Western Optimization Kit

Technical Manual No. 0244 **Version 06192009**

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I. DESCRIPTION

GenScript's Western Optimization Kit allows researchers to quickly configure western and dot blot procedures for optimal precision, reliability, and speed. This kit is particularly useful for western blots that involve unpurified antibodies or antibodies that cause high background. Using GenScript's breakthrough immunodetection technology (patent pending), this kit can shorten total analysis time from the five hours of the traditional three-step western procedure to just under 90 minutes. Transfer the proteins from gel to membrane and incubate it in the Pretreat Solution for five minutes. Then incubate in WB solution with primary antibody for 40 minutes. Lastly, wash the membrane three times for 10 minutes each. The membrane can then be developed with the HRP substrate included in the kit. The ONE-HOUR Western procedure is contrasted with a classical three-step western at right.

The kit contains all the necessary reagents, buffers, nitrocellulose membrane and HRP substrate for optimizing western blots. These include four different Pretreat A solutions (A-a, A-b, A-c, and A-d).

We provide three different Western Optimization Kits for use with rabbit primary antibody (L00257), mouse primary antibody (L00258), and goat primary antibody (L00259), respectively. We also offer customized western kits containing only those regents necessary for specific optimized western analysis systems.

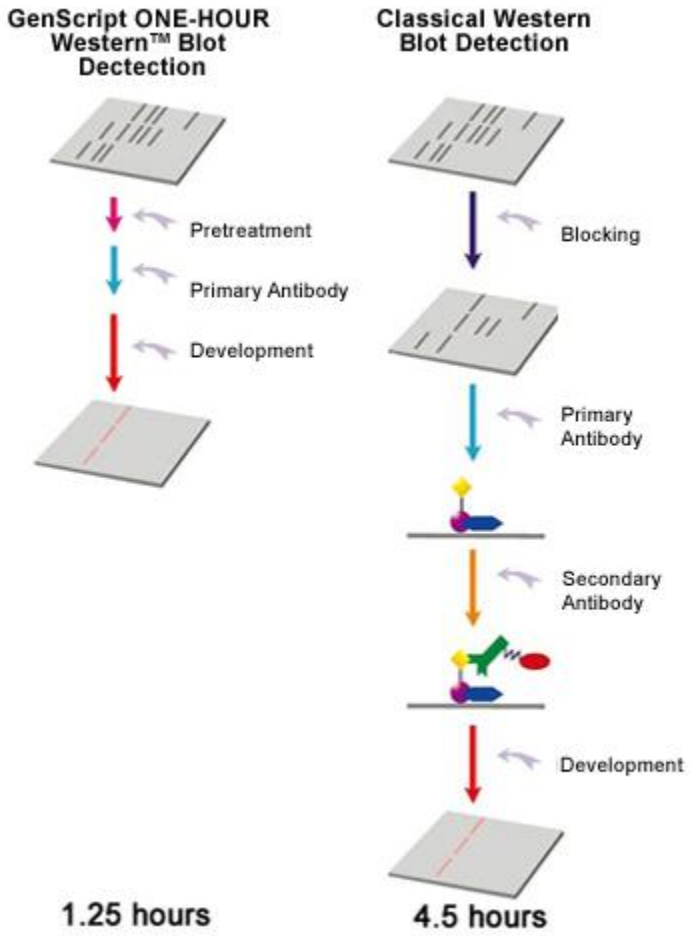


Figure 1. Overview of Western Procedures

II. KIT CONTENTS

Each kit contains enough reagents for ten minigel (7.5 X 8 cm) western blots or dot blots.

Kit Components	10 Assays L00257 (Rabbit)	10 Assays L00258 (Mouse)	10 Assays L00259 (Goat)
Pretreat A-a Solution	100 ml	100 ml	100 ml
Pretreat A-b Solution	100 ml	100 ml	100 ml
Pretreat A-c Solution	100 ml	100 ml	100 ml
Pretreat A-d Solution	100 ml	100 ml	100 ml
Pretreat B Solution	100 ml	100 ml	100 ml
WB-1 Solution	1 ml	1 ml	1 ml
WB Solution	100 ml	100 ml	100 ml
10X Wash Solution	125 ml	125 ml	125 ml
5-Slot Dot Blot Plate	1	1	1
WestClear™ Nitrocellulose Membrane (0.2 µm, 7.5 X 8 cm)	10 sheets	10 sheets	10 sheets
LumiSensor™ Plus Chemiluminescent HRP Substrate	2 X 30 ml	2 X 30 ml	2 X 30 ml
Protocol	1	1	1

III. RELATED PRODUCTS

- WestClear™ Nitrocellulose Membrane L00224A60
- LumiSensor™ Chemiluminescent HRP Substrate Kit L00221V60
- LumiSensor™ Plus Chemiluminescent HRP Substrate Kit L00225
- 10X Wash Solution MB01011

IV. KEY FEATURES

- ♣ Easy to perform: This kit has fewer and simpler steps than regular western kits.
- ♣ Quick optimization: You can optimize your western blot in a few hours instead of a few days.
- ♣ No secondary antibody is needed.

V. STORAGE

Store WestClear™ Nitrocellulose Membrane at room temperature. Store the rest of the kit at 4°C. It will remain stable for six months. **Do not freeze the kit or any of its components**.

VI. ONE-HOUR™ WESTERN OPTIMIZATION PROTOCOL

Reagents not provided:
Primary antibodies, serum, and mouse ascite fluid. All IgG should be whole-molecule.

Before use, prepare the following:
Dilute 10X Wash Solution with distilled or filtered water to make 1X Wash Solution. If any precipitate has formed in the 10X Wash Solution during storage, incubate the bottle in a warm or hot water bath (up to 50°C) with occasional mixing until all the precipitate disappears. Dilute the buffer with ddH_2O to 1X and store it at 4°C. Use 15 ml of 1X Wash Solution for each rinse and 20 ml of 1X Wash Solution for each wash.

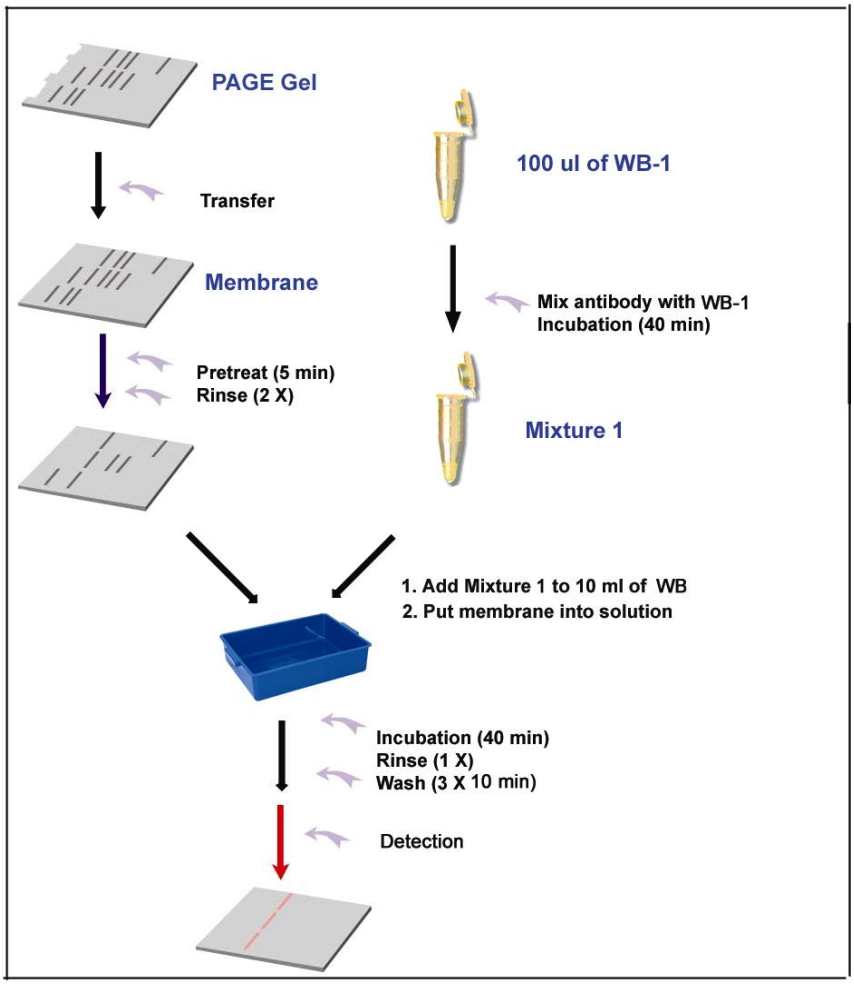
This procedure is optimized for one 7.5 X 8 cm sheet (or five 7.5 X 1.5 cm sheets) of membrane. Reagent volumes may be increased or decreased in proportion to the size of the membrane used.

Western blot optimization procedure:

1. Gel electrophoresis.
Load the same amount of protein sample into four wells of a 10-well minigel. More samples can be loaded into 12-well minigels or 15-well minigels. A 5-Slot Dot Blot Plate can process up to five strips of 7.5 X 1.5 cm membrane.

2. Preparation of Mixture 1
Before or during protein transfer, prepare Mixture 1 by mixing 100 µl of WB-1 with 10 µg of primary antibody in a microcentrifuge tube. Vortex Mixture 1 for a few seconds and then spin down briefly to collect the solution in the bottom of the tube. Incubate Mixture 1 at RT (room temperature) for 40 minutes.

Note: Follow manufacturer's recommendation to use more or less primary antibody than indicated above, reduce or increase the volume of WB- 1 accordingly. For example, mix 50 µL of WB-1 with 5 µg of primary antibody to make Mixture 1. No adjustment of the other reagents is necessary.



3. Pre-Treatment of Membrane
Just before the protein transfer from gel to membrane is complete, mix 2 ml each of Pretreat A-a, A-b, A-c, and A-d Solution with four 2 ml quantities of Pretreat B Solution in the 5-Slot Dot Blot Plate to make the four Pretreat Solutions. Do not wash the membrane after transferring the proteins from the gel. Proceed directly to the steps below. Cut the membrane into four strips (cut off 1, 2, 3, and 4 corners of the 4 strips, respectively, for easy identification) with one, two, or three lanes on each strip and then incubate each strip in a different Pretreat Solution on a shaker for five minutes at RT. After incubation, rinse the strips twice with 3 ml of 1X Wash Solution for each strip.

4. Final Incubation of Pre-Treated Membrane

a. Mix 25 µl or more (depending on the volume of antibody added) of Mixture 1 with 2.5 ml of WB in four slots of the 5-Slot Dot Blot Plate. Incubate all four strips, one in each slot, in this solution (WB containing Mixture 1) on a shaker at RT for 40 minutes.

b. Rinse each strip once with 3 ml of 1X Wash Solution. Then wash each strip on a shaker three times for 10 minutes each with 4 ml of 1X Wash Solution.

5. Signal Development

a. Mix 3 ml of LumiSensor™ Plus Reagent A with 3 ml of LumiSensor™ Plus Reagent B by vortexing for a few seconds to make the Working Solution. Use 0.1 ml of the Working Solution per cm^2 of membrane. The Working Solution remains stable for several hours at room temperature if protected from light.

b. Drain the excess Wash Solution from the strips by holding each strip vertically with forceps and touching its edge against a tissue. Place the strips on clean, flat surface, and cover them with Working Solution.

c. Incubate for **three to five** minutes at room temperature. Place the strips on a soft, clean tissue. Use another tissue to remove excess Working Solution. Arrange the strips in order and wrap them in a clean piece of plastic film.

d. Expose the strips to a sheet of film for one minute and then develop. Repeat with different exposure times to find the best results.

Select the Best Pretreat A Solution

From the western or dot blot results, select the Pretreat A solution that gives the highest signal and lowest background (highest S/N).

Further Optimization

a. The western blot can be further optimized by increasing or decreasing the amount of primary antibody used for the western. (The user must adjust WB-1 volume accordingly).

b. To increase the signal, increase the Step 4 (Final Incubation of Pre-Treated Membrane) incubation time from 40 minutes to several hours, or even overnight at 4°C. Follow the antibody manufacturer's recommended incubation time.

c. The WB solution can be diluted with PBST and then used in western blots.

d. Mix two or three Pretreat A (Aa, Ab, Ac, and Ad) Solutions to make a new Pretreat A Solution.

e. LumiSensor™ Chemiluminescent HRP Substrate Kit (L00221) can be used to further decrease background, however, the signal will be decreased, too.

Order the Customized Kit

From the optimization results, you can choose the appropriate Pretreat A solution and order customized western kits containing only those regents necessary for specific optimized western analysis.

VII. EXAMPLES

1. Western Blot Optimization Using Unpurified Anti-α-Tubulin (Mouse Ascites Fluid, Sigma, T 5168)

Shown below is a western blot performed on five strips using unpurified monoclonal anti-α-Tubulin (Mouse Ascites Fluid, Sigma, T 5168). 10 µg of Hela Cell Lysate (BD Biosciences, #611449) was loaded each of the five wells. After gel electrophoresis, proteins were transferred from gel to nitrocellulose membrane (included in the kit). The membrane was cut into five strips with one loaded lane in each strip. One strip was processed following the classic three-step western procedure. The other four strips were processed following the optimization procedure from the Western Optimization Kit (L00258). The results are shown in Figure 2.

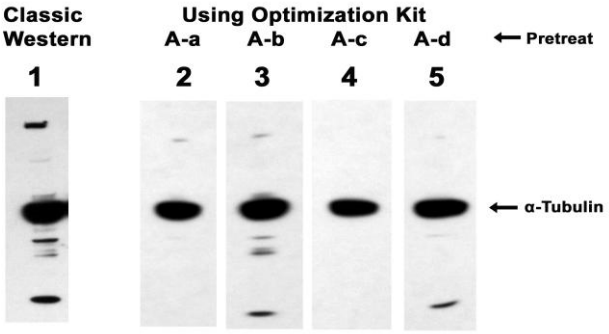


Figure 2. Western blot for the detection of α-Tubulin in Hela cell lysate following both the classical procedure and the optimization procedure from the Western Optimization Kit (L00258) using unpurified mouse antibody. 10 µg of Hela Cell Lysate (BD Biosciences, #611449) was loaded into each of the five wells. The blots were developed with the LumiSensor™ Plus Chemiluminescent HRP Substrate included in the kit.

From the results, it can be seen that Pretreat A-c gave the best results. α-Tubulin is detected without any background or non-specific bands.

2. Western Blot Optimization Using Polyclonal Anti-mTOR (Cell Signaling Technology, #2972)

Shown below is a western blot performed using five western blot strips and purified polyclonal anti-mTOR (Cell Signaling Technology, #2972) to detect mammalian target rapamycin, mTOR. 10 µg of Hela Cell Lysate (BD Biosciences, #611449) was loaded into each of the five wells. After gel electrophoresis, the proteins were transferred from gel to nitrocellulose membrane (included in the kit). The membrane was cut into five strips with one loaded lane in each strip. One strip was processed following the classical three-step western procedure. The other four strips were processed following the optimization procedure from the Western Optimization Kit (L00257). In accordance with the manufacture's recommendation, the antibody incubation was performed overnight on a shaker at 4°C. The results are shown in Figure 3.

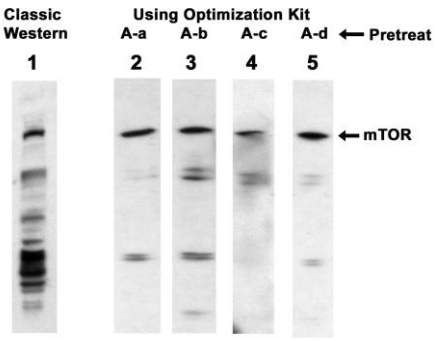


Figure 3. Western blot for the detection of mTOR in Hela cell lysate following both the classical procedure and the optimization procedure from the Western Optimization Kit (L00257) using polyclonal anti-mTOR. 10 µg of Hela Cell Lysate (BD Biosciences, #611449) was loaded into each of the five wells. The blots were developed with the LumiSensor™ Plus Chemiluminescent HRP Substrate included in the kit.

VIII. TROUBLESHOOTING

Use the table below to solve and avoid common problems.

Problem	Probable Cause	Solution
Weak or invisible signal	Too little protein is loaded.	Load more protein(s) onto the SDS-PAGE gel.
	There is poor transfer efficiency.	Optimize the transfer time and/or the electrical current. Make sure that there are no air bubbles between the membrane and the gel.
	The primary antibody has a low affinity for the antigen.	Increase the incubation time of the membrane in WB containing Mixture 1 from 40 to 60 minutes or even overnight at 4°C.
High background	There is too much primary antibody.	Proportionally reduce both the volume of the WB-1 solution and the amount of primary antibody added to it. For example, instead of using 100 µL of WB-1 with 10 µg or more of primary antibody, use 50 µL of WB-1 solution with 5 µg of primary antibody.
	The wash time is too short.	Adding additional washings after primary antibody (in WB) binding can further decrease background.
	The HRP substrate is too sensitive.	Instead of using the LumiSensor™ Plus Chemiluminescent HRP Substrate Kit (L00225), use the regular LumiSensor™ Chemiluminescent HRP Substrate Kit (L00221V60).
	The equipment or reagents have become contaminated.	Use a clean container for each rinse and wash step. Wear gloves and use clean forceps to handle membranes.

IX. ORDERING INFORMATION

Western Optimization Kit: L00257 for use with rabbit primary antibody
L00258 for use with mouse primary antibody
L00259 for use with goat primary antibody

Customized Kits:

Table 1. The customized kits below each contain one of the four Pretreat Solutions:

Kit Components	10 Assays (Rabbit)	10 Assays (Mouse)	10 Assays (Goat)
Pretreat A-a Solution	L00260	L00264	L00268
Pretreat A-b Solution	L00261	L00265	L00269
Pretreat A-c Solution	L00262	L00266	L00270
Pretreat A-d Solution	L00263	L00267	L00271

All of the kits listed above contain all of the following:

Kit Components	10 Assays
Pretreat B Solution	100 ml
WB-1 Solution	1 ml
WB Solution	100 ml
10X Wash Solution	125 ml
5-Slot Dot Blot Plate	1
WestClear™ Nitrocellulose Membrane (0.2 µm, 7.5 X 8 cm)	10 sheets
LumiSensor™ Plus Chemiluminescent HRP Substrate	2 X 30 ml
Protocol	1

Patent Pending.

For Research Use Only.

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