Western Blot Protocol

(updated on 05/20/14)

Required Solutions

10x PBS (1L)
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4 or 22 g $Na_2HPO_47H_2O$
2.4 g KH_2PO_4 or 2 g KH_2PO4
Adjust pH to 7.4
Autoclave

PBST
1xPBS
0.1% Tween-20

TBST
37 mM NaCl
20 mM Tris
0.1% Tween-20
pH 7.6

Buffer C (4ºC)
20 mM Hepes-KOH pH 7.9 (from 0.5 M stock)
0.42 M KCl (from 3 M stock)
25% glycerol (from 80% stock)
0.1 mM EDTA (from 0.5 M stock pH 8.0)
5 mM MgCl2 (from 1 M stock)
0.2% NP40 (from 10% stock)
Use autoclaved stocks for all ingredients.
Store at 4ºC

Add right before use to 10 ml:
10 **µ**l 1 M DTT (store aliquots at -20ºC, thaw once)
50 **µ**l 100 mM PMSF (in iso-propanol -20ºC)
1 **µ**l 10 mg/ml Leupeptin (-20ºC)
1 **µ**l 10 mg/ml Aprotinin (-20ºC)
10 **µ**l 1 mg/ml Pepstatin (-20ºC)
Or, replace the last three protease inhibitors with one mini Roche protease inhibitor tablet (# 11 836 153001) and add DTT and PMSF as above.

For looking at phosphorylated proteins, add the following phosphatase inhibitors to Buffer C:

Na-beta-glycerophosphate to 50 mM (stock 1 M)
NaF to 1 mM (stock 0.4 M)
Na-ortho-vanadate to 1 mM (stock 0.1 M)

Buffer D
20 mM Hepes-KOH pH 7.9
100 mM KCl
25% glycerol
0.1 mM EDTA (cold)

Add right before use, for 250 ml:
250 **µ**l 1 M DTT
1.25 ml 100 mM PMSF

10x Ponceau S
Ponceau S 2% (w/v)
TCA 30% (w/v)
Dilute to 1x before use (can be re-used many times).

2x Laemmli Sample Buffer v.1 (12 ml)

	f.c (1x)
4.4 ml 0.5 M Tris (pH 6.8)	92 mM
4.4 ml Glycerol	18%
2.2 ml 20% SDS	1.8%
0.5 ml 1% Bromophenol Blue	0.02%
0.5 ml Beta-ME (goes off even at -20ºC)	2%

Aliquot and store at -20ºC

2x Laemmli Sample Buffer v.2 (10 ml)

	f.c (1x)
2 ml 0.5 M Tris (pH 6.8)	50 mM
2 ml Glycerol	10 %
4 ml 10% SDS	2%
0.2 ml 1% Bromophenol Blue	0.01%
0.5 ml Beta-ME (goes off even at -20ºC)	2.5%
1.65 ml H_2O	

Aliquot and store at -20ºC

10x Running Buffer (1L)
30.3 g (0.25 M) Tris Base
144 g (1.92 M) Glycine

10 g (1%) SDS or appropriate for concentrated stock

Dilute 1:10 with MillliQ water.
Adjust pH to 8.3 (should be close)

10x Transfer Buffer Stock (1L)
30.3 g Tris Base
144 g Glycine

Transfer Buffer (1L)
100 ml 10x stock
200 ml (20%) Methanol
0.02% SDS

Can be stored at 4ºC for a few weeks.
Don't reuse.

SDS-PAGE
Novex 8% or 10% Tris-Glycine mini Gel (1.5mm, Invitrogen, don't use after expiration dates)

Transfer
Mini Trans-Blot Cell (Bio-Rad)

Nitrocellulose Transfer Membranes
Protran (Perkin Elmer)

Immobilon-P (Millipore) for ATM and delangin

Signal Detection
ECL (GE Healthcare)
BioMax XAR Film (KODAK)

Laemmli Sample Buffer Cell Extracts

Cells can be directly lysed into 2x Laemmli Sample buffer (v.1 or v.2) as follows (not for ubiquitination):

1. Harvest cells by trypsinization, suspend in media with serum and count cells. Keep everything cold after this step.

2. Spin cells in media for 5 min at 1,000 rpm at 4ºC.

3. Aspirate off media and resuspend pellet in 1 ml cold 1x PBS.

4. Transfer 10^6 cells to a 1.5 ml eppendorf tube and spin in Microcentrifuge 5415 for 4 min at 4,000 rpm at 4ºC. Aspriate off PBS. 1 min 6,000 rpm take last bit off.

5. Suspend cells in 100 **µ**l l 2x Laemmli Sample Buffer.

6. Shear DNA through a 28 1/2-gauge insulin syringe 10 times or more, or sonicate in Cold Room (like ChIP).

7. Heat to 100ºC for 5 minutes before loading.

8. Continue to SDS-PAGE or store the samples in the freezer.

Note
For Phospho-proteins (e.g. Chk1-P) cells can also be scraped into 2x Laemmli Sample buffer (v.1 or v.2) with a cell Lifter but this procedure does not allow for a cell count.

Buffer C Whole Cell Extracts

1. Trypsinize cells (minimally one 6 well dish well or one 10 cm plate; keep cells subconfluent). Keep everything cold after this step; chill tubes and solutions on ice.
2. Wash once with medium containing serum (to inactivate the trypsin), 2 times with cold 1x PBS. Cells can be counted in one of these steps.
3. Resuspend cell pellet in 5x the pellet volume of buffer C (4ºC).
4. Incubate on ice for 30 min with occasional mixing (flicking the tube).
5. Spin 15K RPM (microfuge at max setting), 10 min at 4ºC.
6. Set some supernatant aside for a Bradford assay and quick freeze the sample or aliquots on dry ice and store at -80ºC.
7. Optional: Dialyze to 50 vol buffer D for 2 hours at 4ºC before freezing but in this case, spin dialyzed sample for 5 min in microfuge at 4ºC and transfer the supernatant to a new tube before freezing.

Note: Shelterin proteins are in extract, but 53BP1 is not.

SDS-PAGE and Western Blot

1. Load 10 **µ**l (10^5 cells) per well for a 10 well gel.

Note: For a 10 well-comb gel, 10^5 cells for most proteins, $2x10^5$ cells for POT1, Chk1-P,

CtIP, BLM etc.

2. Run on an SDS-PAGE minigel (Invitrogen) with 75V until the blue front is at the bottom of the gel (about 3 hours)

3. While running the gels, prepare Transfer buffer and keep it at 4ºC.

4. Transfer: Blot onto a nitrocellulose membrane. Pre-wet materials in transfer buffer. Stack in the following order:
• case (clear side)
• sponge
• Whatman paper
• membrane
• gel
• Whatman paper
• sponge
• case (black side)

Place in the transfer apparatus with black side facing black.
Bio-Rad Mini-Gel Box Electrotransfer (in the Cold Room):
Use ice-pack to cool down the apparatus

- 90 V x 1 hr
- 70 V x 2 hr
- 75 -100 mA, O/N

Note:
For ATM, 120 mA O/N in the Cold Room.
For smaller size of proteins (<37kD), use less V or A with shorter transfer time.

For POT1 and TPP1, refer to the Guanidine protocol

5. (optional) Stain with 1x Ponceau S for a minute and destain in acidified H_2O (2.3 L of ddH2O+ 4 ml HCl). Wrap in plastic wrap and Xerox. Rinse in 1x PBS.

6. Block the membrane for 30 min in 20-30 ml PBST + 5% non-fat dry milk in a small container on a shaker.

7. Incubate with primary antibody (see Antibodies for Shelterin Components) diluted in 2 ml 1% milk/PBST. Incubate RT 1 to 4 hours or o/n at 4ºC (e.g. POT1) in a Hybri bag on a nutator. Membranes can be stacked back to back in one bag.
(Store primary antibody with NaN3 1:500 for re-use at 4ºC)

Note: For Chk1 and Chk2 blots, use 0.1% milk/PBST
For Phospho proteins (e.g, Chk1-P and Chk2-P), use TBST and 5% BSA (for primary antibody).

8. Wash 3 x for 5 min in ~50 ml PBST at RT in a small container on a shaker.

9. Incubate with secondary antibody for 30 min to 1 hr at RT.
Use HRPO conjugated anti mouse or rabbit secondary antibodies at a dilution of 1:5000 (1:10000 for tublins) in a box or bag.

10. Wash 3 x 5 min to 10 min each in ~50 ml PBST at RT (for goat secondary antibody, wash 4x 10 min)

11. Detect protein with ECL kit (2 ml/membrane). In a separate tube, mix black and white ECL solutions in a 1:1 ratio. Then aliquot solution onto membranes and wait for 1 minute. Drain the ECL, wrap in plastic and expose to film.

Notes:
For ATM and p19ARF, protein must be blotted onto PVDF membranes.
For big size of protein such us ATM, home made 5% gels work better.

Guanidine protocol for POT1 and TPP1

(TPP1 can be detected without Guanidine treatment. but it reduces backgrounds.)

Immediately after transfer:

1. Wash blot for 30 min at RT in 6 M AC buffer.
2. Wash 30 min at RT in 3 M AC buffer.
3. Wash 30 min at 4ºC in 1M AC buffer.
4. Wash 30 min at 4ºC 0.1 M AC buffer.
5. Wash overnight in AC buffer at 4ºC.
6. Proceed with Western protocol, from blocking.

AC Buffers:
Make 8 M stock of Guanidine-HCl

Mix

50% glycerol	25mL
5M NaCl	2.5mL
1M Tris pH7.5	2.5mL
0.5M EDTA	250ul
10% Tween-20	1.25 mL
1M DTT	125 ul
Dry Milk	2.5g

AC buffer	6M	3M	1M	0.1M	0M
8M Guanidine-HCl	18.7mL	9.4mL	3.1mL	310uL	0mL
Mix	6.3mL	6.3mL	6.3mL	6.3mL	6.3mL
H_2O	0mL	9.4mL	15.6mL	18.4mL	18.7mL
	RT	RT	4ºC	4ºC	4ºC