ONE-HOUR Western™ Multiplex Kit III

Technical Manual No. 0257 **Version 06192009**

I	Description	1
II	Kit Contents	2
III	Related Products	2
IV	Key Features	2
V	Storage	2
VI	ONE-HOUR Multiplex Western™ Protocol	2
VII	Examples	4
VIII	Troubleshooting	6
IX	Ordering Information	6

I. DESCRIPTION

Western blot analysis is one of the most common methods of detecting proteins and of determining specific protein concentrations in biological samples. However, loading errors and other human problems can confound this otherwise reliable system. An internal control is often needed to control and correct for such problems and to semi-quantitate protein expression levels. To date, the three most widely used internal controls are housekeeping proteins GAPDH, *β*-actin, and α-tubulin. However, to avoid crossover reactions, researchers usually need to perform two Western blots (by either cutting the membrane into two halves or by stripping the membrane after the first blot) to detect the target protein and the internal control separately. GenScript now introduces the ONE-HOUR Western™ Multiplex Kit III. It can detect both the target protein and α-Tubulin in a single blot on the same membrane. Like other kits employing GenScript's ONE-HOUR Western™ technology, it completes the blot in about one hour.

Using GenScript's breakthrough immunodetection technology (patent pending), the kit replaces the classical three-step Western, which can take nearly five hours. 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, and, lastly, wash three times for ten minutes each. The membrane can then be developed with the HRP substrate included in the kit. The kit contains all the necessary reagents, buffers, nitrocellulose membrane and HRP substrate for performing a Western blot.

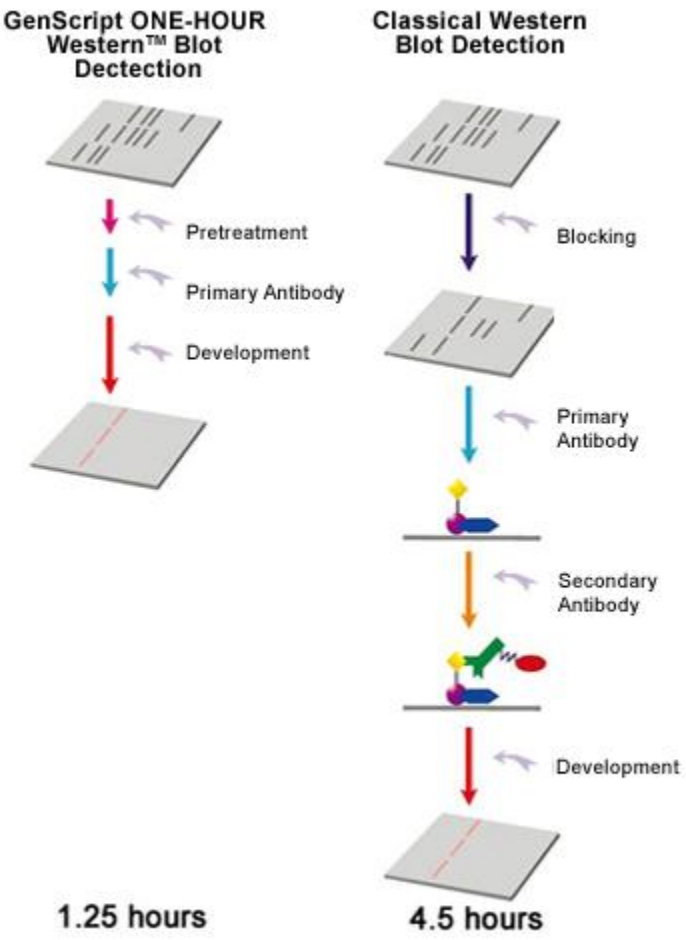


Figure 1. Overview of Western Procedures

The ONE-HOUR Western™ Multiplex procedure is contrasted with a classical three-step Western at right.
The ONE-HOUR Western™ Multiplex Kit III already contains a α-Tubulin antibody that specifically reacts with human, mouse, rabbit and rat α-Tubulin protein. Only a primary antibody for the target protein is required.

The kit contains WestClear™ Nitrocellulose Membrane (0.2 µm) and LumiSensor™ Plus Chemiluminescent HRP substrate optimized for best results. WestClear™ Nitrocellulose Membrane and LumiSensor™ Plus Chemiluminescent HRP Substrate Kits are also available separately.

II. KIT CONTENTS

Three different kinds of ONE-HOUR Western™ Multiplex Kit III are available. GenScript provides kits intended for use with rabbit (L00321), mouse (L00322), and goat (L00323) primary antibodies, respectively. Each kit contains enough reagents for ten minigel (7.5 x 8 cm) Western blots.

Kit Components	10 Assays L00321 (Rabbit)	10 Assays L00322 (Mouse)	10 Assays L00323 (Goat)
Pretreat A 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-2 solution	100 ml	100 ml	100 ml
10X wash solution	125 ml	125 ml	125 ml
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
- Pretreat Solution (A + B) M01013

IV. KEY FEATURES

- Easy to perform: This kit has fewer and simpler steps than other Western kits, leaving fewer chances for human error.
- Low background: The kit contains WestClear™ Nitrocellulose Membrane and LumiSensor™ Plus Chemiluminescent HRP Substrate Kit, optimized for low background.
- High sensitivity: The kit's sensitivity is comparable to or better than that of the classical 4.5-hour procedure, depending on the quality and amount of antibodies used.
- Reproducible results: The kit produces highly reproducible results.
- No secondary antibody is needed: No extra α-Tubulin antibody is needed.
- The ONE-HOUR Western™ needs far less optimization than the classical three-step method.

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**.

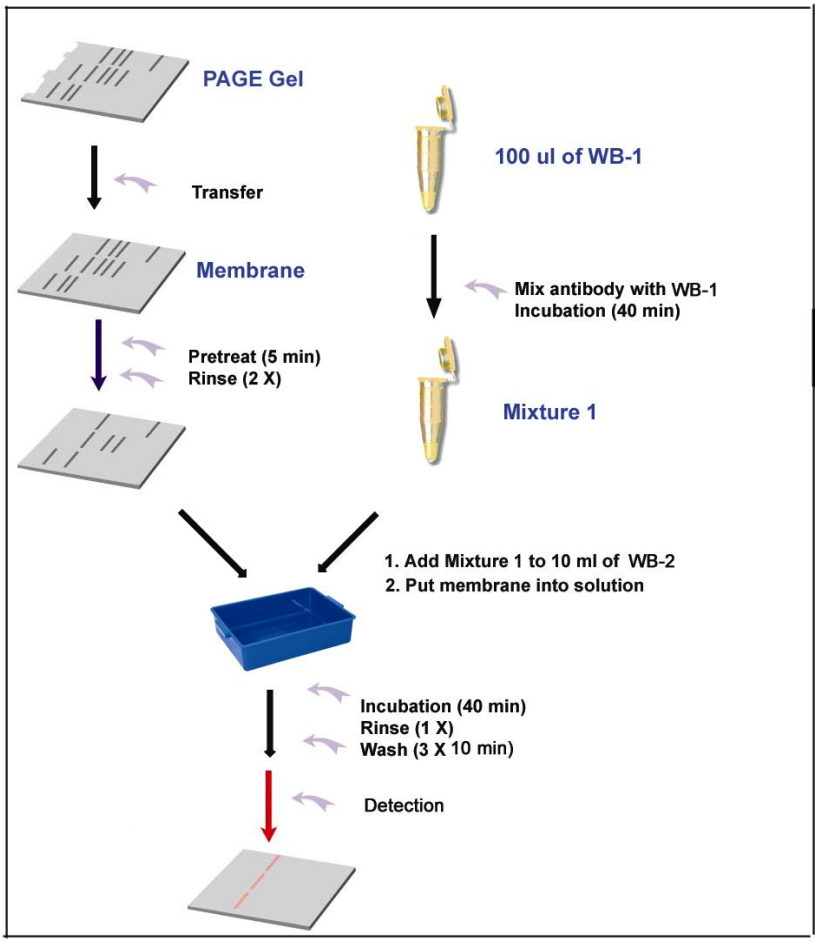
VI. ONE-HOUR WESTERN™ PROTOCOL

This procedure is optimized for a sheet of 7.5 x 8 cm membrane, but reagent volumes can be scaled up or down according to the size of the membrane used.

Reagents not provided:

Purified primary antibodies for target protein. Affinity-purified antibodies are recommended. Further optimization may be needed if the serum containing the antibody is to be used.

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



Western blot procedure:
Do not wash the membrane after transferring the proteins from the gel. Proceed directly to the steps below.

1. Prepare mixture 1

Before or during protein transfer, prepare mixture 1 by mixing 100 µl of WB-1 with 5 to 10 µg of primary antibody in a microcentrifuge tube. Vortex mixture 1 for a few seconds and spin down briefly to collect the solution in the bottom of the tube. Incubate mixture 1 at RT (room temperature) for at least 40 minutes. (Longer incubation is preferred. For overnight incubation, store mixture 1 at 4°C.)

Note: If using less primary antibody, reduce the volume of WB-1 accordingly. For example, mix 50 µL of WB-1 with 2 µg of primary antibody to make mixture 1. No adjustment of the other reagents will be necessary.

2. Pre-treat membrane

Just before the protein transfer from gel to membrane is complete, mix 10 ml of pretreat A solution with 10 ml of pretreat B solution in a plastic container to make the pretreat solution. Incubate the membrane in the pretreat solution mixture 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

a. Add mixture 1 to 10 ml of WB-2 in a plastic container and mix well. Incubate the membrane in this solution (WB-2 containing Mixture 1) on a shaker at RT for 40 minutes.

b. 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. **Use a clean container for each wash step to avoid carryover contamination and to reduce background.**

4. 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 is stable for several hours at room temperature when protected from light.

b. Drain the excess wash solution from the membrane by holding the membrane vertically with forceps and touching the edge against a tissue. Place the membrane on clean, flat surface, and cover the membrane with working solution.

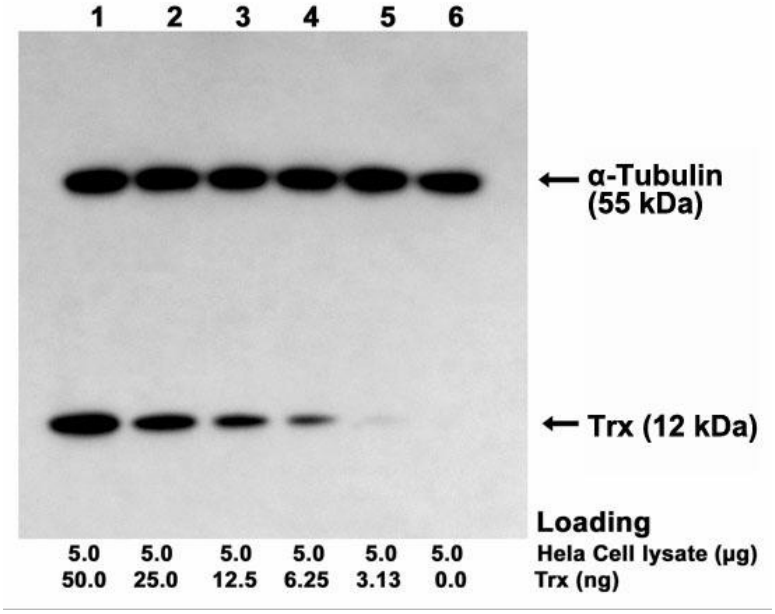
c. Incubate for three minutes at room temperature. Place the membrane on a soft, clean tissue. Use another tissue to remove excess working solution. Wrap the membrane in a clean piece of plastic film.

d. Expose to a sheet of film for 1 minute and then develop. Repeat with different exposure times to find the best results.

VII. EXAMPLES

1. Shown below is a ONE-HOUR Multiplex Western™ Blot performed using rabbit polyclonal antibody.
The ONE-HOUR Western™ Multiplex Kit III (a-Tubulin), Rabbit (GenScript, L00321) was used to detect both α-Tubulin and Trx protein (with the MW of about 12 kDa) in *Hela* cell lysate spiked with Trx protein.

Figure 1. Shown at right is a multiplex Western blot for the detection of both α-Tubulin and Trx protein from *Hela* cell lysate spiked with Trx protein (MW of about 12 kDa) using the ONE-HOUR Western™ Multiplex Kit III (a-Tubulin), Rabbit (GenScript, L00321) and rabbit polyclonal antibody against Trx (Sigma, T0803). 5 µg of *Hela* cell lysate (BD Biosciences, #611449) was spiked with 50.0, 25.0, 12.5, 6.25, 3.13 and 0 ng of Trx protein and loaded into Lanes 1, 2, 3, 4, 5, and 6, respectively. The Western blot was performed following the manufacturer's protocol and developed with the LumiSensor™ Plus Chemiluminescent HRP Substrate Kit (GenScript, L00225) included in the kit.



2. Shown below is a ONE-HOUR Multiplex Western™ Blot performed using mouse monoclonal antibody.
The ONE-HOUR Western™ Multiplex Kit III (a-Tubulin), Mouse (GenScript, L00322) was used to detect both α-Tubulin and GST protein (with the MW of about 26 kDa) in *Hela* cell lysate spiked with GST protein.

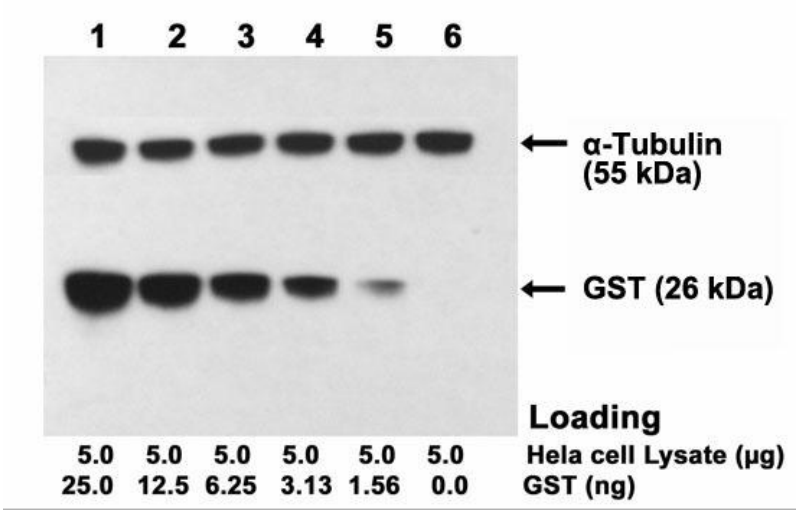


Figure 2. Shown at right is a multiplex Western blot for the detection of both α-Tubulin and GST protein from *Hela* cell lysate spiked with GST protein using the ONE-HOUR Western™ Multiplex Kit III (a-Tubulin), Mouse (GenScript, L00322) and THE™ Anti-GST Monoclonal Antibody (Mouse) (GenScript, A00865). 5 µg of *Hela* cell lysate (BD Biosciences, #611449) was spiked with with 25.0, 12.5, 6.25, 3.13, 1.56 and 0 ng of Glutathione S-Transferase (GST), *Schistosoma japonicum* (GenScript, Z02039) and loaded into Lanes 1, 2, 3, 4, 5, and 6, respectively. The Western blot was performed following the manufacturer's protocol and developed with the LumiSensor™ Plus Chemiluminescent HRP Substrate Kit (GenScript, L00225) included in the kit.

In this case, 2 µg of THE™ Anti-GST Monoclonal Antibody (Mouse) (GenScript, A00865) and 50 µl of WB-1 were mixed to make mixture 1.

3. Shown below is a ONE-HOUR Multiplex Western™ Blot performed using goat polyclonal antibody.

The ONE-HOUR Western™ MultiplexKit III (a-Tubulin), Goat (GenScript, L00323) was used to detect both α-Tubulin and GAPDH protein in *Hela* cell lysate.

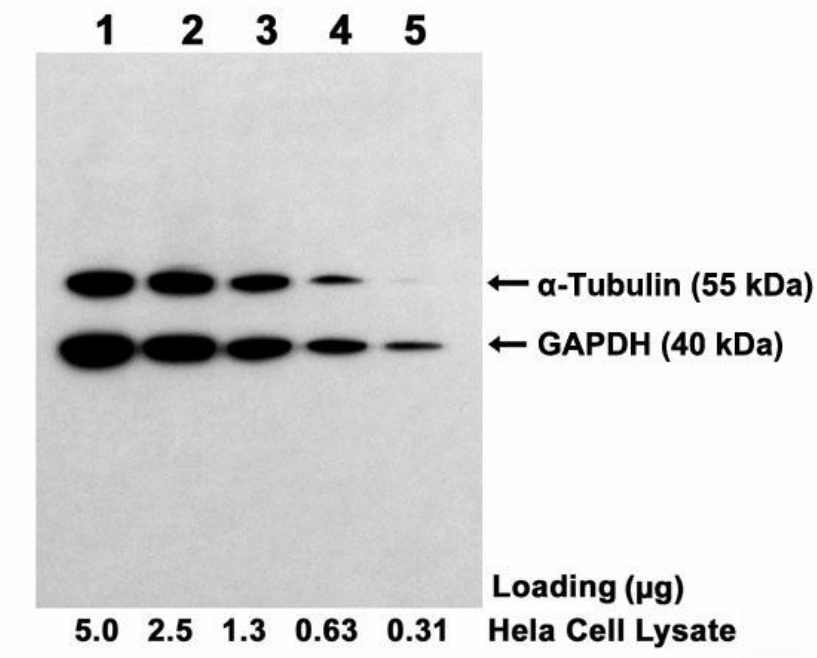


Figure 3. Shown below is a multiplex Western blot for the detection of both α-Tubulin and GAPDH from *Hela* cell lysate using the ONE-HOUR Western™ MultiplexKit III (a-Tubulin),Goat (GenScript, L00323) and Goat Anti-GAPDH Polyclonal Antibody (GenScript, A00191). 5.0, 2.5, 1.3, 0.63 and 0.31 µg of *Hela* cell lysate (BD Biosciences, #611449) were loaded into Lanes 1, 2, 3, 4, and 5, respectively. The Western blot was performed following the manufacturer's protocol and developed with the LumiSensor™ Plus Chemiluminescent HRP Substrate Kit (GenScript, L00225) included in the kit.

In this case, 2 µg of Goat Anti-GAPDH Polyclonal Antibody (GenScript, A00191) and 50 µl of WB-1 were mixed to make mixture 1.

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 from 40 to 50 or 60 minutes.
The background is too high.	The primary antibody shows non-specific binding or cross-reactivity.	Change antibodies. Use a highly specific primary antibody. Affinity-purified primary antibodies are preferred.
	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 5 -10 µg of primary antibody, use 50 µL of WB-1 solution with 2 µg of primary antibody.
	The wash time is too short.	Adding additional washings after primary antibody (in WB) binding can further decrease background.
	The α-Tubulin signal is too strong.	Dilute WB-2 with PBST (0.1% Tween-20). For example, instead of using 10 ml of WB-2 for a mini-gel, use 5 ml of WB-2 and 5 ml of PBST.
The target protein band and α-Tubulin and are too close.	Many factors, such as gel concentration, electrophoresis buffer system and protein modification (phosphorylation, glycosylation, etc.), can affect protein migration in SDS-PAGE.	If the difference between the molecular weight of the target protein and of α-Tubulin is less than 5 kDa, a test Western blot is recommended to make sure that the target protein band and α-Tubulin band are not too close.

IX. ORDERING INFORMATION

ONE-HOUR Western™ Multiplex Kit III: L00321 for rabbit primary antibody
L00322 for mouse primary antibody
L00323 for goat primary antibody

GenScript Corporation
120 Centennial Ave., Piscataway, NJ 08854
Tel: 732-885-9188, 732-885-9688
Fax: 732-210-0262, 732-885-5878
Email: info@genscript.com
Web: www.genscript.com