Western Blot Protocol

1. Reagents

2X SDS-PAGE Sample Buffer

1.5 g	Tris base
4 g	SDS
20 ml	Glycerol
2 ml	β-Mercaptoethanol
0.02 g	Bromophenol blue
100 ml	dd H_2O

Adjust to pH 6.8

Store at -20°

SDS-PAGE Running Buffer

3.03 g	Tris base
24 g	Glycine
1.0 g	SDS
1000 ml	ddH_2O

Store at 4 °C

Note: warm the buffer before use to remove possible precipitates.

Transfer buffer

3.03 g	Tris base
14.4 g	Glycine
200 ml	Methanol
800 ml	ddH_2O

Store at 4 °C

Blocking Buffer

100 ml	PBS Buffer
5 g	Non-fat milk

Store at 4 °C

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Washing Buffer

0.5 ml	Tween 20
1000 ml	PBS Buffer
Store at 4 °C	

PBS Buffer

8.5 g	NaCl
1.4 g	Na_2HPO_4
0.2 g	NaH_2PO_4
1000 ml	dd H_20
Adjust to pH 7.4	
Store at 4°C	

Primary Antibody Dilution Buffer

5 mg	BSA
100 ml	PBS
Adjust to pH 7.4	
Store at 4°C	

ECL kit

2. Procedure

2.1 SDS-PAGE

1. Add 2X SDS-PAGE Sample Buffer to protein samples (1:1) and heat to 100ºC for 5 minutes
   Note: Don't boil too long to avoid proteins getting destroyed.
2. Wash the gel with *SDS-PAGE Running Buffer* and load samples into gel.
   Note: Samples containing multiple proteins require 10-60 ug of protein per well. Purified samples containing a single or very few proteins require less (0.05-1 ug).
3. Run gel at 100 Volts through the stacking part of the gel and turn the volts up to 200V after the proteins have gone through the stack and are migrating through the resolving gel.
4. Allow migration to continue until the blue dye front is at the end of the glass plates, but has not migrated off the gel.

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2.2 Transferring

1. Soak the PVDF membrane in *methanol* for 1 minute, and then place it in deionized water.
2. Place PVDF membrane, two fiber pads, and four Whatman papers (pre-cut) in a shallow tray filled with *Transfer Buffer* for a few minutes.
3. Disassemble gel apparatus and carefully pry plates apart. Then cut off stacking gel with a clean razor blade and soak gel in *Transfer Buffer* for a few minutes.
4. Open transfer apparatus gel cassettes with the black panel lying flat on the bottom of the tray filled with *Transfer Buffer*; the clear panel should be against the side of the tray.
5. Prepare the transfer sandwich on the black panel in the tray filled with *Transfer Buffer*.
   i. one fiber pad
   ii. two Whatman papers
   iii. SDS gel
   iv. PVDF membrane
   v. two Whatman papers
   vi. one fiber pad
   Note: Remove air bubbles by rolling a glass tube on the membrane.
6. Cover the sandwich with the clear panel, fasten with the latch, and insert the gel cassette into the electrode module with the black panel facing the black cathode electrode panel.
7. Insert the bio-ice cooling unit into the buffer chamber, and fill the buffer chamber with *Transfer Buffer*. Transfer for 1-2 hours at 4 ºC, stirring at a constant current of 100 Volts.
8. Stain with 1x Pongee S for one minute and destain in ddH_2O and rinse it with PBS Buffer (This step is optional).

2.3 Blocking and Incubating

1. Incubate the membrane with *Blocking Buffer* on a shaker for 1-2 hours at 37°C or overnight at 4ºC.
2. Dilute primary antibody with *Primary Antibody Dilution Buffer* and incubate the membrane with the diluted primary antibody on a shaker for 1 hour at 37°C or overnight at 4ºC.
3. Wash the membrane four times with *Washing Buffer* on the Shaker for 5-10 minutes each time.
   Note: For HRP conjugated primary antibody, get rid of step 4 and step 5.
4. Dilute secondary antibody with *Blocking Buffer* and incubate the membrane with the diluted secondary antibody on a shaker for 1 hour at 37°C or overnight at 4ºC.
5. Wash the membrane four times with *Washing Buffer* on the Shaker for 5-10 minutes each time.

2.4 Detection

a. For HRP conjugated secondary antibody

1. Detect protein with ECL kit (2 ml/membrane). In a separate tube, mix black and white ECL solutions in a 1:1 ratio.

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2. Aliquot solution onto membranes and wait for 1 minute. Drain the ECL, wrap in plastic and expose to film.

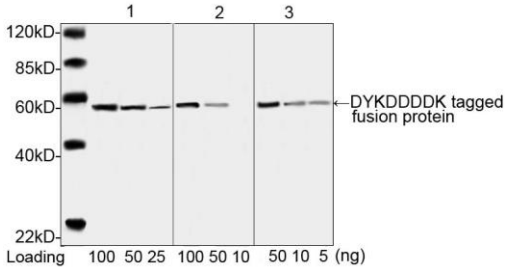
Note: Expositon time to the blots for 10 seconds, 1 minute, 5 minutes, and 20 minutes to visualize the chemiluminescence signal corresponds to the specific antibody-antigen reaction. Optimal dilutions of Genscript HRP conjugated secondary antibody should also be determined. Suggested starting dilutions to test are at the range of 1:5000 ~ 1:10,000

b. For IRDye™ 800 Conjugated secondary antibody

Rinse the membrane with *PBS Buffer* and develop the signal with Odyssey® Infrared Imaging System according to its accompanying manual.

Note: Optimal dilutions of dye-conjugated secondary antibodies should also be determined. Suggested starting dilutions to test are 1:5000, 1:10,000, and 1:20,000.

3. Western Blot examples



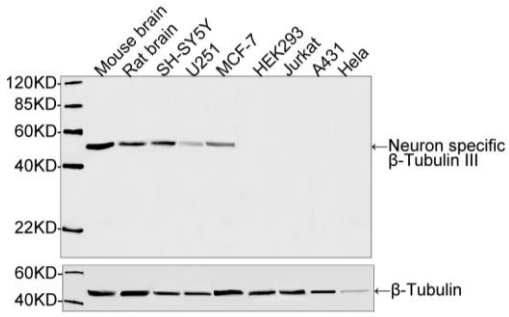
Western blot analysis of DYKDDDDK tagged-fusion protein using THE™ DYKDDDDK Tag Antibody, mAb, Mouse (GenScript, A00187, 1 μg/ml).

Lane 1 N-terminal DYKDDDDK-tagged fusion protein

Lane 2 Internal DYKDDDDK-tagged fusion protein

Lane 3 C-terminal DYKDDDDK-tagged fusion protein

The signal was developed with IRDye™ 800 Conjugated Goat Anti-Mouse IgG.



Western blot analysis of cell and tissue lysates using **Neuron Specific β-Tubulin III Antibody, pAb, Rabbit** (GenScript, A01627, 1 μg/ml)

The signal was developed with IRDye™ 800 Conjugated Goat Anti-Rabbit IgG.

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4. Recommended Products

Loading control Antibodies

Name	Cat. No.	Size	Price
α-Actin-1 Antibody, pAb, Rabbit	A00885	40 ug	$50.00
GAPDH Antibody, pAb, Goat	A00191	40 ug	$50.00
GAPDH Antibody [HRP], pAb, Goat	A00192	40 ug	$60.00
GAPDH Antibody [Biotin], pAb, Goat	A00915	40 ug	$60.00
THE™ beta Actin Antibody, mAb, Mouse	A00702	40 ug	$69.00
THE™ beta Actin Antibody [HRP], mAb, Mouse	A00730	40 ug	$69.00
THE™ beta Actin Antibody [Biotin], mAb, Mouse	A01546	100 ug	$335.00
THE™ alpha Tubulin Antibody, mAb, Mouse	A01410	100 ug	$225.00
THE™ alpha Tubulin Antibody [HRP], mAb, Mouse	A01490	100 ug	$335.00
THE™ alpha Tubulin Antibody [Biotin], mAb, Mouse	A01545	100 ug	$335.00

Negative control Antibodies

Name	Cat. No.	Size	Price
Human IgG Control(Whole Molecule), Purified	A01006	4 mg	$50.00
Mouse IgG control (Whole Molecule), Purified	A01007	1 mg	$50.00
Rabbit IgG Control (Whole Molecule), Purified	A01008	4 mg	$50.00
Goat IgG Control (Whole Molecule), Purified	A01009	4 mg	$50.00
Chicken IgY Control (Whole Molecule), Purified	A01010	4 mg	$50.00

Secondary Antibodies

Name	Cat. No.	Size	Price
Goat Anti-Rabbit IgG Antibody (H&L) [HRP], pAb	A00098	1 mg	$60.00
Goat Anti-Mouse IgG Antibody (H&L) [HRP], pAb	A00160	1 mg	$60.00
Goat Anti-Chicken IgY Antibody (H&L) [HRP], pAb	A00165	1 mg	$63.00
Goat Anti-Human IgG Antibody (H&L) [HRP], pAb	A00166	1 mg	$60.00
Goat Anti-Rat IgG (H&L) [HRP] Antibody, pAb	A00167	1 mg	$60.00
Donkey Anti-Goat IgG Antibody (H&L) [HRP], pAb	A00178	1 mg	$63.00

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5. Troubleshooting Guide

Problem	Possible Cause	Solution
High background	Antibody concentrations too high	Optimize primary and secondary antibody dilutions.
	Insufficient washing	Increase number of washes and buffer volume.
	Inadequate antibody volume used	Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times. Do not allow any area of membrane to dry out.
	Membrane contamination	Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags, and trays for incubations.
Weak or no signal	Insufficient antibody used	Increase amount of primary or secondary antibody, optimizing for best performance.
	Insufficient incubation time	Extend primary antibody incubation time.
	Primary or secondary antibody lost reactivity	Use new antibody
Additional band	Non-specific band	Use immunogen as blocking material to confirm whether the band is labeled by primary antibody and whether the band is modified pattern of target protein.

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