Western Blot Protocol

Required Solutions

10x PBS (1L)
80 g NaCl
2 g KCl
14.4 g Na₂HPO₄ or 22 g Na₂HPO₄·7H₂O
2.4 g KH₂PO₄ or 2 g KH₂PO₄
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 (4°C)
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 4°C

Add right before use to 10 ml:
10 µl 1 M DTT (store aliquots at -20°C, thaw once)
50 µl 100 mM PMSF (in iso-propanol -20°C)
1 µl 10 mg/ml Leupeptin (-20°C)
1 µl 10 mg/ml Aprotinin (-20°C)
10 µl 1 mg/ml Pepstatin (-20°C)
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 µl 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)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 ml 0.5 M Tris (pH 6.8)</td>
<td>92 mM</td>
</tr>
<tr>
<td>4.4 ml Glycerol</td>
<td>18%</td>
</tr>
<tr>
<td>2.2 ml 20% SDS</td>
<td>1.8%</td>
</tr>
<tr>
<td>0.5 ml 1% Bromophenol Blue</td>
<td>0.02%</td>
</tr>
<tr>
<td>0.5 ml Beta-ME (goes off even at -20ºC) 2%</td>
<td></td>
</tr>
</tbody>
</table>

Aliquot and store at -20ºC

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml 0.5 M Tris (pH 6.8)</td>
<td>50 mM</td>
</tr>
<tr>
<td>2 ml Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>4 ml 10% SDS</td>
<td>2%</td>
</tr>
<tr>
<td>0.2 ml 1% Bromophenol Blue</td>
<td>0.01%</td>
</tr>
<tr>
<td>0.5 ml Beta-ME (goes off even at -20ºC) 2.5%</td>
<td></td>
</tr>
<tr>
<td>1.65 ml H₂O</td>
<td></td>
</tr>
</tbody>
</table>

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 MilliQ 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 4°C 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 4°C.

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 4°C. Aspirate off PBS. 1 min 6,000 rpm take last bit off.

5. Suspend cells in 100 µl 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 100°C 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.

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**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 (4°C).

4. Incubate on ice for 30 min with occasional mixing (flicking the tube).

5. Spin 15K RPM (microfuge at max setting), 10 min at 4°C.

6. Set some supernatant aside for a Bradford assay and quick freeze the sample or aliquots on dry ice and store at -80°C.

7. Optional: Dialyze to 50 vol buffer D for 2 hours at 4°C before freezing but in this case, spin dialyzed sample for 5 min in microfuge at 4°C and transfer the supernatant to a new tube before freezing.

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

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**SDS-PAGE and Western Blot**

1. Load 10 µl ($10^5$ 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 Electrottransfer (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 H₂O (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 as 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 4°C in 1M AC buffer.
4. Wash 30 min at 4°C 0.1 M AC buffer.
5. Wash overnight in AC buffer at 4°C.
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
H₂O                0mL  9.4mL 15.6mL 18.4mL 18.7mL

RT  RT  4°C  4°C  4°C