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Western Blot

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Protocol status: Working
We use this protocol and it's working.

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Abstract

Western Blot - protein expression assessment

Materials

RECOMMENDED SOLUTIONS
Gel casting Reagents

Acrylamide/Bis (30%)
Acrylamide (29.2g/100 mL) – 87.60 g
N'N'-bis-methylene-acrylamide– 2.40 g
diH_2O– to 300 mL
Filter and store at 4ºC in the dark (30 days).

1.5M Tris-HCl pH 8.8 (150 mL)
Tris base (18.15g/ 100 mL) – 23.23 g
diH_2O – 80 mL
Adjust to pH 8.8 with 6N HCl.
diH_2O – to 150 mL
Store at 4ºC.

0.5M Tris-HCl pH 6.8 (100 mL)
Trisbase – 6.00 g
diH_2O – 60 mL
Adjust to pH 6.87 with 6N HCl.
diH_2O – to 100 mL
Store at 4ºC.

10% SDS (100 mL)
SDS– 10.00 g
diH_2O– 690 mL Dissolvewith gentle stirring.
diH_2O– to 100 mL

10% APS (fresh daily)
Ammoniumpersulfate – 0.10 g
diH_2O– 1 mL

Running, transfer and blocking buffers

Laemmli 2X buffer/loading buffer
– 4% SDS
– 10%2-mercaptoethanol
– 20% glycerol
– 0.004% bromophenolblue
– 0.125 M Tris-HCl

Check the pH and adjust to 6.8

Running buffer (Tris-Glycine/SDS)
– 25 mM Tris base
– 190 mM glycine
– 0.1% SDS

Check the pH and adjust to 8.3

Transfer buffer (wet)
– 25 mM Tris base
– 190 mM glycine
– 20% methanol

Check the pH and adjust to 8.3. For proteins larger than 80 kDa, we recommend that SDS is included at a final concentration of 0.1%.

Blocking buffer
1–5% milk or BSA
(bovine serum albumin). Add to TBSTw buffer. Mix well and filter. Failure to filter can lead to spotting, where tiny dark grains will contaminate the blot during color development.

Tris buffered saline (TBS) Stock 10´ pH 7,6 (Vol. final 1L)
- 24,2g Tris (200 mM)
- 80g NaCl
- 38 ml HCl 1M

TBS-Tw – Blocking, incubation with antibodies and wash
- 100ml TBS 10´
- 1ml Tween
- diH_2O – until 1L

Before start

Before start please check the antibody datasheet provided by the manufacturer.

Sample preparation

1 Protein Quantification - BCA, Quibit measurment

2 After determining how much protein to load, add an equal volume of **2X Laemmli sample** buffer, in order to reduce and denature the samples, unless the online antibody datasheet indicates that non-reducing and non-denaturing conditions should be used.

2.1 Do a spindown for 10 s.

3 Boil each cell lysate in sample buffer at 70°C for 10 min. Place the samples at room temperature and wait for a few seconds.

Loading and running the gel eletrophoresis

4 Gel loading and eletrophoresis

4.1 Assemble the electrophoresis:

4.2 a. Remove the comb from the gels

4.3 b. Fill the inner and outer buffer chambers with running buffer. Fill the inner buffer chamber of each core with 200 mL of Running buffer.

4.4 Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker (5-10µL).

4.5 Connect the electrophoresis cell to the power supply and perform electrophoresis.

4.6 Run the gel at 150V for 1h 15min, or until the blue reaches the end of the gel.

4.7 After electrophoresis is complete, turn the power supply off and disconnect the electrical leads. Pop open the gel cassettes and remove the gel by floating it off the plate into Transfer buffer.

Transfer

5 Prepare the transfer buffer according to the manufacturer's instructions.

6 The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 30sec and wash heavily with water. Rinse with transfer buffer before preparing the stack.

7 Prepare the sandwich according to the manufacturer's instructions. Make sure you wet the BioRad white paper filter in transfer buffer before you prepare the sandwich. IMPORTANT: The gel should be on the black side of the sandwich and the membranes should be on the red side. Be sure you remove the air bubbles with a BioRad roller.

8 Start the run at 370 Å for 1h30min in an ice cold bath (put a magnet inside the cassette before starting the transfer and surround the cassette with ice after closing).

Antibody staining

9 Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step. If necessary, use Ponceau S during 30 min (under slow agitation). IMPORTANT: After 30 min wash the membrane with H2O three times, 5 min each (under mid-fast agitation). Take a print of the membrane. If necessary, prepare a solution of 0.1M NaOH in H2O in order to remove the remaining Ponceau. Wash the membrane with H2O to remove the NaOH solution.

10 Block the membrane for 1 h at room temperature using blocking buffer, Milk 5% (under slow agitation).

11 Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer, Milk 5%. The recommend condition is overnight incubation at 4°C (under slow agitation); other conditions can be optimized.

12 Wash the membrane three times with TBST, 10 min each (under mid-fast agitation).

13 Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer, Milk 5%, at room temperature for 1 h (under slow agitation).

14 Wash the membrane three times with TBST, 10 min each (under mid-fast agitation).

15 For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.

16 Acquire image using darkroom development techniques for chemiluminescence.