ONE-HOUR Western™ Detection System

Update Date: June 25, 2010

User Manual

GenScript
The Biology CRO

Table of Contents

Kit Contents

Type of products

Product	Cat. No.
ONE-HOUR Western™ Basic Kit (Rabbit)	L00204
ONE-HOUR Western™ Basic Kit (Mouse)	L00205
ONE-HOUR Western™ Basic Kit (Goat)	L00399
ONE-HOUR Western™ Standard Kit (Rabbit)	L00204C
ONE-HOUR Western™ Standard Kit (Mouse)	L00205C
ONE-HOUR Western™ Standard Kit (Goat)	L00228
ONE-HOUR Western™ Standard Kit with TMB (Rabbit)	L00204T
ONE-HOUR Western™ Standard Kit with TMB (Mouse)	L00205T
ONE-HOUR Western™ Standard Kit with TMB (Goat)	L00228T
ONE-HOUR Western™ Advanced Kit (Rabbit)	L00241
ONE-HOUR Western™ Advanced Kit (Mouse)	L00242
ONE-HOUR Western™ Advanced Kit (Goat)	L00243
ONE-HOUR IP-Western Kit (Rabbit)	L00231
ONE-HOUR IP-Western Kit (Mouse)	L00232
ONE-HOUR IP-Western Kit (Goat)	L00233
ONE-HOUR Western™ Fluorescent Kit	L00397
ONE-HOUR Western™ Multiplex Fluorescent Kit	L00398

Contents

ONE-HOUR Western™ Detection Kits

Components	Basic Kits	Standard Kits		Advanced Kits
		With TMB	Standard	
Pretreat Solution A	50 ml	50ml	50 ml	50 ml
Pretreat Solution B	50 ml	50 ml	50 ml	50 ml
WB-1 Solution	0.5 ml	0.5 ml	0.5 ml	0.5 ml
WB-2 Solution	50 ml	50 ml	50 ml	50 ml
5X Wash Solution	125 ml	125 ml	125 ml	125 ml
WestClear™ Nitrocellulose Membrane (0.2 μm, 7.5 x 8 cm)		5 sheets	5 sheets	5 sheets
ChromoSensor™ One Solution TMB Substrate		15 ml		
LumiSensor™ Chemiluminescent HRP Substrate			2 x 7.5 ml	
LumiSensor™ Super Chemiluminescent HRP Substrate				5 + 10 ml
User Manual	1	1	1	1

Kit Contents, continued

ONE-HOUR Western™ Fluorescent Kits

Component	Fluorescent Kit	Multiplex Fluorescent Kit
Pretreat Solution A	100 ml	100 ml
Pretreat Solution B	100 ml	100 ml
WB-1 Solution	2 ml	2 ml
WB-2 Solution	100 ml	
WB-M Solution		100 ml
10X Wash Solution	125 ml	125 ml
User Manual	1	1

ONE-HOUR IP-Western Kits

Components	L00231(Rabbit)	L00232(Mouse)	L00233(Goat)
Pretreat Solution A	50 ml	50 ml	50 ml
Pretreat Solution B	50 ml	50 ml	50 ml
Protein A&G blocker (100X)		0.5 ml	0.5 ml
Protein G blocker (100X)	0.5 ml		
IP-WB 1 solution	0.5 ml	0.5 ml	0.5 ml
IP-WB 2 solution	0.5 ml	0.5 ml	0.5 ml
IP-WB 3 solution	50 ml	50 ml	50 ml
5X Wash solution	125 ml	125 ml	125 ml
WestClear™ Nitrocellulose Membrane (0.2 µm, 7.5 × 8 cm)	5 Sheets	5 Sheets	5 Sheets
LumiSensor™ Chemiluminescent HRP Substrate	2 × 7.5 ml	2 × 7.5 ml	2 × 7.5 ml
User Manual	1	1	1

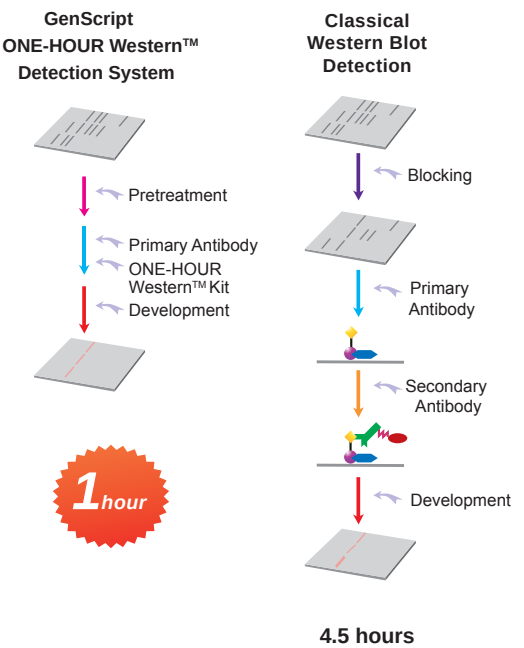
ONE-HOUR QuickBlock Kit

Component	L00276
Pretreat Solution A	2 × 50 ml
Pretreat Solution B	2 × 50 ml
User Manual	1

Introduction

The ONE-HOUR Western™ Detection System is designed to produce a high signal with a low background for quick and clear western analysis of proteins. GenScript's breakthrough ONE-HOUR Western™ technology simplifies the classical western blot analysis by skipping the secondary antibody binding and washing steps. The kits reduce the total western blot analysis time from 4.5 hours down to only one hour.

- **Easy to perform:** Quick and simple procedure.
- **High sensitivity:** The sensitivity of the ONE-HOUR Western™ is comparable to or better than that of the classical 4.5-hour procedure, depending on the quality and quantity of antibodies used.
- **Highly reproducible results.**
- **Less optimization needed than the classical method.**
- **Secondary antibody is included.**
- **No special labeling required for primary antibody.**

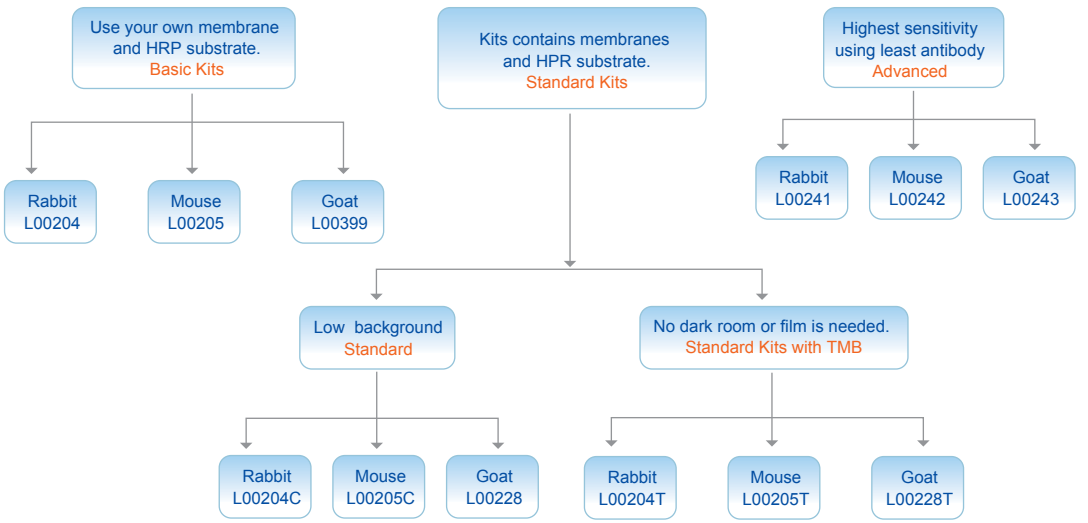


Storage

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.**

Quick Selection Guide

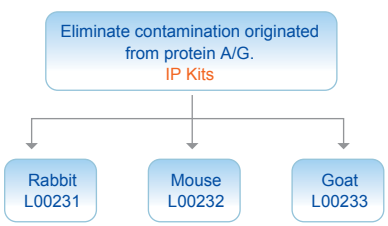
ONE-HOUR Western™ Detection Kits



	Standard Kits	Advanced Kits
Sensitivity	High	Very High
Amount of antibody	2 – 10 ug	0.5 – 2.5 ug
Total time	1.25 hrs	1.25 hrs

ONE-HOUR Western™ Fluorescent Kits

ONE-HOUR IP-Western Kits



*Note: Customers need to provide both the primary and secondary antibody.

Protocols

ONE-HOUR Western™ Basic/ Standard/Advanced Kits **Cat. No: L00204, L00205, L00399/L00204C, L00205C, L00228, L00204T, L00205T, L00228T/L00241, L00242, L00243**

Reagents Needed

This procedure is optimized for a sheet of 7.5 x 8.0 cm membrane. However, reagent volumes can be scaled up or down according to the size of the membrane used.

Reagents not provided:

Purified primary antibodies: 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:

1X wash solution: Dilute 25 ml of 5X wash solution with 100 ml of distilled or filtered water to make 125 ml of 1X wash solution. If any precipitate forms in the 5X 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. Use 15 ml of 1X wash solution for each rinse and 20 ml of 1X wash solution for each wash.

Prepare Mixture 1

Before or during protein transfer, prepare Mixture 1 by mixing the primary antibody with WB-1 in a microcentrifuge tube. Vortex Mixture 1 gently for a few seconds and centrifuge briefly. Incubate Mixture 1 at room temperature for at least 40 minutes.

Mixture 1 Preparation	L00204, L00205, and L00399	L00204T, L00205T, and L00228T	L00204C, L00205C, and L00228	L00241, L00242, and L00243
WB-1 Solution	20 - 100 µl	50 - 100 µl	20 - 100 µl	5 - 25 µl
Primary Antibody*	2 - 10 µg	5 - 10 µg	2 - 10 µg	0.5 – 2.5 µg
Ratio of WB-1: Antibody	10 µl : 1 µg	10 µl : 1 µg	10 µl : 1 µg	10 µl : 1 µg
For Antibody without Known Titer	Mix 5 µg of Ab with 50 µl WB-1	Mix 5 µg of Ab with 50 µl WB-1	Mix 5 µg of Ab with 50 µl WB-1	Mix 1 µg of Ab with 10 µl WB-1

* Refer to manufacturer's recommendations of appropriate amounts of antibody. With ONE-HOUR Western™ Advanced Kits, use 1/4 to 1/2 of the recommended amount. For antibodies without known titers, start with 1 µg for Advanced Western Kits and 5 µg for other ONE-HOUR Western™ Kits.

Protocols, continued

ONE-HOUR Western™ Basic/Standard/Advanced Kits, continued

Pretreat 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 wash box (GenScript, M00100)) to make the pretreat solution mixture. Always prepare and use fresh solution mixture. Place the membrane directly in the pretreat solution mixture and incubate on a shaker for five minutes at room temperature. After incubation, rinse the membrane twice with 15 ml of 1X wash solution.
Final Incubation of Pretreated Membrane	
	a. Add Mixture 1 to 10 ml of WB-2 in a Western blot box and mix well. Incubate the membrane in this solution (WB-2 containing Mixture 1) on a shaker at room temperature for 40 minutes. This solution (WB-2 containing Mixture 1) may be recovered and reused up to three times if stored at 4°C. However, this may cause variations to arise due to changes in antibody concentration and carryover contamination. 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. When using the TMB substrate, wash the membrane three times for just five 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.
Signal Development with Chemiluminescent HRP Substrate	
	a. When using LumiSensor™ Chemiluminescent HRP Substrate, mix 1.5 ml of Reagent A with 1.5 ml of Reagent B by vortexing for a few seconds to make the working solution. When using LumiSensor™ Super Chemiluminescent HRP Substrate, mix 1.0 ml of reagent A with 2.0 ml of reagent B by vortexing for a few seconds to make the working solution. Anout 0.05 ml of the working solution is sufficient to cover 1 cm^2 of membrane. When protected from light, the working solution (A+B) remains stable for several hours at room temperature. Summary of Working Solution Preparation: 0.05 ml is needed per cm^2 of membrane.

Protocols, continued

ONE-HOUR Western™ Basic/Standard/Advanced Kits, continued

Signal Development with Chemiluminescent HRP Substrate, continued

Working Solution Preparation	L00204C, L00205C, and L00228	L00241, L00242, and L00243
Reagent A	1.5 ml	1.0 ml
Reagent B	1.5 ml	2.0 ml
Total Volume	3.0 ml	3.0 ml

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 a 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 (not provided) for 30 seconds and then develop. Repeat with different exposure time to find the best results. An imager capable of detecting chemiluminescent signals can also be used to record the results.

Signal Development with TMB Substrate

a. ChromoSensor™ One Solution TMB Substrate is a ready-to-use working solution, and 0.05 ml is sufficient to cover 1 cm^2 of membrane. 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 a clean plate and cover it with TMB.

b. Incubate for 5 to 10 minutes at room temperature until the desired color intensity is reached. Stop the reaction by rinsing the membrane three times for thirty seconds each in 20 ml of deionized water.

c. Drain off the excess water and transfer the membrane to a piece of paper towel. Air-dry the membrane in a dark place.

Protocols, continued

ONE-HOUR Western™ Basic/Standard/Advanced Kits, continued

Examples

Comparison of the two ONE-HOUR Western™ Kits of different sensitivities using monoclonal antibodies:

Two similar blots were processed with the same procedures using different ONE-HOUR Western™ Kits: Standard (L00205C) and Advanced (L00242). 10 μg and 2.5 μg of THE™ Anti-GST Monoclonal Antibody (Mouse) (GenScript, A00865), respectively, were used with these two kits to detect GST protein. The results are shown in Figure 1.

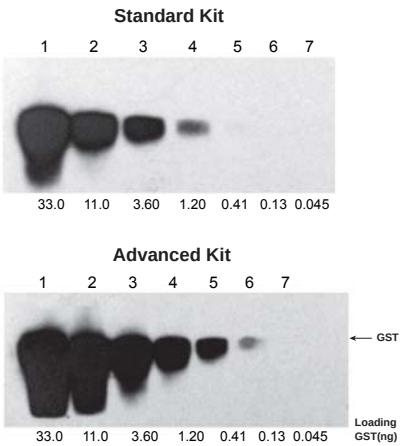


Figure 1. Western blots for the detection of GST protein using different ONE-HOUR Western™ Kits: Standard (L00205C) and Advanced (L00242). 33.0, 11.0, 3.60, 1.20, 0.41, 0.13 and 0.045 ng of GST protein were loaded onto Lane 1, 2, 3, 4, 5, 6, and 7 respectively.

Protocols, continued

ONE-HOUR Western™ Basic/Standard/Advanced Kits, continued

Examples, continued

Comparison of the two ONE-HOUR Western™ Kits of different sensitivities using polyclonal antibodies:

Two similar blots were processed with the same procedures using different ONE-HOUR Western™ Kits: Standard (L00204C) and Advanced (L00241). 10 μg and 2.5 μg of Rabbit Anti-GST-tag Polyclonal Antibody (GenScript, A00097), respectively, were used with the two kits to detect GST protein. The results are shown in Figure 2.

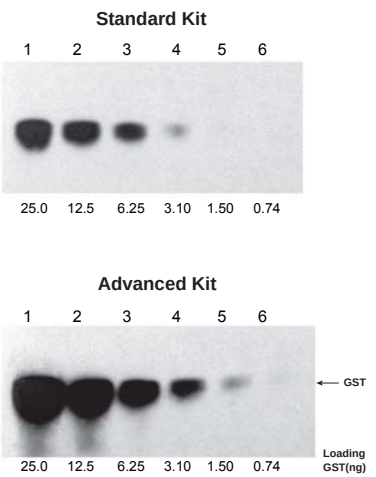


Figure 2. Western blots for the detection of GST protein using different ONE-HOUR Western™ Kits: Standard (L00204C) and Advanced (L00241). 25.0, 12.5, 6.25, 3.10, 1.50 and 0.74 ng of GST protein were loaded onto Lane 1, 2, 3, 4, 5, and 6 respectively.

Protocols, continued

ONE-HOUR IP-Western Kits

Cat. No: L00231, L00232, L00233

Reagents Needed

1. This procedure is optimized for a sheet of 7.5 x 8.0 cm membrane. The reagent volumes can be scaled up or down according to the size of the membrane used.
2. The product is optimized to block up to 2 µg of antibody per lane. Do not load more than 2 µg of antibody per lane. Theoretically 2 µg of antibody can pull down 1.33 µg of a 50 kDa antigen.
3. If using a mouse (L00232) or goat kit (L00233) with Protein A, G or A/G MagBeads, use the Protein A&G blocker to prevent leaked protein A, G or A/G from interfering with the Western results. If using a rabbit kit (L00231), use Protein G blocker to prevent leaked protein G or A/G from interfering with the western results. Protein A does not affect the Western results in the case of rabbit antibodies. All the kits are optimized to block up to 50 ng of protein A, G, or A/G per lane.

Reagents not provided:

Primary antibodies. Affinity-purified antibodies are recommended. Rabbit polyclonal antibodies should be whole-molecule. Fab fraction gives a significantly low signal. GenScript has a complete portfolio of antibodies for signal pathways and other applications. It may be viewed online here: http://www.genscript.com/cgi-bin/products/rec_antibody.cgi

Before use, prepare the following:

1X wash solution: Dilute 25 ml of 5X wash solution with 100 ml of distilled or filtered water to make 125 ml of 1X wash solution. If any precipitate forms in the 5X 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. Use 15 ml of 1X wash solution for each rinse and 20 ml of 1X wash solution for each wash.

Protocols, continued

ONE-HOUR IP-Western Kits, continued

Prepare Mixture 1	
	Before or during protein transfer, prepare Mixture 1 by mixing 100 µl of IP-WB 1 with 10 µg or more of the 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 room temperature for at least 40 minutes. Longer incubation is preferred. For overnight incubation, store Mixture 1 at 4°C. **Note:** If less than 10 µg of primary antibody is to be used in Western blot, the volume of IP-WB 1 should be reduced accordingly. For example, mix 50 µl of IP-WB 1 with 5 µg of primary antibody to make Mixture 1. The other reagents do not need to be adjusted.
Pretreatment of Membrane and Preparing Mixture 2	
	Mix 10 ml of Pretreat Solution A with 10 ml of Pretreat Solution B in a plastic container to make the pretreat solution mixture. Incubate the membrane after protein transfer into the pretreat solution mixture on a shaker for five minutes at room temperature. After incubation, rinse the membrane twice with 15 ml of 1X wash solution. Meanwhile prepare Mixture 2 by adding 100 µl of IP-WB 2 to Mixture 1. Vortex Mixture 2 for a few seconds and spin down briefly to collect the solution in the bottom of the tube. Incubate Mixture 2 at room temperature for five minutes.
(Optional) Protein A and Protein G Active Site Blocking	
	For mouse and goat kits: If Protein A, G or A/G MagBeads is used during immunoprecipitation, dilute 100 µl of Protein A&G blocker with 10 ml of 1X wash solution and incubate the membrane from step 2 in this diluted blocker on a shaker for five minutes at room temperature. Do not wash or rinse. For rabbit kits: If Protein G or A/G MagBeads is used during immunoprecipitation, first add Mixture 2 to 10 ml of IP-WB 3 and then add 100 µl of Protein G blocker directly to the combined solution. Mix well.

Protocols, continued

ONE-HOUR IP-Western Kits, continued

Final Incubation of Pretreated Membrane
a. Add Mixture 2 to 10 ml of IP-WB 3 in a plastic container and mix well. Incubate the membrane in the IP-WB 3 containing Mixture 2 on a shaker for 40 minutes at room temperature. b. Rinse the membrane once with 15 ml of 1X wash solution. Wash the membrane three times on a shaker for five minutes each with 20 ml of 1X wash solution. Use a clean container for each rinse and wash step to avoid carryover contamination and to reduce background.
Signal Development
a. Mix 1.5 ml of Reagent A with 1.5 ml of 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 a 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 one minute and then develop. Repeat with different exposure times for best results. An imager capable of detecting chemiluminescent signals can also be used to record the results.

Protocols, continued

ONE-HOUR IP-Western Kits, continued

Examples

1. Comparison of ONE-HOUR IP-Western blot and classical Western blot using rabbit primary antibody:

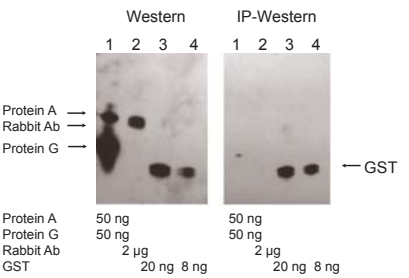


Figure 1. Western blot detection of GST protein by both classical western and ONE-HOUR IP-Western (using kit L00231). Both blots are developed using Rabbit Anti-GST-tag Polyclonal Antibody (GenScript, A00097) and the LumiSensor™ Chemiluminescent HRP Substrate included in kit L00231.

2. Comparison of ONE-HOUR IP-Western blot and classical western blot using mouse primary antibody:

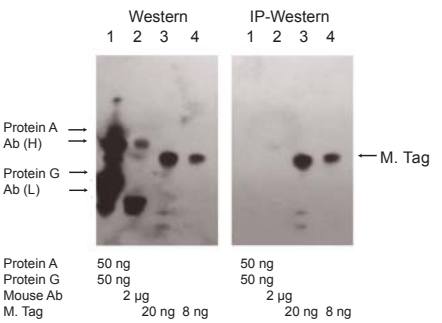


Figure 2. Western blot detection of multiple-tag fusion protein by both classical western and ONE-HOUR IP-Western (using kit L00232). Both blots are developed using Mouse Anti-Trx-tag Monoclonal Antibody (GenScript, A00180) and the LumiSensor™ Chemiluminescent HRP Substrate that is included in kit L00232.

Protocols, continued

ONE-HOUR IP-Western Kits, continued

Examples, continued

3. Comparison of ONE-HOUR IP-Western blot with classical western blot using goat primary antibody:

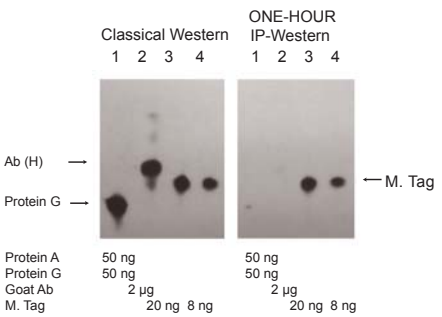


Figure 3. Western blots for the detection of multiple-tag fusion protein by both classical Western and ONE-HOUR IP-Western (using kit L00233). Both blots are developed using goat antibody anti-HA (GenScript, A00168) and the LumiSensor™ Chemiluminescent HRP Substrate included in kit L00233.

Protocols, continued

ONE-HOUR Western™ Fluorescent Kit **Cat. No: L00397**

Reagents Needed

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 these kinds 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. Use 15 ml of 1X wash solution for each rinse and 20 ml of 1X wash solution for each wash.

Prepare Mixture 1

Before or during protein transfer, prepare Mixture 1 by mixing primary antibody and fluorescent dye labeled secondary antibody in WB-1. Add 2—10 µg of primary antibody* to 100 µl of WB-1 in a microcentrifuge tube, then add 1—5 µg of 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 Mixture 1 in the dark at room temperature for at least 40 minutes.

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

Protocols, continued

ONE-HOUR Western™ Fluorescent Kit, continued

Pretreat 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, M00100) to make the pretreat solution mixture. Always prepare and use a fresh solution mixture. Place the membrane directly in the pretreat solution mixture and incubate on a shaker for five minutes at room temperature. After incubation, rinse the membrane twice with 15 ml of 1X wash solution.
Final Incubation of Pretreated Membrane	
	a. Add Mixture 1 to 10 ml of WB-2 in a Western blot box (GenScript Western Blot Box, Black, M00103) and mix well. Incubate the membrane in this solution (WB-2 containing mixture 1) on a shaker at room temperature for 40 minutes. Protect this box (or bag) from light during incubation. This solution (WB-2 containing mixture 1) may be recovered and reused up to three times if stored at 4°C. However, this may cause variations to arise due to changes in antibody concentration and carryover contamination. 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. Protect box (or bag) from light during wash. Use a clean container for each wash to reduce background.
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.

Protocols, continued

ONE-HOUR Western™ Fluorescent Kit, continued

Examples

1. Fluorescent Western blot detection of GST-tag Antibody, pAb, Rabbit (GenScript, A00097)

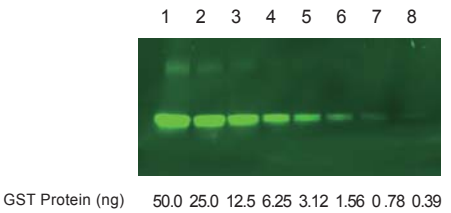


Figure 1. Fluorescent Western blots for the detection of GST protein using the ONE-HOUR Western™ Fluorescent Kit (L00397). 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 ng of GST protein were loaded into Lane 1, 2, 3, 4, 5, 6, 7 and 8 respectively.

2. Fluorescent Western blot detection of GAPDH Antibody, pAb, Goat (GenScript, A00191)

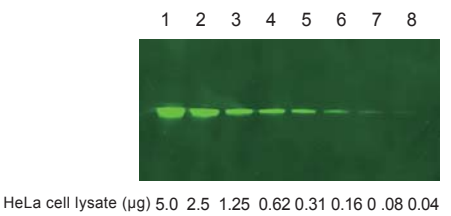


Figure 2. Fluorescent Western blots for the detection of GAPDH using the ONE-HOUR Western™ Fluorescent Kit (L00397). 5.0, 2.5, 1.25 0.62, 0.31, 0.16, 0.08 and 0.04 µg of Hela cell lysate were loaded into Lane 1, 2, 3, 4, 5, 6, 7 and 8 respectively.

Protocols, continued

ONE-HOUR Western™ Multiplex Fluorescent Kit **Cat. No: L00398**

Reagents Needed

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 these kinds 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. Use 15 ml of 1X wash solution for each rinse and 20 ml of 1X wash solution for each wash.

Prepare Mixture 1

Before or during protein transfer, prepare Mixture 1 by mixing the 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 for at least 40 minutes.

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

Protocols, continued

ONE-HOUR Western™ Multiplex Fluorescent Kit, continued

Pretreat 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 mixture. Always prepare and use a fresh solution mixture. Place the membrane directly in the pretreat solution mixture and incubate on a shaker for five minutes at room temperature. After incubation, rinse the membrane twice with 15 ml of 1X wash solution.
Final Incubation of Pretreated Membrane	
	a. Just after setting up the pre-treatment step, **add 1 ml of WB-M to each of the Mixture 1's and mix well by inverting the tubes several times. Incubate all the tubes at room temperature 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, M00100 or 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.** 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. **Protect box (or bag) from light during wash. Use a clean container for each wash to reduce background.**
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.

Protocols, continued

ONE-HOUR Western™ Multiplex Fluorescent Kit, continued

Examples

1. Multiplex Fluorescent Western blot detection of four proteins 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

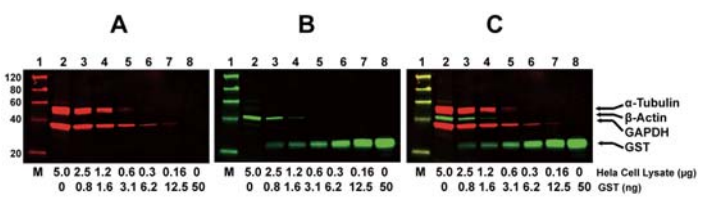


Figure 1. Multiplex Fluorescent Western blots for the detection of α-Tubulin, β-Actin, GAPDH, and GST proteins using the ONE-HOUR Western™ Multiplex Fluorescent Kit (L00398). A: 700 nm fluorescence image; B: 800 nm fluorescence image; C: The two fluorescence colors were imaged simultaneously in a single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

Troubleshooting

ONE-HOUR Western ™
Basic/Standard/Advanced
Kits

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 washing for 10 min x 3, wash for 5 min x 3 to increase signal.
There is high background.	Too much primary antibody was 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 pretreat A-b (M01052). Customers can also use the Quick Block Optimization Kit to find the best blocking reagent.
	The wash time is too short.	Adding additional washing steps can further decrease background.
	The signal development time is too long.	Reduce the exposure time. If both the signal and background are high, wait for a few minutes for background signal to go down before exposing the film.
	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.

Troubleshooting, continued

ONE-HOUR
IP-Western Kits

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.
There is high background.	There is non-specific binding/ cross-reactivity of primary antibody.	Change antibodies. Use a highly specific primary antibody. Affinity-purified primary antibodies are preferred.
	The blot shows protein A, G or A/G carryover contamination.	Increase the Protein A/G blocking time to ten minutes or longer. Add some Protein A&G blocker to the IP-WB 3 solution. Instead of 100X, try 200X.
	The heavy chain or light chain of the antibody is still visible.	If using the rabbit kit, use more protein G blocker. Load less sample to reduce antibody loading. Use the same amount of primary antibody but less WB-1 solution. For example, mix 10 µg of primary antibody with 80 µl of WB-1 solution.
	There is too much primary antibody.	Reduce both the volume of the WB-1 solution and the amount of primary antibody added to it in step 1 while keeping the proportions the same. 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 signal development time is too long.	Reduce the exposure time. If both the signal and background are high, wait for a few minutes before exposing the film.

Troubleshooting

ONE-HOUR™
Fluorescent Kit

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 washing for 10 min x 3, wash for 5 min x 3 to increase signal.
There is high background.	Too much primary antibody was 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.

Troubleshooting, continued

ONE-HOUR™
Multiplex Fluorescent Kit

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 washing for 10 min x 3, wash for 5 min x 3 to increase signal.
There is high background.	Too much primary antibody was 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.

Technical Support

Web Resources

Visit the GenScript Web site at www.genscript.com for:

1. Technical resoures, including manuals, MSDS, FAQ, *etc*
2. Online 2010-2011 Product Catalog
3. Additional promotions and special offers

Contact Us

GenScript USA Inc.

860 Centennial Ave,

Piscataway, NJ 08854

Tel: 732-885-9188, 732-885-9688

Fax: 732-210-0262, 732-885-5878

Email: product@genscript.com

Patent Pending

Limited Use Label license: This product may be the subject of one or more patents filed by GenScript USA Inc.. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for any commercial purposes. For commercial use, please contact GenScript at info@genscript.com

GenScript USA Inc.

860 Centennial Ave.

Piscataway, NJ 08854

Tel: 732-885-9188, 732-885-9688

Fax: 732-210-0262, 732-885-5878

Email: product@genscript.com