ONE-HOUR Complete IP-Western Kit

Technical Manual No. 0218 **Version 06192009**

I. DESCRIPTION

ONE-HOUR Complete IP-Western Kit contains all the necessary reagents, buffers, nitrocellulose membrane and HRP substrate for performing an IP-western blot. It was developed using breakthrough immunodetection technology (patent pending), allowing the user to perform an IP-western blot in a fraction of the usual time. After transferring the proteins from gel to membrane, simply incubate the membrane in pretreat solution for five minutes and follow with incubation in WB solution (prepared from primary antibody, IP-WB 1, IP-WB 2 and IP-WB 3) for 40 minutes. Wash the membrane three times for five minutes each and then the blot can be developed using the HRP substrate included in the kit. The ONE-HOUR Western™ blot procedure is compared to the classical three-step procedure at right.

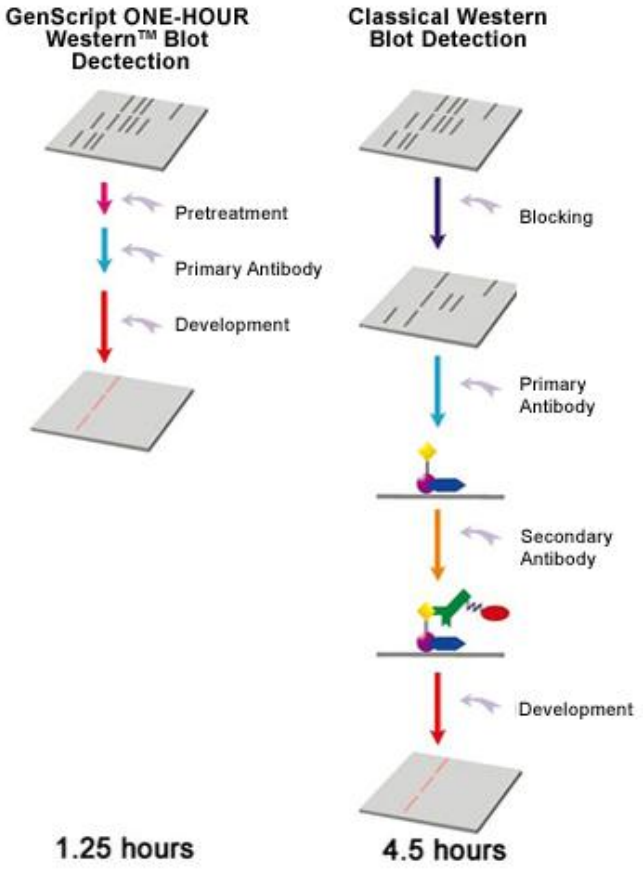


Figure 1. Overview of Western Procedures

The ONE-HOUR Complete IP-Western Kit, available with rabbit, mouse, and goat primary antibody, allows for the detection of even nanograms of antigen without showing quantities of immunoprecipitation antibody under 2 µg per lane, yielding a clearer, more visible result. The A&G blocker in the mouse and goat kits takes only five minutes to use and gives clean IP-Western results even in cases of serious protein A, G or A/G carryover contamination. Regarding the rabbit kit, protein G blocker is only needed if protein G or A/G resin is used during immunoprecipitation. The protein A carryover does not affect the western results.

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

II. KIT CONTENTS

Three different kinds of ONE-HOUR Complete IP-Western Kits are available. L00231, L00232 and L00233 are for use with rabbit primary antibody, mouse primary antibody and goat primary antibody, respectively. Each kit contains enough reagents for ten minigel (7.5 x 8 cm) IP-western blots.

Kit Components	10 Assays L00231 (Rabbit)	10 Assays L00232 (Mouse)	10 Assays L00233 (Goat)
Pretreat A solution	100 ml	100 ml	100 ml
Pretreat B solution	100 ml	100 ml	100 ml
A&G blocker (100X)		1 ml	1 ml
Protein G blocker (100X)	1 ml		
IP-WB 1 solution	1 ml	1 ml	1 ml
IP-WB 2 solution	1 ml	1 ml	1 ml
IP-WB 3 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™ 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
10X Wash Solution	MB01011
A&G Blocker (100X)	M01014
Protein G Resin	L00209
Protein A Resin	L00210

IV. KEY FEATURES

Easy to perform: Fewer steps mean fewer chances for human error.

- Secondary antibody, protein A, G and A/G are blocked to give clean IP-Western results.
- Low background: The kit contains WestClear™ Nitrocellulose Membrane and LumiSensor™ Chemiluminescent HRP Substrate Kit, optimized for low background.
- High sensitivity: The kit's sensitivity is comparable with 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.
- The ONE-HOUR Western™ Kit needs far less optimization than the classical three-step method.

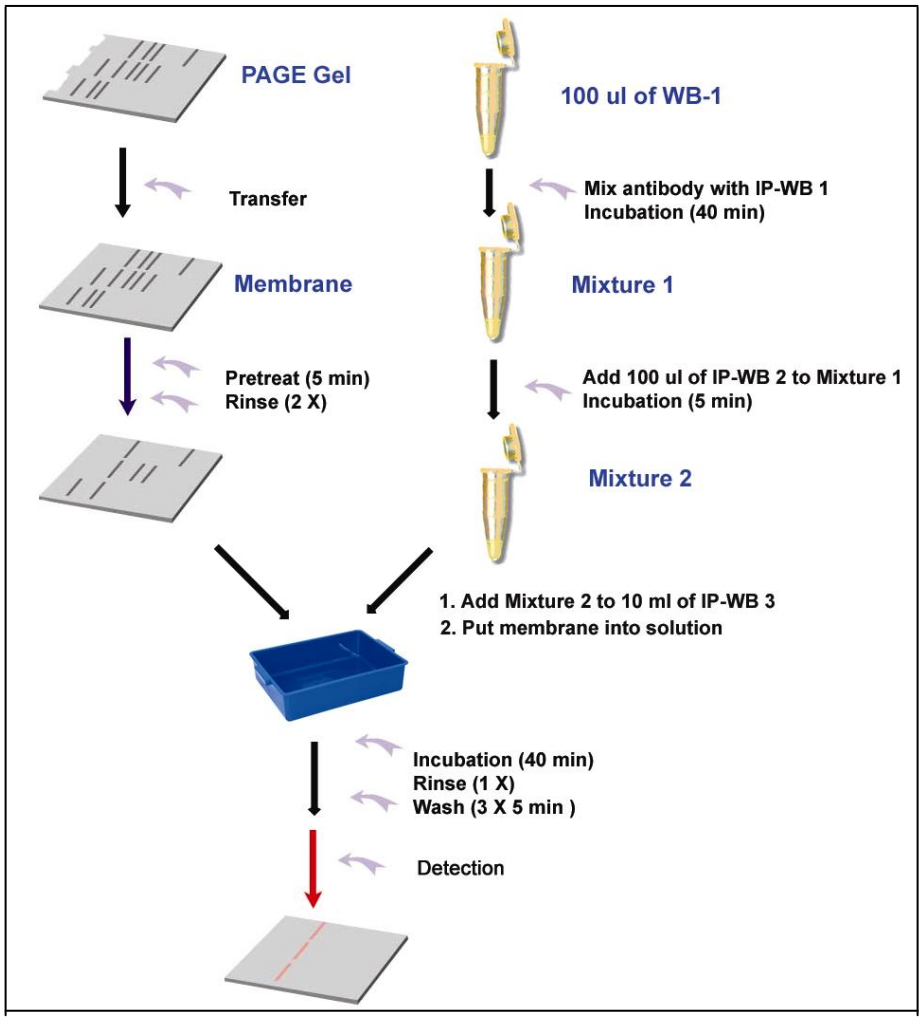
V. STORAGE

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™ KIT PROTOCOL

Specifications:

1. This procedure is optimized for a sheet of 7.5 x 8 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 resin, use the 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 application. It may be viewed online here: http://www.genscript.com/cgi-bin/products/rec_antibody.cgi.

Before use, prepare the following:

Dilute 12.5 ml of 10X wash solution with 112.5 ml of distilled 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 dH_2O to 1X and store it at 4°C.

Western Blot Procedure:

1. **Preparing Mixture 1**

   Before or during protein transfer, prepare Mixture 1 by mixing100 µl of IP-WB 1 with 10 µg or more 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 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.

2. Pretreatment of Membrane and Preparing Mixture 2

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 after protein transferring in 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.

3. (Optional) Protein A and Protein G Active Site Blocking

For mouse and goat kits: If protein A, G or A/G resin is used during immunoprecipitation, dilute 100 µl of A&G blocker with 10 ml of 1X wash solution and incubate the membrane from step 2 in this diluted A&G blocker on a shaker for five minutes at room temperature. Do not wash or rinse.

For rabbit kits: If protein G or A/G resin 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.

4. 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.**

5. Signal Development

a. Mix 3 ml of reagent A with 3 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 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.

VII. EXAMPLES

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

ONE-HOUR IP-Western blot is compared to the classical Western blot detection of GST protein using Rabbit Anti-GST-tag Polyclonal Antibody (GenScript, A00097): Classical western blot detection (4.5 hours, left panel, Figure 2) and ONE-HOUR IP-Western blot (one hour, right panel, figure 2).

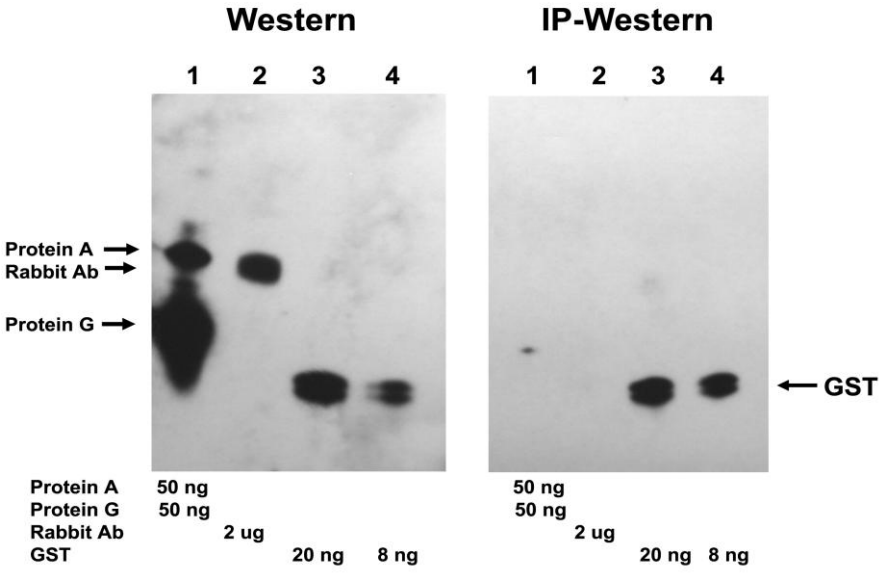


Figure 2. Western blot detection of GST protein by both classical western and ONE-HOUR IP-Western (using kit L00231). Both blots are developed using the LumiSensor™ Chemiluminescent HRP Substrate included in kit L00231.

2. Comparison of ONE-HOUR IP-Western blot and classical western blot using mouse primary antibody: The ONE-HOUR IP-Western blot is compared to the classical Western blot detection of purified multiple-tag (M. Tag) fusion protein. Two similar blots are processed with different procedures using Mouse Anti-Trx-tag Monoclonal Antibody (GenScript, A00180): Classical Western blot detection (4.5 hours, left panel, figure 3) and ONE-HOUR IP-Western blot (one hour, right panel, figure 3).

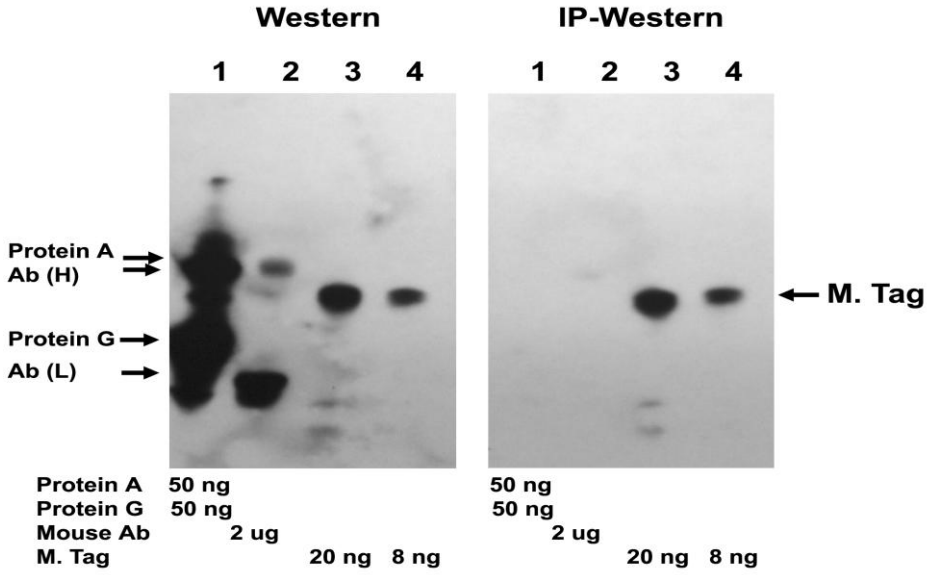


Figure 3. Western blot detection of multiple-tag fusion protein by both classical western and ONE-HOUR IP-Western (using kit L00232). Both blots are developed using the LumiSensor™ Chemiluminescent HRP Substrate that is included in kit L00232.

3. Comparison of ONE-HOUR IP-Western blot with classical western blot using goat primary antibody: ONE-HOUR IP-Western blot is compared to the classical western blot detection of purified multiple-tag (M. Tag) fusion protein. Two similar blots are processed with different procedures using goat antibody anti-HA (GenScript, A00168): Classical Western blot detection (4.5 hours, left panel, figure 4) and ONE-HOUR IP-Western blot (one hour, right panel, figure 4).

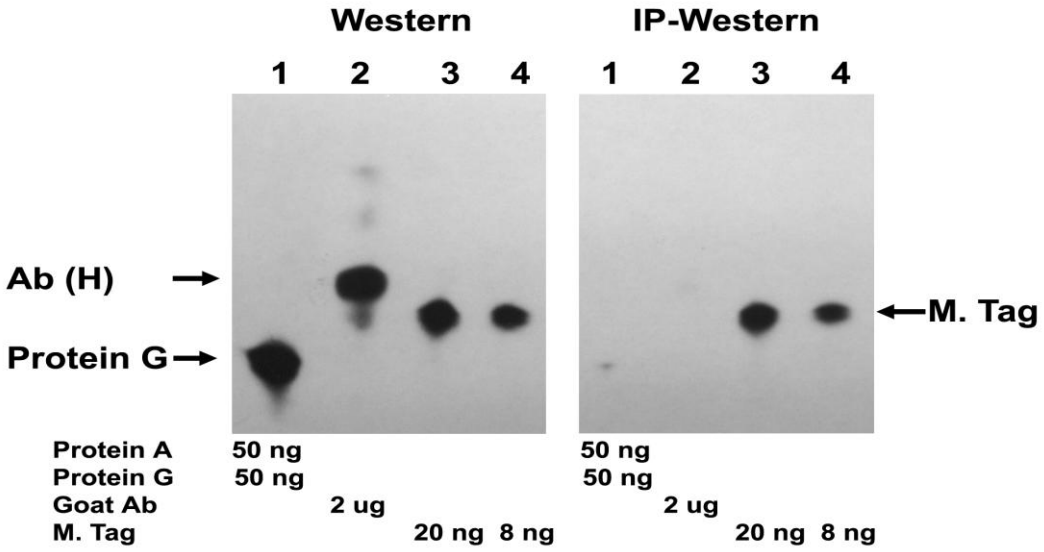


Figure 4. Western blots for the detection of multiple-tag fusion protein by both classical Western and ONE-HOUR IP-Western (using kit L00233). Both blots are developed using the LumiSensor™ Chemiluminescent HRP Substrate included in kit L00233.

VIII. TROUBLESHOOTING

Use the table below to solve and avoid common problems.

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 A&G blocker to the IP-WB 3 solution. Instead of 100X, try 200X. If using the rabbit kit, use more protein G blocker.
	The heavy chain or light chain of the antibody is still visible.	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 wash time is too short.	Increase wash time from five minutes to ten for each wash. Adding additional washings after primary antibody (in WB) binding 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 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.

IX. ORDERING INFORMATION

ONE-HOUR Complete IP-Western Kit: Cat. No. L00231 for rabbit primary antibody
Cat. No. L00232 for mouse primary antibody
Cat. No. L00233 for goat primary antibody

Patent Pending.

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