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	ddH_2O

Adjust to pH 6.8

Store at -20 °C

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 to remove possible precipitates before use.

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 dry 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	ddH_2O

Adjust to pH 7.4

Store at 4 °C

Primary Antibody Dilution Buffer

1 g	BSA
100 ml	Washing Buffer

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 100 ºC for 5 minutes.
   Note: Don't boil too long to avoid proteins getting destroyed.
2. Wash a gel with *SDS-PAGE Running Buffer* and load samples into the gel.
   Note: Samples containing multiple proteins require 10-60 µg of protein per well. Purified target protein requires 0.05-1 µg.
3. Run the gel at 100V 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.

2.2 Transferring

1. Soak a PVDF membrane in *methanol* for 30 seconds, and then place it in distilled water.
2. Place the PVDF membrane, two fiber pads, and four Whatman papers (pre-cut) in a shallow tray filled with

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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 100V.
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 4 ºC.
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 4 ºC.
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 4 ºC.
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.
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 HRP

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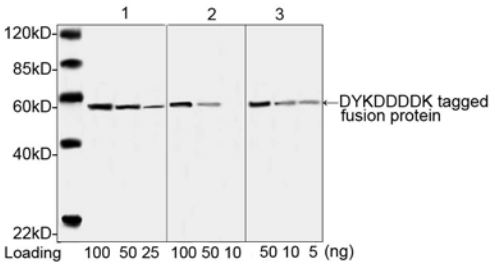
conjugated secondary antibody should also be tested. Suggested starting dilutions to test are at the range of 1:5000 ~ 1:10,000.

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 tested. Suggested starting dilutions to test are 1:5000, 1:10,000, and 1:20,000.

3. Western Blot Examples



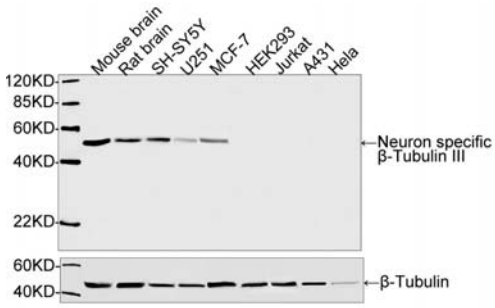
Western blot analysis of DYKDDDDK tagged-fusion protein using THE™ DYKDDDDK Tag Antibody, mAb, Mouse (GenScript, A00187, 1 μg/ml).

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.
α-Actin-1 Antibody, pAb, Rabbit	A00885
GAPDH Antibody, pAb, Goat	A00191
GAPDH Antibody [HRP], pAb, Goat	A00192
GAPDH Antibody [Biotin], pAb, Goat	A00915
THE™ beta Actin Antibody, mAb, Mouse	A00702
THE™ beta Actin Antibody [HRP], mAb, Mouse	A00730
THE™ beta Actin Antibody [Biotin], mAb, Mouse	A01546
THE™ alpha Tubulin Antibody, mAb, Mouse	A01410
THE™ alpha Tubulin Antibody [HRP], mAb, Mouse	A01490
THE™ alpha Tubulin Antibody [Biotin], mAb, Mouse	A01545

Negative control antibodies

Name	Cat. No.
Human IgG Control(Whole Molecule), Purified	A01006
Mouse IgG control (Whole Molecule), Purified	A01007
Rabbit IgG Control (Whole Molecule), Purified	A01008
Goat IgG Control (Whole Molecule), Purified	A01009
Chicken IgY Control (Whole Molecule), Purified	A01010

Secondary antibodies

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

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5. Troubleshooting Guide

Problem	Possible Cause	Solution
High background	Membrane contamination	Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags and trays for incubations.
	Blocking of non-specific binding might be absent or insufficient	Increase the blocking incubation period and consider changing blocking agent, such as 3% BSA.
	Primary antibody concentration may be too high	Optimize the primary antibody concentration.
	Secondary antibody concentration may be too high	Optimize the secondary antibody concentration.
	Insufficient washing	Increase number of washes and buffer volume.
	Exposure time for film is too long	Reduce exposure time.
Additional band	Too much protein per lane	Reduce the amount of total protein loaded on gel.
	Protein target may form multimers	Boil the sample for 10 minutes rather than 5 minutes to disrupt multimers.
	Antigen non-specific bind to IgG (primary antibody or secondary antibody)	Run WB assay with isotype control.
	Protein is degraded	Prepare protein sample again. Minimize freeze/thaw cycles of sample. Add protease inhibitors to sample before storage. Make fresh samples.
	Protein variants	Many factors can alter a protein molecular weight and include post-translational modifications (such as glycosylation), protein processing (cleavage from a pro-form to a mature form), isoforms from alternative splicing, multimer formation, and amino acid charge. To confirm specificity, use a control such as recombinant protein or overexpression lysate, a downregulated knockdown/knockout lysate, a peptide competition of the

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		primary antibody, or a treatment known to effect the expression of the protein.
	Non-specific binding of primary antibody.	Decrease the antibody concentration and/or the incubation period.
	Non-specific binding of secondary antibody.	Decrease the concentration. Run a secondary antibody control (without the primary antibody). If bands develop, choose an alternative secondary antibody. Increase NaCl concentration in buffer used for antibody dilution and wash steps (recommended range 0.15 M - 0.5 M).
	Secondary antibody reacting with the blocking reagent	Run a secondary control without primary antibody. If bands still occur, use new secondary antibody.
	Contamination of reagents	Check buffers for particulate or bacterial contamination. Make fresh reagents. When washing buffer was polluted, use new washing buffer. Increase washing times.
Weak signal	Incomplete transfer	Optimize transfer time. High MW protein may require more time for transfer.
	Non-fat dry milk prevent antibody binding	Reduce the milk percentage in Block Buffer or use 1% BSA to replace milk.
	Insufficient antibody	Increase concentration of primary or secondary antibody, optimizing for best performance. Increase antibody incubation time.
	Primary or secondary antibody lost reactivity	Use fresh antibody.
	Something wrong with ECL reagents	Use new ECL reagents.
	The amount of loading protein is insufficient.	Load at least 20-50 μg protein per lane. Use protease inhibitors. Run the recommended positive control.
No signal	Small proteins	Reduce transfer time. Use a high percentage SDS-PAGE gel.

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	Large proteins	Increase transfer time. Use a low percentage SDS-PAGE gel.
	Protein of interest is not abundantly present	Use an enrichment step to maximize the signal (e.g. Enrichment of the antigen by fractionation or by immunoprecipitation should be considered, etc.). Use alternative expression system or optimize protein expression system. Use a positive control lysate known to express the target protein, an overexpression lysate, or a recombinant target protein.
	Poor transfer of protein to membrane	Check the transfer with a reversible stain such as Ponceau S; check that the transfer was not performed the wrong way
	Primary antibody too dilute	Increase the concentration of the antibody
	Over-use of the primary antibody	Use fresh antibody as the effective concentration is lowered upon each re-use.
	Secondary antibody too dilute	Increase the concentration of the antibody
	Old antibody	If antibody is expired or past manufacturer warranty, purchase fresh antibody.
	Secondary antibody inhibited by sodium azide	Do not use sodium azide together with HRP-conjugated antibodies.
	Excessive washing	Do not over wash the membrane.

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