Western Blot Protocol

Required Solutions

10x PBS
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 g KH_2PO_4
dissolve into 800 ml ddH_2O
Adjust pH to 7.4
qs to 1000 ml
autoclave
make a 1x working solution

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 μl 1 M DTT (store aliquots at -20ºC, thaw once)
50 μl 100 mM PMSF (in iso-propanol -20ºC)
1 μl 10 mg/ml Leupeptin (-20ºC)
1 μl 10 mg/ml Aprotinin (-20ºC)
10 μl 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 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).

4x Laemmli Buffer
4.4 ml 0.5 M Tris (pH 6.8)
4.4 ml Glycerol
2.2 ml 20% SDS
0.5 ml 1% Bromophenol Blue
0.5 ml Beta-ME
Aliquot and store at -20ºC. Dilute to 2x before use.

10x Running Buffer
30.3 g (0.25 M) Tris Base
144 g (1.92 M) Glycine
10 g (1%) SDS or appropriate for concentrated stock
qs 1000 ml ddH_2O
Dilute 1:10 with ddH_2O. pH will be 8.3

10x Towbin's Electrotransfer Buffer
30.3 g Tris Base
144 g Glycine
qs 1000 ml ddH_2O

1x Towbin's
100 ml 10x stock
200 ml (20%) Methanol
qs 1000 ml ddH_2O
1 ml (0.02%) 20% SDS– optional

Laemmli Buffer Cell Extracts

Cells can be directly lysed into Laemmli buffer as follows:

1. Harvest cells by trypsination and count cells. Keep everything cold after this step.
2. Spin cells in media for 5 min at 1,000 g at 4ºC.
3. Aspirate off media and resuspend pellet in 1 ml cold 1x PBS.
4. Transfer 10^6 cells to an eppendorf tube and spin in microfuge for 4 min at 4,000 g at 4ºC. Aspriate off PBS.
5. Suspend cells in 100 µl hot Laemmli buffer.
6. Heat for 5 min (80- 100ºC).
7. Shear DNA through a 28 1/2 gauge insulin syringe 5 times, or sonicate.
8. Heat to 80-100ºC for 5 minutes before loading.
9. Load 10 µl (10^5 cells) per lane.

Cells can also be scraped into Laemmli buffer with a rubber policeman 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.

SDS-PAGE Gel and Western Blot

Run the gel according to the instructions below. Refer to *SDS-PAGE Gels* on page 7 for details.

1. Measure protein concentration in duplicate using Bradford using a BSA standard curve. Run up to 70 µg/lane (expect 300-400 µg protein from a subconfluent 10 cm plate).
2. Mix extract 1:1 with Laemmli buffer and heat to 80-100ºC for 5 min (don't boil too long, proteins get destroyed).
3. Run on an SDS-PAGE minigel until the blue front is at the bottom of the gel (refer to *SDS-PAGE Gels* for gel composition).

   Bio-Rad Mini-Gel Box Running Conditions: 75 V x 3 hrs (dye about in the bottom of the gel)

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:
   - 90 V x 1 hr (use ice-pack to cool down the apparatus)
   - 70 V x 2 hr (use ice-pack to cool down the apparatus)

   Transfer at 0.5 A-Hour (0.5 A for one hour, or 0.05 A for 10 hrs.)

   For POT1 and TPP1 refer to the Detection of POT1 and TPP1 (mouse and human) protocol on page *5.*

5. Stain with 1x Ponceau S for a minute and destain in acidified H_2O (2.3 l of ddH_2O + 4 ml concentrated HCl). Wrap in plastic wrap and Xerox. Rinse in 1x PBS.
6. Block the membrane for 30 min in 20-30 ml 1x PBS + 5% non-fat dry milk + 0.1% Tween 20, in a small Tupperware dish on a shaker.
7. Incubate with primary antibody (see *Antibodies for Shelterin Components* on page *8*) diluted in 2 ml 1x PBS + 5% milk + 0.1% Tween 20. Incubate o/n at 4ºC or 4 hrs in a seal-a-meal bag on a nutator at RT. Membranes can be stacked back to back in one bag. The primary antibody mix can be re-used (store at 4ºC.)
8. Wash 3 x for 5-10 min in ~50 ml 1x PBS + 0.1% Tween 20 at RT in a small Tupperware on a shaker.
9. Incubate with secondary antibody for 30 min to 1 hr at RT in 2 ml 1x PBS + 5% milk + 0.1% Tween 20 in a seal-a-meal bag on a nutator. Use Amersham anti-mouse or rabbit HRPO conjugated at a dilution of 1:2500.
10. Wash 3 x 10 min each in ~50 ml 1x PBS + 0.1% Tween 20 at RT in a small Tupperware on a shaker.
11. Rinse with ddH_2O.
12. 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 RB, proteins must be run on a 5% gel & the 50 kD MW marker should run off gel.

Stripping/Re-probing Western Blot

1. Shake filters in 2M Glycine (pH 2.2) for 20-30 minutes.
2. Rinse the filters in 1x PBS + 0.1% Tween 20 (several changes).
3. Re-block with 1x PBS + 10% non-fat dry milk + 0.1% Tween 20. The filter can now be used for the next antibody.

Note:
Some strong signals cannot be stripped of. In such case, use the membrane for probing proteins at very different molecular weight. You can strip the membrane 2-6 times.

Detection of POT1 and TPP1 (mouse and human)

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 2 hours or overnight in AC buffer at 4ºC.
6. Proceed with Western protocol, step 6 (blocking) on page 4.

AC Buffers:
(Make 8 M stock of Guanidine.)

Guanidine-HCl	**6 M**	**3 M**	**1 M**	**0.1 M**	**AC**
Glycerol (50%)	5 ml	5 ml	5 ml	5 ml	5 ml
5 M NaCl	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml
1 M Tris 7.5	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml
0.5 M EDTA	50 µl	50 µl	50 µl	50 µl	50 µl
Tween 20 (10%)	250 µl	250 µl	250 µl	250 µl	250 µl
8 M Guanidine-HCl	18.75 ml	9.3 ml	3.1 ml	0.31 ml	---
Milk Powder	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
1 M DTT	25 µl	25 µl	25 µl	25 µl	25 µl
ddH_2O	-	10.3 ml	15.6 ml	18.4 ml	18.7 ml
Total Volume:	25 ml	25 ml	25 ml	25 ml	25 ml

***Note**: The milk in the 1 M AC buffer does not go into solution well.*

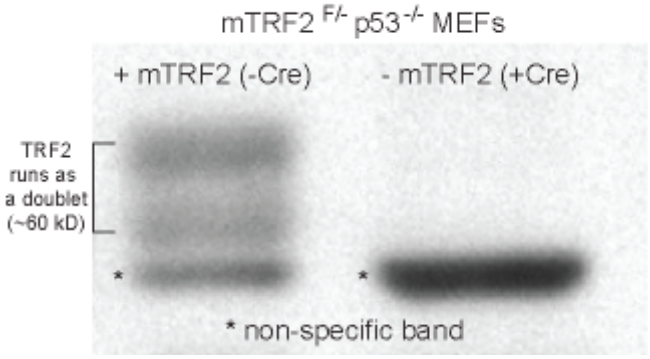
mTRF2 1254 Western Blot

The key to getting good resolution of the TRF2 band is to use a homemade gel and to run the gel slowly (65 V) until the 37 kD marker is at the bottom of the gel.

Blocking Buffer
0.1% Tween 20
5% powdered milk in 1x PBS

Incubation Buffer
0.1% Tween 20
0.1% milk in 1x PBS

1. Run gel at 65 V until the 37 kD marker is at the bottom of the gel (~3 hours).
2. Transfer the gel to nitrocellulose at 90 V for 1.5 hours (use an ice pack to cool down the apparatus) or at 100 mA overnight in the cold room.
3. (Optional) Stain blot with Ponceau to visualize bands; destain with water; make a copy on the copier (wrap in plastic wrap).
4. Block blot for 30 min at RT (in a small tupperware on a shaker).
5. Incubate blot with αmTRF2 1254 at a concentration of 1:5,000 for 2 hours at RT or overnight in the cold room on a nutator (in a seal-a-meal bag).
6. Wash blot 3 x 10 min in incubation buffer or PBST (in a small Tupperware on a shaker).
7. Incubate blot with secondary antibody (rabbit) at a concentration of 1:2,500 in incubation buffer for 30-45 min at RT on a nutator (in a seal-a-meal bag).
8. Wash the blot 3 x 10 min in incubation buffer or PBST, wash for 5 min in 1x PBS, and rinse with water (in a small Tupperware on a shaker).
9. Place blot on a piece of plastic wrap on your bench. Add ECL on top of the blot for 1 min (use equal parts of solution 1 and solution 2).
10. Shake off excess solution from the blot and wrap securely in a new piece of plastic wrap.
11. Expose blot for 1-5 min.



mTRF2 blot with 1254 antibody

SDS-PAGE Gels

6% Gel	10 ml	20 ml	30 ml	60 ml
40% Acrylamide/Bis (29:1)	1.5 ml	3 ml	4.5 ml	9 ml
1.5 M Tris (pH 8.8)	2.5 ml	5 ml	7.5 ml	15 ml
ddH_2O	5.8 ml	11.6 ml	17.4 ml	34.8 ml
10% SDS	100 µl	200 µl	300 µl	600 µl
10% APS	100 µl	200 µl	300 µl	600 µl
TEMED	10 µl	20 µl	30 µl	60 µl

8% Gel	10 ml	20 ml	30 ml	60 ml
40% Acrylamide/Bis (29:1)	2 ml	4 ml	6 ml	12 ml
1.5 M Tris (pH 8.8)	2.5 ml	5 ml	7.5 ml	15 ml
ddH_2O	5.3 ml	10.6 ml	15.9 ml	31.8 ml
10% SDS	100 µl	200 µl	300 µl	600 µl
10% APS	100 µl	200 µl	300 µl	600 µl
TEMED	10 µl	20 µl	30 µl	60 µl

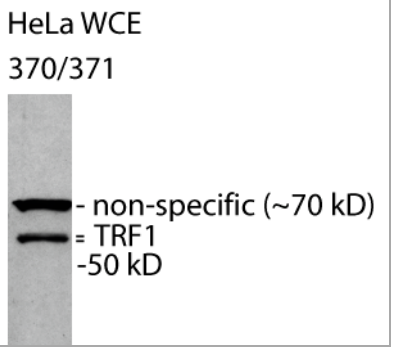
10% Gel	10 ml	20 ml	30 ml	60 ml
40% Acrylamide/Bis (29:1)	2.5 ml	5 ml	7.5 ml	15 ml
1.5 M Tris (pH 8.8)	2.5 ml	5 ml	7.5 ml	15 ml
ddH_2O	4.8 ml	9.6 ml	14.4 ml	28.8 ml
10% SDS	100 µl	200 µl	300 µl	600 µl
10% APS	100 µl	200 µl	300 µl	600 µl
TEMED	10 µl	20 µl	30 µl	60 µl

12% Gel	10 ml	20 ml	30 ml	60 ml
40% Acrylamide/Bis (29:1)	3 ml	6 ml	9 ml	18 ml
1.5 M Tris (pH 8.8)	2.5 ml	5 ml	7.5 ml	15 ml
ddH_2O	4.3 ml	8.6 ml	12.9 ml	25.8 ml
10% SDS	100 µl	200 µl	300 µl	600 µl
10% APS	100 µl	200 µl	300 µl	600 µl
TEMED	10 µl	20 µl	30 µl	60 µl

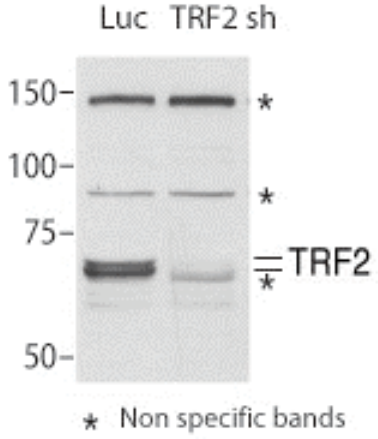
Stacking Gel (4%)	5 ml	10 ml	15 ml
40% Acrylamide/Bis (29:1)	0.5 ml	1.0 ml	1.5 ml
0.5 M Tris (pH 6.8) (4x)	1.25 ml	2.5 ml	3.75 ml
ddH_2O	3.2 ml	6.4 ml	9.6 ml
10% SDS	50 µl	100 µl	150 µl
10% APS	50 µl	100 µl	150 µl
TEMED	5 µl	10 µl	15 µl

Antibodies for Shelterin Components

Protein:	h Trf1
Antigen:	Baculovirus full length TRF1
Serum:	Rabbit Polyclonal Aff. Pur
Number:	370/371
Concentration:	1:1000-2000
Reference:	Van Steensel and de Lange, 1997

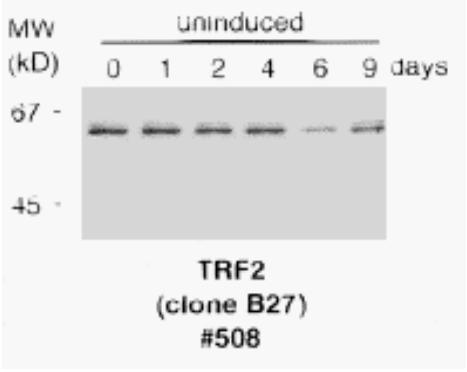


Protein:	h Trf2
Antigen:	Baculovirus full length hTRF2
Serum:	Rabbit Polyclonal Aff. Pur
Number:	647
Concentration:	1:4000
Reference:	Zhu et al, 2000

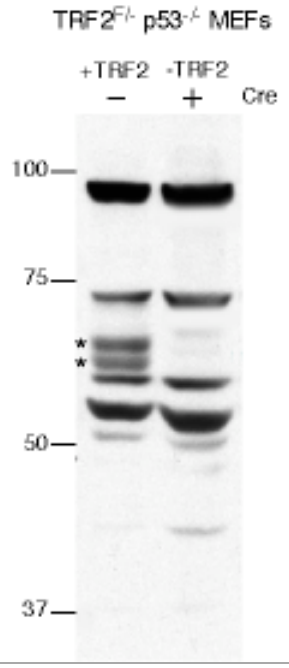


105 HTC75 cells/lane
anti hTrf2 (647) 1:4000 1 hr RT

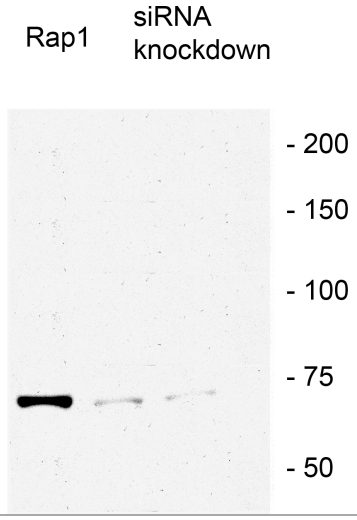
Protein:	h Trf2
Antigen:	aa16-42 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	508
Concentration:	1:300
Comments:	Basic domain
Reference:	Van Steensel et al., Cell 1998



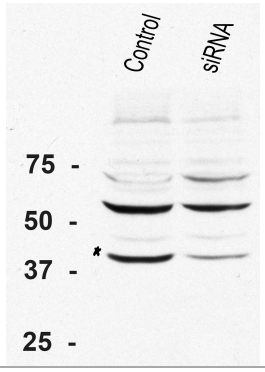
Protein:	m Trf2
Antigen:	GST -mTRF2
Serum:	Rabbit Polyclonal Aff. Pur
Number:	1254
Concentration:	1:5000



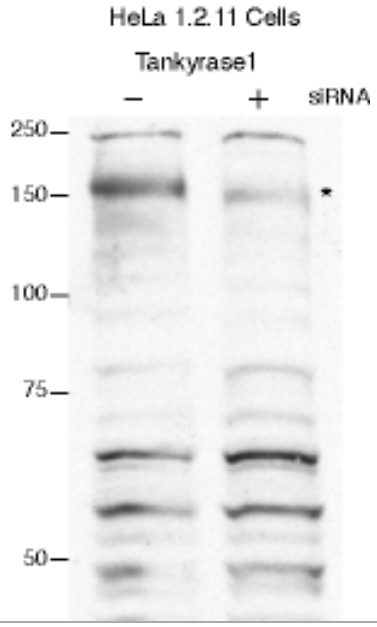
Protein:	h Rap1
Antigen:	Baculovirus full length Rap1
Serum:	Rabbit Polyclonal Aff. Pur
Number:	765
Concentration:	1:2000
Cross reacts with:	mouse, hamster
Comments:	Centrosomal staining



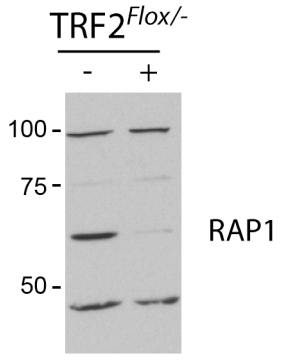
Protein:	h Tin2
Antigen:	Baculovirus full length hTin2
Serum:	Rabbit Polyclonal Aff. Pur
Number:	864
Concentration:	1:2000
Comments:	run long on a 10% gel



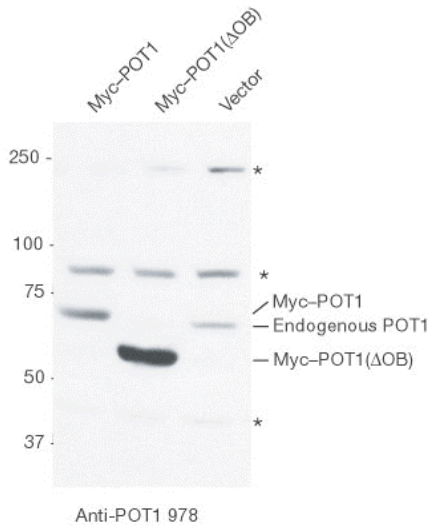
Protein:	Tankyrase
Antigen:	aa973-1149 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	465
Concentration:	1:500
Cross reacts with:	mouse, rat, hamster



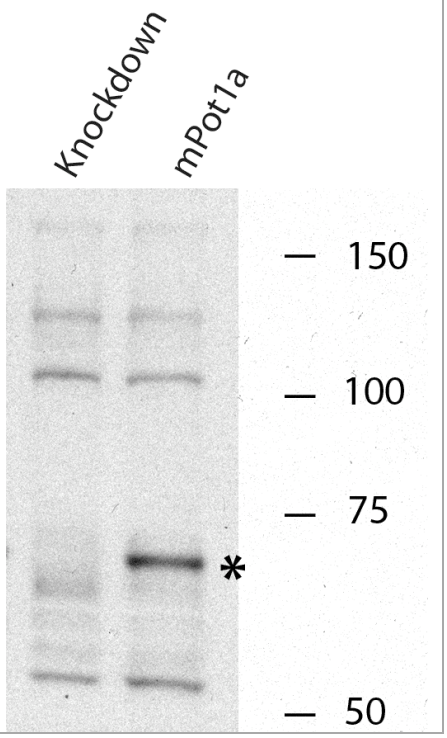
Protein:	m Rap1
Antigen:	GST - mRap1
Serum:	Rabbit Polyclonal Aff. Pur
Number:	1252
Concentration:	1:10000



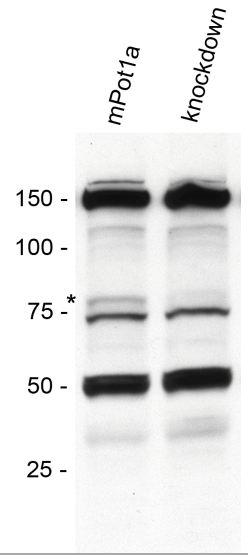
Protein:	h Pot1
Antigen:	aa271-294 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	978
Concentration:	1:1000
Comments:	Guanidine renat
Reference:	Loayza and de Lange, Nature 2003



Protein:	m Pot1a
Antigen:	aa395-421 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	1221
Concentration:	1:1000
Comments:	Guanidine renat



Protein:	m Pot1b
Antigen:	aa285-307 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	1223
Concentration:	1:1000
Comments:	Guanidine renat



Protein:	H Rif1
Antigen:	Aa2076-2101 peptide
Serum:	Rabbit Polyclonal Aff. Pur
Number:	1060
Concentration:	1:1000
Comments:	Guanidine renat
Reference:	Silverman et al., 2004