bio-Rad)
- FluorChem and Chemilmager (Alpha Innotech)
- Chemi, BioChemi and OptiChemi (UVP)
- Image Station 2000MM (Kodak)
- FOTO/Analyst Luminary (Fotodyne)
- LAS-3000 (Fujifilm)
- UVIchemi and UVIprochemi (UVItec Ltd.)
- G:BOX and GeneGnome (Syngene)

Westar is manufactured in compliance with our ISO 9001 certified quality management system. Westar is intended for research use only, and shall not be used in any clinical procedures, or for diagnostic purposes.

For more information, MSDS or to download product instructions, please visit www.cyanagen.com or mail to info@cyanagen.it

Troubleshooting:

a) High Background:

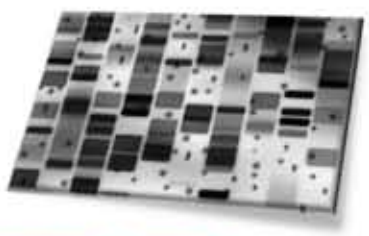


a. Insufficient blocking. Incubation time may be extended. Try different blocking conditions. Increase Tween-20 in **TBS-T Buffer** (0.1%÷0.5% v/v).
b. Insufficient wash. Increase number of washes. Use sufficient volumes to submerse the membrane.
c. Non-specific binding of primary antibody. Increase NaCl in **TBS-T Buffer** (100mM÷350mM) used for dilution of primary antibody and wash steps. Add 3% milk in **TBS-T Buffer.**
d. Film overexposed. Reduce exposure time.
e. Excessive signal generated. Due to the extreme sensitivity of the system it is always crucial to use the recommended antibody dilution. Very often further dilution of the secondary antibody helps to overcome this problem.
f. Poor quality of antibodies. Quality and age of primary and secondary antibody may lead to background problems.
g. Interaction with plastic wrap.

b) White Bands Or "Ghost Bands":

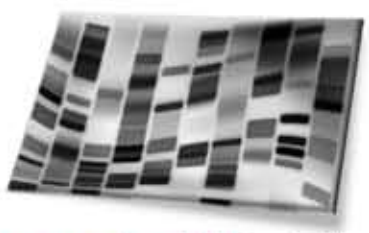
a. Excessive signal generated. Excessive antibody or protein can cause extremely high levels of localized signal (usually at a single band). This results in rapid, complete consumption of substrate at this point. Since there is no light production after the completion of this reaction, white bands are the result when exposed to film. Visibly glowing bands in darkroom and brown bands on membrane after the reaction are indicators of overloading.

c) Patchy Uneven Black/White Spots:



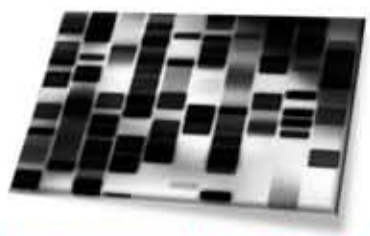
a. Contamination of reagents. Check buffers for particulate or bacterial contaminate. Use fresh reagents.
b. Not enough solution during incubation or washing. Make sure that the membrane is fully immersed during washes and antibody incubations.
c. Air bubble trapped in membrane. Remove air bubbles by gently rolling a clean pipette or a test-tube during sandwich assembling.
d. Uneven agitation during incubations. Ensure uniform agitation by placing the membrane on a rocker.
e. Contaminated equipment. Make sure that the electrophoresis unit is properly washed. Protein or pieces of gel remaining on the unit may stick to the membrane. Wash membrane thoroughly.
f. Formation of aggregates in HRP-conjugate. Filter secondary antibody solution through a 0.2 µm filter.
g. Unevenly hydrated membrane. Hydrate the membrane properly. The membrane should always stay wet.
h. Interaction with plastic wrap.

d) Uneven/Jagged Bands:



a. Voltage or current is too high. Band smiling and band smearing (diffuse bands) are commonly seen effects.
b. Uneven gel run. Load all available slots to avoid band distortions.
c. Effects of high salt in samples. Reduce the number of washes to minimum. Reduce NaCl concentration in **TBS-T Buffer** (recommended range 100mM÷350mM).

e) Non-Specific Bands:



a. Antibody is not specific for the protein of interest. Use monospecific or antigen affinity purified antibodies. Run a control with the secondary antibody alone. If bands develop choose an alternative secondary antibody.
b. Proteolytic breakdown of the antigen. Addition of protease inhibitors could help prevent this effect.
c. Concentration of antigen too low. Signal enhancement may then lead to the appearance of artificial bands. Enrichment of the antigen by fractionation or by immunoprecipitation should be considered.
d. Non-specific binding of secondary antibody. Add 3% non-fat dry milk in **TBS-T Buffer.**
e. Aggregation of analyte. Increase amount of TCEP or β-Mercaptoethanol to ensure complete reducing of disulfide bonds.
f. Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin systems. Substitute with **5% BSA Blocking Buffer.**

f) No Signal/Weak Signal:

a. Too much HRP. The enzyme in the system depleted the substrate and caused the signal to fade quickly. Further dilute HRP-conjugate.
b. Insufficient quantities of antigen or antibody. Increase amount of antibody or antigen.
c. Insufficient protein. Increase the amount of total protein loaded on gel.
d. Antibody may have lost activity. Perform a Dot Blot. Follow manufacturer's recommended storage and avoid freeze/thaw cycles.
e. Inefficient transfer. Always wet PVDF membrane in methanol or nitrocellulose membrane in transfer buffer before use. Ensure that there is good contact between PVDF or nitrocellulose membrane and gel. High MW protein may require more time for transfer.
f. Over transfer. Reduce voltage or time of transfer for low molecular weight proteins (< 10 kDa).
g. Incorrect secondary antibody used. Confirm host species and Ig type of primary antibody.
h. Sodium azide contamination. Make sure buffers do not contain sodium azide as this will quench HRP signal.
i. Low protein-antibody binding. Reduce the number of washes to minimum. Reduce NaCl in **TBS-T Buffer** (100mM÷350mM).
j. Weak or old chemiluminescent substrate. Use new Westar reagents.
k. Non-fat dry milk may mask some antigens. Decrease milk percentage in **Blocking Buffer** or substitute with **5% BSA Blocking Buffer.**
l. Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin systems. Substitute with **5% BSA Blocking Buffer.**

Dealer Contact:

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"Società soggetta a direzione e coordinamento da parte di Finance & Technology Srl - art.2497 bis cc".
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For more information, MSDS or to download product instructions, please visit www.cyanagen.com or mail to info@cyanagen.it

Westar EtaC - Reference Tables and Suggestions

1) Sample Preparation:

Table 1 – 2× Laemmli Buffer	
2×Laemmli Buffer (final):	**2× Laemmli-odorless Buffer (final):**
• 125 mM Tris-HCl • 4% (w/v) SDS • 20% (v/v) Glycerol • 0.02% (w/v) Bromophenol blue-sodium salt • 10% (v/v) β-Mercaptoethanol	• 31.25 mM Tris-HCl • 1 % (w/v) SDS • 12.5% (v/v) Glycerol • 0.02% (w/v) Bromophenol blue-sodium salt • 25 mM TCEP
2× Laemmli Buffer 25 ml:	**2× Laemmli-odorless Buffer 25 ml:**
• 3.6 ml of 1 M Tris-HCl in Milli-Q water • 5 ml of 20% (w/w) SDS in Milli-Q water • 5 ml Glycerol (pipet slowly) • 100 µl of 5% (w/w) Bromophenol blue-sodium salt in Milli-Q water • 2.5 ml β-Mercaptoethanol • Aliquot with Milli-Q water • Check the pH and bring it to pH 6.8 • Store at -20°C in 1 ml aliquots	• 1.3 ml of 1 M Tris-HCl in Milli-Q water • 1.3 ml of 20% (w/w) SDS in Milli-Q water • 3.1 ml Glycerol (pipet slowly) • 100 µl of 5% (w/w) Bromophenol blue-sodium salt in Milli-Q water • 156 mg TCEP • Aliquot with Milli-Q water • Check the pH and bring it to pH 6.8 • Store at -20°C in 1 ml aliquots
NOTE: For best results, do not store 2× Laemmli Buffers with β–mercaptoethanol. Moreover, its odor is unpleasant and is considered a toxicant.	**NOTE:** TCEP is a powerful, odorless and multi-purpose reducing agent. It is non-volatile and resistant to air oxidation.

2) Gel Casting:

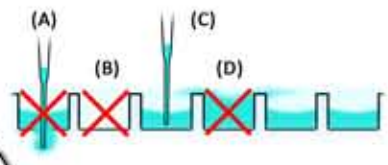
- The best gels are poured and polymerized the evening before the actual run is supposed to take place.
- SDS binds to proteins giving each a negative charge proportional to its mass.

Table 2 – 5% Stacking Gels								
Solution components	**Component volumes (ml) per gel mold volume of**							
	1 ml	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
Milli-Q water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
20% Acrylamide Mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris base (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% Ammonium Persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Table 3 – 8% Resolving Gels (for 25÷200 kDa protein size)								
Solution components	**Component volumes (ml) per gel mold volume of**							
	5 ml	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
Milli-Q water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% Acrylamide Mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris base (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% Ammonium Persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03

3) Perform SDS-PAGE:

(A) - Do not go too deep into the well to avoid puncturing the gel.
(B) - Load all available wells to avoid band distortions.
(C) - Put the tip into the well as vertically as possible.
(D) - Avoid overloading and cross-contamination.



Run at 90÷130 V constant voltage until the dye front reaches the bottom of gel.

If the current is too high band smiling and smearing (diffuse band) are commonly seen effects.

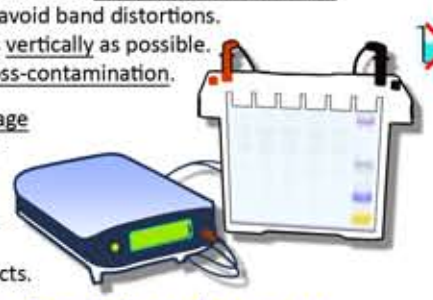


Table 4 – 1× Running Buffer
• 25 mM Tris base • 192 mM Glycine • 0.1% (w/v) SDS • Final pH 8.3

4) Prepare Transfer Membrane:

If using PVDF membrane activate it with methanol for 30 seconds.

If using nitrocellulose membrane place into Milli-Q 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.

- For proteins >15 kDa use membrane pore size 0.45 µm.
- For proteins <15 kDa use membrane pore size 0.2 µm.

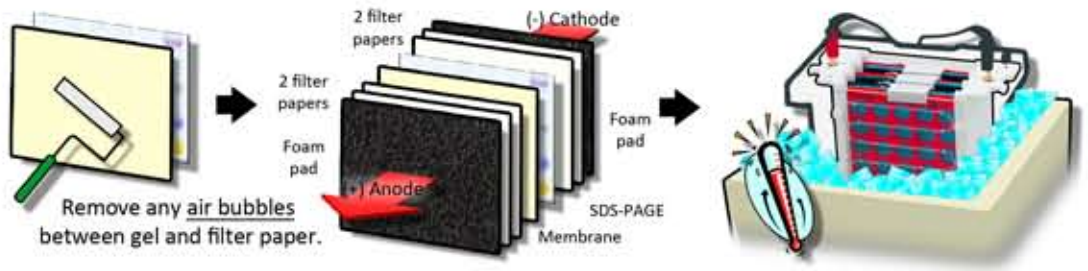
NOTE: Low molecular weight proteins (< 15kDa) are sometimes transferred through nitrocellulose membranes, therefore may be not visible on the blot. PVDF membrane has higher protein binding capacity than nitrocellulose membrane and is recommended for best detection sensitivity.

5) Transfer To Membrane:

Table 5 – Transfer Buffer		
10× Transfer Buffer 2 L:	**1× Transfer Buffer 4 L:**	**1×Transfer Buffer (Final):**
• 60.6 g Tris base • 288 g Glycine • Dissolve in 2 L Milli-Q water	• 400 ml 10× Transfer buffer • 800 ml Methanol • 2800 ml Milli-Q water	• 25 mM Tris base • 192 mM Glycine • 20% (v/v) Methanol

NOTE: For best results do not store Transfer Buffer with methanol.

Cooling the gel during transfer is crucial.



Remove any air bubbles between gel and filter paper.

NOTE: The proteins will transfer as soon as the gel is placed on the membrane, its repositioning can generate a smeared image.

Run the transfer with the following settings:

- **Wet transfer:** 80÷100 V for 30÷60min.
- **Semi-dry transfer:** 15÷25 V for 20÷30min.

Constant Voltage or Current?

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):

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

Table 6 – Ponceau S Staining Solution
• Dissolve 1 g Ponceau S in 50 ml glacial acetic acid • Bring volume to 100 ml with Milli-Q water • Final solution is 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid • Wrap the bottle in foil to protect the solution from light • Do not freeze • Stain solution can be re-used up to 10 times

NOTE: Be sure to handle the membrane only with gloves and use tweezers. Remeber that touching any part of the membrane even with gloves may result in some undesired background.

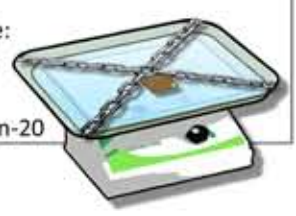
- Always use clean plastic trays to avoid any type of cross-contamination.
- Handle membrane with clean plastic forceps and non-powdered gloves.
- Check buffers for particulate or bacterial contaminate.

7) Blocking The Membrane:

NOTE: Non-fat milk contains biotin and phosphoproteins. If you are working with anti-phospho proteins or with biotinylated antibodies substitute with 5% BSA.

Table 7 – Blocking Buffer	
5% (w/v) Non-fat milk:	**5% (w/v) BSA:**
• 5 g Non-fat dry milk • 100 ml 1× TBS-T Buffer	• 5 g BSA (Cohn fraction V) • 100 ml 1× TBS-T Buffer

Table 8 – TBS-T Buffer	
10× TBS 2L:	**1× TBS-T (Final) 1L:**
• 48.4 g Tris base • 160 g NaCl • 1600 ml Milli-Q water Add concentrated HCl (about 20 ml required) until pH is about 7.8. When solution reaches room temperature adjust pH to 7.5 with 6 N HCl and bring volume to 2 L with Milli-Q water.	• 100 ml 10×TBS • 900 ml Milli-Q water • While stirring, add 1 ml Tween-20 Final concentrations are: • 20 mM Tris-HCl • 136 mM NaCl • 0.1% (v/v) Tween-20



- A maximum blocking time of 2 hours at RT should not be exceeded.
- Don't save TBS-T for a long time because it is not very stable.

8) Antibody Incubation:

- Do not increase the incubation time of the secondary Ab, as this will usually leads to higher background.
- Do not use sodium azide in any blocking buffers or wash solutions as it is a potent inhibitor of HRP activity.

a) 1°Ab incubation **b) Multiple washing** **c) 2°Ab-HRP incubation** **d) Multiple washing**

Overnight incubation at 4°C to increase sensitivity.

Wash 4 times for 3 to 5 min. each with TBS-T Buffer.

Incubate for 30 min. to 1 hour at RT.

Wash 4 times for 3 to 5 min. each with 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 optimise both primary and secondary Ab dilutions for best results with high signal and low background. Optimal Ab dilutions may be determined by Dot-Blot assay.

Suggested Ab Dilutions (with TBS-T Buffer):

	For highly expressed proteins		For poorly expressed proteins	
Product:	**WESTAR Nova 2011**	**WESTAR EtaC**	**WESTAR EtaC Ultra**	**WESTAR Supernova**
Detection limit:	Low-Picogram (10^{-12} g)	High-Femtogram (10^{-13} g)	Mid-Femtogram (10^{-14} g)	Low-Femtogram (10^{-15} g)
Suggested antibody dilutions @ 1mg/ml:	Primary: 1/500÷1/5,000 Secondary: 1/20,000÷1/100,000	Primary: 1/1,000÷1/15,000 Secondary: 1/25,000÷1/150,000	Primary: 1/5,000÷1/50,000 Secondary: 1/50,000÷1/250,000	Primary: 1/5,000÷1/100,000 Secondary: 1/100,000÷1/500,000
Excellent alternative to:	ECL™ SuperSignal®WestPico Lumi-Light Western Lightning®PLUS Luminata™ Classico	ECL™ Prime SuperSignal®WestDura Lumi-Light®PLUS Western Lightning®PRO Luminata™ Crescendo	ECL™ Prime SuperSignal®WestDura Lumi-Light®PLUS Western Lightning®PRO Luminata™ Forte	ECL™ Select SuperSignal®WestFemto LumiGLO Reserve™ Western Lightning®Ultra Luminata™ Forte

NOTE: Add 3% non-fat dry milk in TBS-T Buffer when diluite 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.

9) Chemiluminescent Detection:

Use 0.1 ml of Westar WS per cm^2 of membrane.

Just pipet the volume required directly onto the membrane and incubate for 1 min (protein side up).

The membrane should always stay wet.

Autoradiography film detection

Digital image detection

- For reproducible performance allow the detection solutions to equilibrate to RT before using.
- Make sure that the caps on the detection solution bottle have not been switched.
- Do not contaminate the solutions with the same pipette tips.

Re-probing the membrane or not?

- You will never get rid of all background from bands entirely.
- You will always loose a huge amount of proteins and the stripping can be uneven.

Autoradiography Film vs. Imaging Device

Nowadays, Western Blot 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÷4 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.

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