Western Blot and chemiluminescent immunodetection of rho1D4-tagged proteins

Overview

This protocol describes the blotting of proteins from an SDS-PAGE gel onto western blot membranes, and the subsequent detection of rho1D4-tagged proteins using the Cube Rho1D4 antibody and chemoluminescence detection reagents. The Rho1D4 antibody specifically recognizes the epitope TETSQVAPA.
Note that this protocol was optimized for this particular antibody. Other antibodies might require different buffers, antibody dilutions, and incubation times.

Please refer to the dedicated protocols for detection of his-tagged and GST-tagged proteins. All our protocols are available for free download at www.cube-biotech/protocols.

Please contact us at contact@cube-biotech.com if you have questions or need assistance optimizing a protocol for your application.

Equipment

- ☐ Western Blot equipment
- ☐ Micropipetting tips
- ☐ Shaking device (room temperature)
- ☐ 50 mL Falcon Tube or plastic box that fits the size of the western blot membrane
- ☐ Chemiluminescence detector instrument
- ☐ Optional: Plastic bag sealer

Materials

- ☐ Rho1D4 antibody (Cube cat.no 40020)
- ☐ HRP-coupled goat anti-mouse secondary antibody (e.g. Dianova 115-035-003)
- ☐ Sodium chloride (NaCl)
- ☐ Tris base
- ☐ Sodium dodecyl sulfate (SDS)
- ☐ Glycine
- ☐ Methanol
- ☐ Blot paper (e.g. Whatman)
- ☐ Western blot membrane (nitrocellulose)
- ☐ Tween 20 (e.g. Cube cat. 61135)
- ☐ Triton X-100
- ☐ Hydrochloric acid (HCl)
- ☐ Ponceau S dye
- ☐ Acetic acid
- ☐ Milk powder
- ☐ Luminol (3-aminophthalhydrazide)
- ☐ p-Cumaric acid (trans-4-hydroxycinnamic acid)
- ☐ Hydrogen peroxide
- ☐ Optional: sealable plastic bag that fits the size of the membrane

Solutions and buffers

Western Blot Transfer Buffer (1000 mL)

Component	Final concentration	Stock	Amount needed for solution
Methanol	20 % (v/v)	100 % (v/v)	200 mL
Tris base	25 mM	Solid powder, 121.14 g/mol	3.02 g
Glycine	192 mM	Solid powder, 75,07 g/mol	14.42 g
SDS	0.037 % (w/v)	10 % (w/v) 10 g in 100 mL	3.7 mL
Instructions: Mix all components and fill up volume to 900 mL with double distilled water. Set pH to 8.0 using HCl and fill up to 1000 mL.			

Ponceau S solution (100 mL)

Component	Final concentration	Stock concentration	Amount needed for solution
Ponceau S	0.5 % (w/v)	powder	0.5 g
Acetic acid	1 % (v/v)	100%	1 mL
Instructions: Mix all components and fill volume up to 100 mL using double distilled water.			

TBS Buffer (500 mL)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaCl	150 mM	58.44	5 M	146.1 g/500 mL	15 mL
Tris base, pH 7.0	50 mM	121.14	1 M	60.57 g/500 mL Set pH to 7.5 using HCl	25 mL
Instructions: Mix all components and fill volume up to 500 mL using double distilled water.					

Blocking Buffer (100 mL)

3% (w/v) milk powder
Instructions: Dissolve 3 g of milk powder in 100 ml TBS buffer.

TBS-TT Buffer (200 mL)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
NaCl	150 mM	58.44	5 M	146.1 g/500 mL	15 mL
Tris base, pH 7.0	50 mM	121.14	1 M	60.57 g/500 mL Set pH to 7.5 using HCl	25 mL
Tween 20	0.05% (v/v)		100%		0.1 mL
Triton X-100	0.05% (v/v)		100%		0.1 mL
Instructions: Mix all components and fill volume up to 500 mL using double distilled water. Alternatively, take 200 ml TBS buffer and add Tween 20 and Triton X-100.					

CL 1 (Chemiluminescence Detection Solution 1) (50 mL)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris base, pH 8.5	100 mM	121.14	1 M	60.57 g/500 mL Set pH to 8.5 using HCl	5 mL
p-Cumaric acid	4 µM	164.16	9 mM	0.15 g in 10 mL DMSO Store as 220 µL aliquot at -20°C	220 µL
Luminol	2.48 mM	177.16	248 mM	0.44 g in 10 mL DMSO Store as 500 µL aliquot at -20°C	500 µL
Instructions: Mix all components and fill volume up to 50 mL using double distilled water.					

CL 2 (Chemiluminescence Detection Solution 2) (50 mL)

Component	Final concentration	Molecular weight (g/mol)	Stock concentration	Amount needed for stock	Stock needed for buffer
Tris base, pH 8.5	100 mM	121.14	1 M	60.57 g/500 mL Set pH to 8.5 using HCl	5 mL
H_2O_2	0.02%		30 % (v/v)		37 µL
Instructions: Mix all components and fill volume up to 50 mL using double distilled water.					

Procedure

1. Perform SDS-PAGE.
2. Set up a western blot sandwich in a semi-dry blotter as follows:
   - 3 layers blotting paper
   - Nitrocellulose blot membrane
   - SDS-PAGE Gel
   - 3 layers blotting paper

   Note: Most proteins, including membrane proteins, blot well on nitrocellulose membranes. Alternatively, PVDF membranes can be used.
3. Place a heavy weight on the blot chamber. Run the western blot at 400 mA constant electric current for 30-60 min.

   Note: It is important to adjust the blotting time to the protein of interest. Allow 1 min per kDa of protein and add 5-10 min (e.g. blotting time for a 30 kDa protein would be 35-40 min). To ensure that you are not losing any protein, put two membranes on top of each other.
4. Dismantle the western blot sandwich and stain the membrane with Ponceau solution. Remove excess staining solution with double distilled water and check for successful protein transfer.
5. Place membrane in a 50 mL Falcon tube or a suitably sized plastic box and place it on a shaker. Wash membrane twice for 10 min each time with 10 mL TBS buffer.

   Note: Perform all incubation steps at room temperature (15-25°C)
6. Incubate for 1 h in blocking buffer.
7. Wash twice for 10 min each with 10 mL TBS-TT buffer.
8. Wash for 10 min with 10 mL TBS buffer.
9. Dilute Rho1D4 antibody 1:1000 in blocking buffer and incubate the membrane in the diluted antibody solution for 1 h.

   Note: Depending on the antibody, other dilutions of primary and secondary antibodies might be required, e.g. 1:2000 - 1:5000.
10. Wash twice for 10 min each with 10 mL TBS-TT buffer.
11. Wash for 10 min with 10 mL TBS buffer.
12. Dilute the secondary antibody 1:10.000 in blocking buffer or according to the manufacturers' instructions. Incubate the membrane in the diluted secondary antibody solution for 1 h.

    Note: To reduce the amount of antibody solution required, the membrane can be sealed in a plastic bag.
13. Wash twice for 10 min each with 10 mL TBS-TT buffer.
14. Wash for 10 min with 10 mL TBS buffer.
15. Mix 5 mL CL solution 1 and 5 mL CL solution 2 and apply them to the blot.
16. Detect chemiluminescence signal immediately in the imager.