Western Blot Analysis

Section of Cancer Genomics, Genetics Branch, NCI
National Institutes of Health

Reagents

- 2-Mercaptoethanol (Sigma, M6250)
- 96 well plates (Corning, 3596)
- BCA Protein Assay Reagent Kit (Pierce, Cat. 23225)
- Blot module for XCELLII (Invitrogen, Cat. EI9051)
- Bromophenol blue (Fisher, Cat. B-392)
- Cell scrapers (Sarstedt, 83.1830)
- Glycerol (Invitrogen, Cat. 15514-011)
- Hyperfilm ECL (VWR scientific, Cat. 95017-655)
- Methanol (Mallinckrodt, Cat. 3016-02)
- NaCl (Mallinckrodt, Cat. 7581)
- Non-fat Dry Milk (NFDM) (Apex, Cat. 20-241 or Carnation brand, at grocery stores)
- Nupage MES SDS Running Buffer (20X) (Invitrogen, Cat. NP0002)
- Nupage precast gels (Invitrogen, Cat. NP0321BOX (4-12%) or Invitrogen, Cat. NP0301BOX (10%))
- Nupage LDS sample buffer 4X (Invitrogen, Cat. NP0008)
- Nupage Transfer Buffer (20X) (Invitrogen, Cat. NP0006-1)
- Phosphate Buffered Saline (PBS), 1X (Gibco, 10010-023)
- Plastic bag pouches (Kapak, 404)
- PMSF (Sigma, P7626)
- PVDF Membrane (IMMOBILON-P roll) Millipore, Cat. IPVH00010
- RIPA buffer (10x) (Cell Signaling, 9806**)**
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- SDS (Biorad, Cat. 161-0301)
- SDS PAGE apparatus (SURELOCK XCELL) (Invitrogen, Cat. EI0001)
- Supersignal West Pico chemiluminescent substrate (Thermo Scientific, 34080)
- Tris 1 M, pH 7.5, (Quality Biological, Inc., Cat.351-006-100)
- Tris 2 M, pH 6.8, (Quality Biological, Inc., Cat.351-091-101
- Tween-20 (Sigma, Cat. P1379)

Preparation

RIPA Buffer (1X) - 1ml:
RIPA buffer (10X) -100µl
PMSF -10µl
dH_2O -890µl
Cool on ice

BSA Standards:
Make standards by diluting supplied BSA stock solution [2 mg/ml] with RIPA buffer (1X) following the chart (Table 1).
Notes: if another lysis buffer than RIPA was used to prepare cell lysates, use that same lysis buffer to prepare dilutions. Mix well by vortexing 10-15 sec. before taking volumes from tubes and at the end when the standards are ready.

Table 1. BSA standards preparation.

Tube Name	Volume of BSA	From Tube	Volume Lysis Buffer (RIPA (1X))	Final [BSA]
A	30.0 µl of	BSA stock	0 µl	2000 µg/ml
B	37.5 µl of	BSA stock	12.5 µl	1500 µg/ml
C	32.5 µl of	BSA stock	32.5 µl	1000 µg/ml
D	17.5 µl of	B	17.5 µl	750 µg/ml
E	32.5 µl of	C	32.5 µl	500 µg/ml
F	32.5 µl of	E	32.5 µl	250 µg/ml
G	32.5 µl of	F	32.5 µl	125 µg/ml
H	10.0 µl of	G	40.0 µl	25 µg/ml
I	0 µl of	-	40.0 µl	0 µg/ml=Blank

Laemmli buffer (4X) (sample buffer alternative to Nupage LDS sample buffer) - 10ml:
SDS, 0.8g - final [8%]
Tris 2M (pH6.8), 1.25ml - final [250mM]
Glycerol, 4ml - final [40%]
2-Mercaptoethanol, 2ml - final [20%]
Bromophenol blue, 0.001g - final [0.01%]
Complete to 10ml with dH_2O. Mix well to dissolve. Aliquot and store at -20C.

Running Buffer (1X) - 1L:
Running Buffer (20X), 50 ml
Milli-Q Water, 950 ml

Transfer Buffer (1X) - 1L:
Transfer Buffer (20X), 50ml
Methanol, 200ml
Milli-Q water, 750ml

TBS (10X) - 500ml:
NaCl, 43.83g - final [1.5 M]
Tris 1M (pH 7.5), 100 ml - final [0.2 M]
Bring volume to 500ml with dH_2O.

TBS-T 0.1% (1X) - 2L:
TBS (10X), 200 ml
Milli-Q Water, 1800 ml
Tween-20, 2ml

LDS sample buffer 1X - 1ml:
NuPage LDS sample buffer (4X), 250µl
RIPA buffer (1X), 750µl

Laemmli buffer (1X) - 1ml:
Laemmli buffer (4X), 250µl
RIPA buffer (1X), 750µl

Preparation of protein extracts form cell cultures

1. Wash cells once with 5ml room temp PBS.

2. After aspirating PBS put the cells on ice.

Important: keep the cells and lysates on ice from this point on.

3. Put 350µl of cold RIPA buffer (1X) on each plate of 100mm or T75 flask.
Note: the volume of RIPA can be adjusted if using other plates or depending of the expected protein concentration.

4. Leave on ice for 10 min.

5. Scrape cells with a cell scraper and collect them in a microcentrifuge tube. Keep on ice.
Notes: a different cell scraper should be used (or the same one washed) for each sample to prevent cross contamination. At this point, samples can be stored at -20˚C until further processing (max 1 month).

6. Sonicate at 40-50% intensity 3 X 10 sec. Let sample cool on ice for at least 10 sec. between each sonication.
Note: clean sonicator head between each sample with dH_2O to prevent cross contamination.

7. Centrifuge samples at 20,000g-40,000g for 30 min at 4˚C in ultracentrifuge.

8. Transfer supernatants containing soluble proteins to new tubes. Keep on ice.
Notes: by using the same volume for each sample it is easier to calculate the amount of sample buffer to add later. At this point, samples can be stored at -20˚C until further processing (max 1 month).

Protein quantification

1. If frozen, thaw cell lysates on ice.

2. Use Pierce BCA Protein Assay Reagent Kit, following directions inside.

3. For each cell lysate, make 60µl dilutions 1:5 and 1:10 with lysis buffer (RIPA (1X)).
(1:5 dilutions = 12µl sample + 48µl lysis buffer; 1:10 dilutions = 6µl sample + 54µl lysis buffer).

4. Put 25 µl of each standard and duplicates for each diluted samples (2 wells for 1:5 and 2 wells for 1:10) in a clear bottom 96 well plate.
Note: if sample size is limited use only 10 µl for each standards and samples.

5. Calculate the amount of working solution (WS) needed following the formula:
((9 standards + (# of samples X 4 duplicates)) X 200µl). Make WS by combining 50:1 Soln A:B (example: 2500 µl Soln. A + 50 µl Soln.B) from BCA Protein Assay Reagent Kit. Mix well.

6. Add 200µl WS in all wells for standards and samples.

7. Incubate in the dark at 37˚C for 30 min.

8. Read absorption at 562nm with a plate reader.
Note: After protein quantification if samples concentrations need to be adjusted use Lysis buffer (RIPA (1X)) to do so.

9. Add 1/3 the volume of sample (example: if sample volume 300µl use 100 µl) Nupage LDS sample buffer (4x) (or alternatively Laemmli buffer (4X)) to samples. Final concentrations of sample buffer should be 1X. Vortex well.
Note: samples in sample buffer can be store for long term at -20 ℃.

SDS PAGE, Blotting and Chemiluminescence

1. If needed thaw samples on thermomixer (37°C).

2. Prepare desired total amount of protein to be loaded in equal total volumes for each sample. Adjust with LDS sample buffer (1X) (or Laemmli buffer (1X)).

3. Load samples and protein markers (SeePage blue and MagigMarker).

4. Run gels in Running buffer (1X) at 200V for 40 to 90 min. depending on gel type and protein to be resolved.
Note: bigger protein need more time to be resolved. Adjust time with the help of the protein marker.

5. Transfer to PVDF membrane:
Pre-wet PVDF in 100% MeOH for few seconds and equilibrate in Transfer Buffer (1X) for ~5 min. Set-up transfer apparatus (see Nupage Blot Module manual) and transfer in Transfer buffer (1X) at 30V for 1h.
Note: Transfer can be done for 40 min. for small proteins or 1h30 for large proteins.

6. Block membrane in 5% non fat dry milk (NFDM)/TBS-T (1X) for 30 min. on shaker.
Note: It is also possible to block o/n at 4°C.

7. Put membrane with 5 ml-10ml primary antibody (Ab') diluted in 5% NFDM/TBS-T (1X) in a sealed bag on rocker o/n at 4°C.
Note: Some antibodies can be done 2h at room temperature (RT).

8. Rinse membrane 1X with TBS-T (1X) and wash 2 x 10 min with TBS-T (1X).

9. Put membrane with 20ml-30ml secondary antibody (Ab") HRP-conjugated diluted in 5% NFDM/TBS-T (1X) for 60 min. at RT with shaking.

10. Rinse membrane 1X with TBS-T (1X) followed by 2 x 10 min wash with TBS-T (1X) and rinse 1X with TBS (1X).

11. Mix equal volumes of Supersignal West Pico chemiluminescent substrate solutions A & B (4ml total detection mixture/membrane).

12. Put the membrane in a small clean plate and add the detection mixture.
Note: If it is known that the signal if strong or need to save detection reagent: remove excess TBS from membrane and put it between two part of a plastic bag. Add detection mixture (0.5-1ml / membrane) and put back plastic bag to cover it (make sure there is no bubbles by removing them gently through the plastic bag with a folded paper).

13. Incubate for 5 min at RT.

14. Drain off detection solution and put membrane between two parts of a new clear plastic bag, again removing air bubbles.

15. Expose membranes to Hyperfilm ECL for desired length of time.
Note: test one exposition of 2-3 min. and one of 10-20 sec. on the same film by turning the film between expositions. Depending on the result adjust time of exposition.