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I. DESCRIPTION

GenScript ONE-HOUR Western™ Multiplex Fluorescent Kit yields journal-quality Western results in about one hour. Using GenScript breakthrough immunodetection technology (patent pending), the kit replaces the classical three-step western process, which can take nearly five hours. Furthermore, this kit allows researchers to use multiple antibodies to detect multiple antigens on the same membrane at the same time. Transfer the proteins from gel to membrane and incubate it in the pretreat solution for five minutes. Then incubate in WB-M solution with primary antibodies and the corresponding fluorescent dye labeled secondary antibodies for 40 minutes. Then wash three times for ten minutes each. The membrane can then be scanned on a LI-COR Odyssey Infrared Imaging Systems. The ONE-HOUR Western™ procedure is contrasted with a classical three-step Western at right.

The ONE-HOUR Western™ Multiplex Fluorescent Kit is designed to produce high signal with low background for quick and clear Western analysis of multiple proteins.

This kit can be used for any primary antibodies raised from these three species: rabbit, mouse, and goat. The corresponding fluorescent-dye labeled secondary antibodies are also needed.

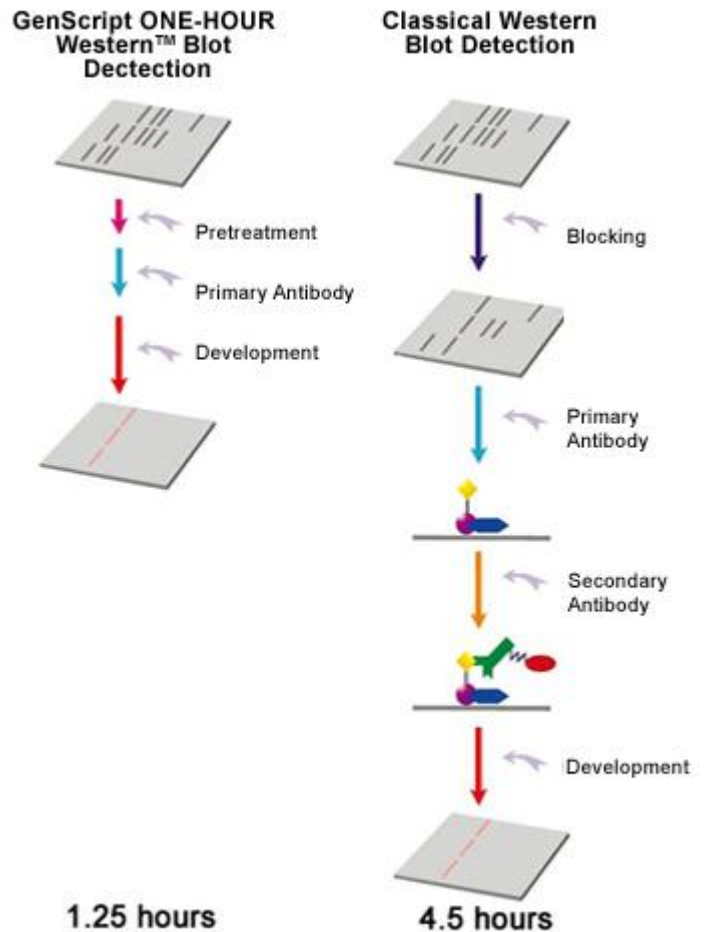


Figure 1. Overview of Western Procedures



II. KEY FEATURES

- ◆ Antibodies from all three major species (rabbit, mouse and goat) can be used.
- ◆ No cross-reaction between primary antibodies and secondary antibodies.
- ◆ Reproducible results: The kit produces highly reproducible results.

III. KIT CONTENTS

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

In some rare cases, the primary antibody (including some antibodies against phosphoproteins) may not be compatible with Pretreat Solution A, resulting in very high background. For these cases, GenScript provides an alternate pretreat solution A (Pretreat A-b) to solve this problem. This solution (GenScript, M01057) is available separately.

Pretreat A-b (100 ml) M01057

Kit Components	Quantity
Pretreat Solution A	100 ml
Pretreat Solution B	100 ml
WB-1 Solution	2 ml
WB-M Solution	100 ml
10X Wash Solution	125 ml
Protocol	1

Either Nitrocellulose or PVDF membrane (not provided) can be used with the kit.

IV. STORAGE

Store the kit at 4°C. It will remain stable for six months.

V. RELATED PRODUCTS

- WestClear™ Nitrocellulose Membrane (0.2 μm) L00224A60
- 10X Wash Solution MB01011
- Pretreat A-b M01057
- GenScript Dot Blot Box M00108
- Western Blot Box (Black) M00103
- Protein marker for Fluorescent Western M00124
- 5X Sample Buffer for SDS-PAGE MB01015

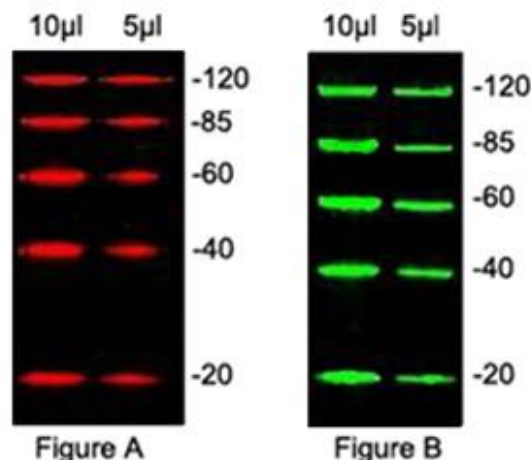
Protein marker for Fluorescent Western

GenScript also provides protein marker for Fluorescent Western (M00124), the performance of the marker is demonstrated at right.

10 μl and 5 μl of reconstituted Protein Marker for Fluorescent Western Blot were loaded in 12% Express SDS-PAGE gel (GenScript, MG012W10).

Fig. A shows the results when IRDye 680-conjugated Goat Anti-Rabbit IgG (LI-COR) is used in the Western blot.

Fig. B shows the results when IRDye 800CW-conjugated Goat Anti-Mouse IgG (LI-COR) is used in the Western blot.





Images were acquired using Odyssey Infrared Imaging system (LI-COR).

VI. ONE-HOUR MULTIPLEX FLUORESCENT WESTERN PROTOCOL

This procedure is optimized for a sheet of 7.5 X 8.0 cm membrane, but reagent volumes can be scaled according to the size of the membrane used.

Reagents not provided:

1. Purified primary antibodies: Affinity-purified antibodies are recommended.
2. Fluorescent-dye labeled secondary antibodies. Several vendors provide this kind of antibodies. LI-COR and Rockland provide IRDye® 680/800 labeled secondary antibodies. Pierce provides DyLight 680/800 labeled secondary antibodies. Invitrogen provides Alexa Fluor® 680 labeled secondary antibodies.

Before use, prepare the following:

1X wash solution: Dilute 12.5 ml of 10X wash solution with 112.5 ml of distilled or filtered water to make 125 ml of 1X wash solution. If any precipitate forms 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 ddH₂O 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.

Western blot procedure:

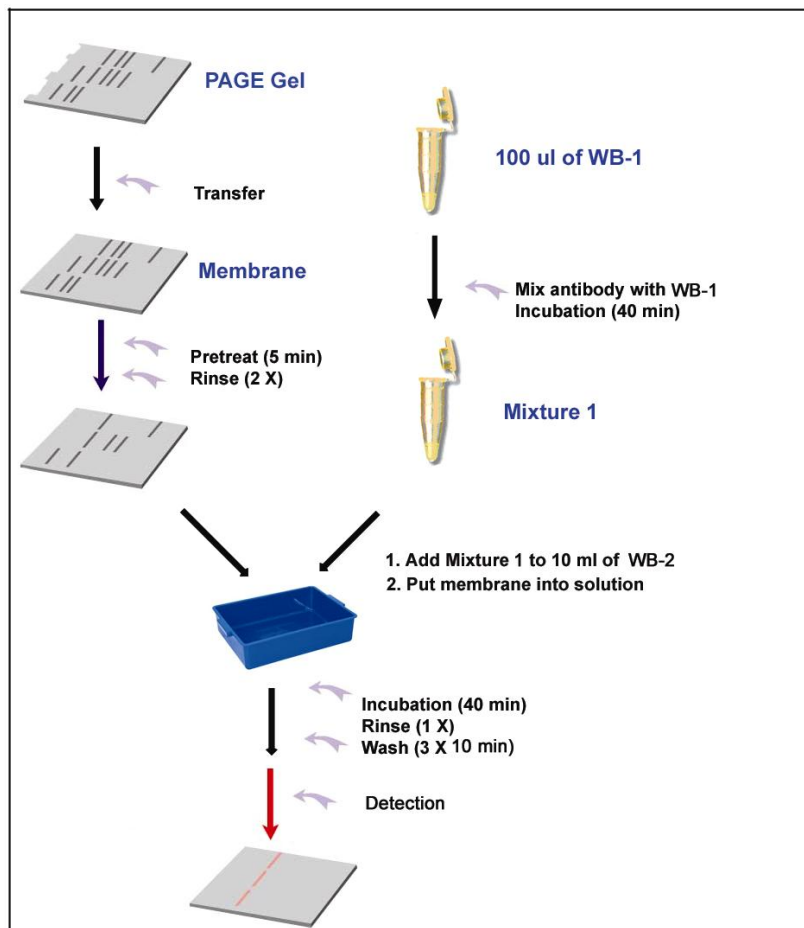
1. Prepare Mixture 1

Before or during protein transfer, prepare Mixture 1 by mixing primary antibody and fluorescent-dye labeled secondary antibody in WB-1. For multiple primary antibodies, **multiple Mixture 1's need to be prepared separately in different tubes**. For each primary antibody, add 2 – 10 µg of the antibody* to 50 µl of WB-1 in a microcentrifuge tube, then add 1 – 5 µg of the corresponding fluorescent-dye labeled secondary antibody (the amount of secondary antibody is 50% of the primary antibody used) to the same tube. Vortex Mixture 1 gently for a few seconds and centrifuge briefly. Incubate all the Mixture 1's in the dark at room temperature (RT) for at least 40 minutes.

* Refer to manufacturer's recommendations when using appropriate amounts of antibody.

2. Pre-Treat Membrane

Just before the protein transfer from gel to membrane is complete, mix 10 ml of Pretreat Solution A with 10 ml of Pretreat Solution B in a plastic container (Western Blot Box, GenScript, M00103) to make the pretreat solution. Always prepare and use fresh mixture. Place the membrane directly in the pretreat solution mixture and incubate on a shaker for five minutes at RT. After incubation, rinse the membrane twice with 15 ml of 1X wash solution.





3. Final Incubation of Pre-Treated Membrane

- Just after setting up the pre-treatment step, **add 1 ml of WB-M to each of Mixture 1's and mix well by inverting the tubes several times, incubate all the tubes at RT for 5 minutes.** Then add all of the Mixture 1's one by one to appropriate volume of WB-M in a Western blot box (GenScript Western Blot Box, Black, M00103) and mix well. The total volume of the WB-M solution should be 10 ml. For example, if 2 ml of WB-M are already used to make 2 Mixture 1's, another 8 ml is needed to make the final solution. Incubate the membrane in this solution (WB-M containing all the Mixture 1's) on a shaker at RT for 40 minutes. **Protect box (or bag) from light during incubation.**
- Rinse the membrane once with 15 ml of 1X wash solution. Wash the membrane on a shaker three times for ten minutes each with 20 ml of 1X wash solution. **Protect box (or bag) from light during wash. Use a clean container for each wash to reduce background.**

4. Imaging or Scanning

After final wash, transfer the membrane to a container containing 20 ml of distilled or filtered water. Rinse the membrane for 1 minute and then scan the membrane on a LI-COR Odyssey Infrared Imaging Systems following the Odyssey Operation Manual.

VII. EXAMPLES

Multiplex Fluorescent Western blot detection of four proteins at the same time on the same membrane.

Hela cell lysate was spiked with GST protein as shown in Figure 1. All the primary antibodies and secondary antibodies are listed in the following table.

Antigens	Primary Antibodies	Amount	Secondary Antibodies	Amount
α-Tubulin	Mouse Anti-α-Tubulin Monoclonal Antibody (Sigma, T6074)	6 µg	IRDye®680 Donkey Anti-Mouse (LI-COR, 926-32222)	3 µg
β-Actin	THE™ Anti-β-actin Monoclonal Antibody (Mouse) (GenScript, A00702)	6 µg	IRDye®800CW Goat Anti-Mouse (LI-COR, 926-32210)	3 µg
GAPDH	Goat Anti-GAPDH Polyclonal Antibody (GenScript, A00191)	4 µg	IRDye®680 Donkey Anti-Goat (LI-COR, 926-32224)	2 µg
GST	Rabbit Anti-GST Polyclonal Antibody (GenScript, A00097)	6 µg	IRDye®800CW Goat Anti-Rabbit (LI-COR, 926-32211)	3 µg

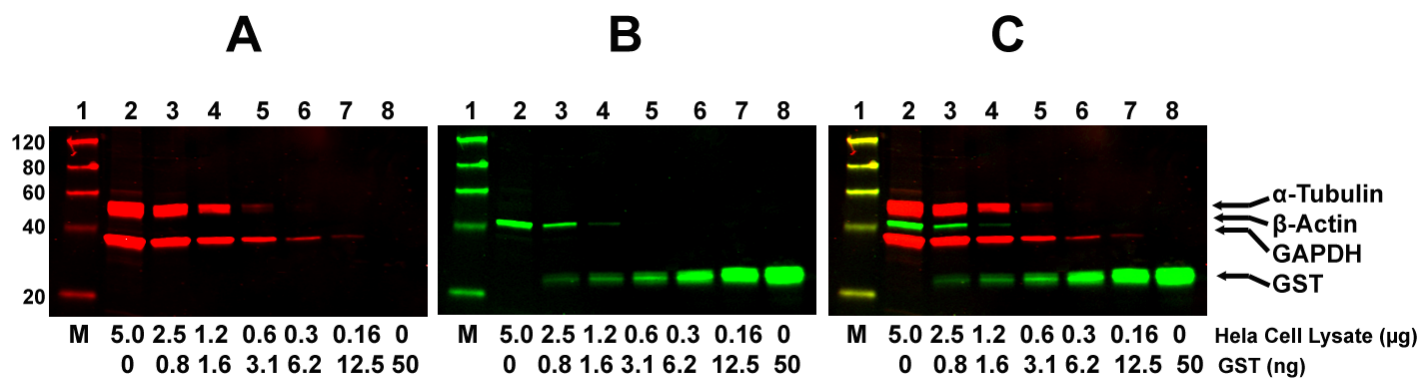




Figure 1. Multiplex Fluorescent Western blots for the detection of α -Tubulin, β -Actin, GAPDH, and GST proteins using the ONE-HOUR Western™ Multiplex Fluorescent Kit (L00398). A: 700 nm fluorescence image; B: 800 nm fluorescence image; C: The two fluorescence colors were imaged simultaneously in a single scan on a LI-COR Odyssey Infrared Imaging Systems. M is the Protein Marker for Fluorescent Western (GenScript, M00124).

VIII. TROUBLESHOOTING

Problem	Probable Cause	Solution
The signal is weak or invisible.	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-2 containing mixture 1. Increasing antibody concentration can also improve signal.
	The primary antibody has a low affinity for the antigen.	Reducing wash time can increase the signal for low-affinity antibody. Instead of wash for 3 x 10 min, wash for 3 x 5 min to increase signal.
There is high background.	Too much primary antibody is used.	Reduce the amount of primary antibody, and reduce WB-1 accordingly.
	The primary antibody has non-specific binding or cross-reactivity with the blocking reagent.	Use an alternate Pretreat A-b (M01057).
	The wash time is too short.	Adding additional washing steps can further decrease background.
	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.
There is cross-reaction between primary antibody and secondary antibody.	The WB-M solution containing all the Mixture 1's is not mixed well.	Add Mixture 1 (with 1 ml of WB-M added) one by one to WB-M solution. Mix well after each addition of Mixture-1.

Patent Pending.

For Research Use Only.

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