WESTERN

Solutions

1) TBS/TBST: (Roche recipe – added by Paul)

1x TBS (Tris buffered saline) – 1 liter	➢ Dissolve 6.05 g Tris (50 mM) and 8.76 g NaCl (150 mM) in 800 ml H_2O. ➢ Adjust pH to 7.5 with 1 M HCl (~9.5 ml). ➢ Adjust volume to 1 liter with H_2O. ➢ TBS is stable at +2 to+8°C for three months. ➢ **Note:** Since sodium azide inhibits peroxidase activity, it is not recommended for use as an antimicrobial reagent.
1x TBST (Tris buffered saline Tween) – 1 liter	➢ Dissolve 1 ml Tween 20* in 1 liter TBS buffer. ➢ TBST is stable at +2 to +8°C for three months. ➢ **Note:** Tween 20 is suitable for most applications, but depending on the type of membrane and antibodies used, different detergents, such as like SDS*, Triton X100*, or Nonidet P40*, and concentrations between 0.01 – 1% may lead to better results.

2) RIPA Buffer

20 mM	Tris pH7.5
150 mM	NaCl
1%	Nonidet P-40
0.5%	Sodium Deoxycholate
1 mM	EDTA
0.1%	SDS

For 100ml RIPA Buffer:
0.24g Tris to 75 ml distilled water
Add 0.877g NaCl
Stir
Adjust pH to 7.5 with HCl
Add 10 ml 10% Nonidet P-40
Add 5 ml 10% Na-deoxycholate stock*
Stir until solution is clear
Add 1 ml 100 mM EDTA**
Add 1 ml 10%SDS***
Adjust to 100 ml
Store buffer at 2-8º until ready to use

* 10% Na-deoxycholate stock solution

5g into 50 ml
Protect from light

**100 mM EDTA stock solution
Ethylene diamine tetraacetic acid, disodium salt dehydrate MW 272: 1.86g into 40 ml water
Add NaOH to dissolve and adjust pH to 7.4
Adjust total volume to 50 ml

***10%SDS stock
5g into 50ml water

NB: Before use, add proteinase inhibitor and PMSF (1mM final).
PMSF stock solution: 100mM PMSF in EtOH (100X)

Sample preparation

1. Cells lysed in RIPA:

Rinse the cells with PBS

Lysis in ripa buffer (60-70µl for 1 well of 6 well plate) in ice for 1/2h, mix by inverting the tube few times during the incubation (or perform in cold room, in rocker platform).

Centrifuge the lysate, 2' in microcentrifuge (RT is ok) and keep the supernatant. Concentration of protein is determined by Bradford assay (Use 10-20µg of protein per lane.)

* RC DC assay (Biorad # 5000121) is compatible with detergent therefore is better in my experience – Paul.

Mix sample and loading buffer* (loading buffer is 3X). Loading buffer (Biolabs #B7703S) is prepared by adding dTT (dTT 30X is provided). The loading buffer w/o dTT is stored at RT, and dTT is stored at -20º.

Incubate the sample at 95º for 1 minute.

* Lysates can also be mixed with 6X Laemmli sample buffer (see below) to be 1-2X and heat at 95º for 10 min - Paul.

2. Cells directly lysed in Laemmli sample buffer (Paul added on 1-6-2017):

a. Cell pellets or cells grown on plates can be directly lysed in 1-2X Laemmli sample buffer. To do this, take off the medium and directly put appropriate amount of 1-2X sample buffer on the cells (100-120 μl buffer for one well of 6-well plate. It can be adjusted according to the confluence or cell types). Scrape the well with cell lifter and collect the lysate to eppendorfs. Heat samples for 10 min. The lysate can be directly loaded to the SDS-PAGE.

b. Recipe to prepare 10 ml **6X** Laemmli sample buffer:
   - 1.2g SDS (sodium dodecyl sulfate)
   - 6mg bromophenol blue
   - 4.7ml glycerol
   - 1.2ml Tris 0.5M pH6.8
   - 2.1ml ddH2O

   warm it a little bit and shake it till everything is dissolved. It takes time.
   - add 0.93g DTT
   Wait until it is completely dissolved.
   Aliquot and keep frozen at -20ºC.

Protein detection

Rinse the membrane in TBST

Incubate the membrane with the primary antibody in TBST (5% milk) over-night in cold room on the rocker platform.

Wash the membrane with 10 ml TBST 3 times for 5 minutes at RT on the rocker platform to remove the excess of primary antibody.

Incubate the membrane with the secondary antibody 1:4000 in TBST (5%milk is optional) for 1-2 hours at RT.

Wash the membrane with 10 ml TBST 3 times for 5 minutes at RT on the rocker platform to remove the excess of secondary antibody.

Incubate the membrane with the HRP substrate for 5 minute.

Develop the membrane in the dark room.

Tips for Western blot (Paul 2017):

1. In general, I like to load 12-20 μl samples per well. Loading bias will be more pronounced if you load <10 μl.

2. I personally prefer thick comb and small well.

3. Try to keep every sample with the same amount of glycerol, which influences the width of the band. When you need to re-run the samples based on the previous endogenous controls (which happens in most cases), adjust the samples with the same dilution of sample buffers. For examples, if the samples are in the Laemmli sample buffer with the final 2X concentration, dilute them with 2X sample buffer.

4. Clean up the gel residues when you remove the comb.

5. Rinse the wells with SDS running buffer with syringe & needle before loading the samples.

6. Use Gilson-type 20 μl pipettor for sample loading.

7. Check the volume mark on the pipettor after you put on the tip (because the mark may move when punch the tip box).

8. Because the lysate is viscous, while the tip is still in the lysate, wait an extra, consistent time (for example, 2 sec) for each sample after you releasing the vacuum.

9. To load the sample, try to put the tip directly to the near-bottom of the well and start to load as soon as possible. Searching for the well will make you losing the samples (because the lysate has higher density).

10. Load the sample in the moderate speed.

11. After expelling the lysate, be slow when you pull the tip out of the well. Avoid the formation of the water channel of turbulence.

12. After transferring, check the whole amount of protein loading with Ponceau S (0.1% Ponceau S in 5% acetic acid) before blocking. It is simple, fast, and will not influence the result. It can also be used to judge if the endogenous control is appropriate for the experiment. Ponceau S, once prepared in the brown bottle, can be reused for multiple times.