Lysis protocol

Reagents and materials

- Radioimmunoprecipitation assay (RIPA) buffer
- Cocktail 2&3
- Complete-EDTA
- Phosphate-buffered saline (PBS)
- Scraper
- Eppendorf tubes
    - Non SafeLock
    - SafeLock
- Centrifuge
- Vortex
- Bradford reagent
- mQ water
- Cuvettes
- Absorbance reader
- Lämmli buffer
- Heating block

All the steps should be performed on ice.

Step 0

Prepare RIPA buffer (the lysis buffer):

- 1:100 cocktail 2&3
- 1:25 complete-EDTA
- The rest with RIPA buffer (already prepared in the fridge)
    - For 1mL of RIPA buffer with cocktail 2&3 and complete-EDTA:
        - 10µL cocktail 2
        - 10µL cocktail 3
        - 40µL complete-EDTA
        - 940µL RIPA

Pre-cool down the centrifuge (4°C) and turn on the heating block (95°C).

Step 1

Aspirate the media of the cells and wash 2 times with ice cold PBS using the vacuum pump. Draw off PBS after the last washing step by tilting the plate.

Step 2

Add 200µL of RIPA buffer to each plate. Scrape the cells and lyse them by pipetting up and down. Transfer the cells into an Eppendorf tube (1.5mL, non SafeLock).

Step 3

Centrifuge 10 minutes at 6.000g (4°C). Transfer the supernatant into a fresh Eppendorf tube (1.5mL, non SafeLock) and discard the pellet.

Step 4

Make the Bradford OD measurement for each sample at 595nM in the absorbance reader:

- Prepare Bradford reagent by diluting it 1:5 in mQ water
- Use 1mL of diluted Bradford reagent per sample (plus a blank)
- Add 4µL of sample to the 1mL of Bradford reagent (the blank contains JUST 1mL of Bradford)
- Vortex and measure the absorbance at 595nM

Step 5

Adjust the samples with the corresponding volume of Lämmli and RIPA buffer. Use the document "Bradford template". Mix well.

Step 6

Heat the samples 5 minutes at 95°C in the heating block. Vortex and spin down. Proceed with SDS-PAGE and western blot.

SDS-PAGE and Western blot

Reagents and materials

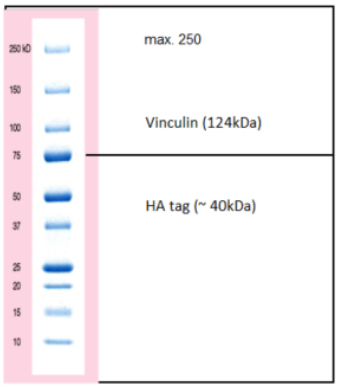
- Protein markers: All Blue and Dual Colour
- Acrylamide gels (already prepared and stored in the fridge) with the running buffer
    - 2 x 10% gel per group will be used
- Running buffer
- Transfer buffer
- PVDF membranes
- Whatmann paper, sponges
- Running/blotting chambers
- BSA solution
- Tris-buffered saline with Tween20 (TBST)
- Shaker
- Antibodies (HA tag, GFP, vinculin)
- PURECL - chemiluminescence substrate Vilber

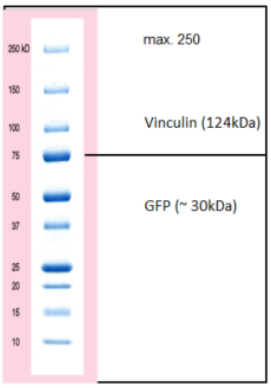
Step 1

Load the samples on the gels with new Running buffer. Follow the scheme showed in the picture below:

western blot

10%		
HepG2		
lane	sample (µL)	vol (µL)
1	Blue marker	4
2	Ctrl no induced	15
3	Ctrl induced	15
4	G6PCwt no induced	15
5	G6PCwt induced	15
6	R83C no induced	15
7	R83C induced	15
8	T255I no induced	15
9	T255I induced	15
10	Dual color marker	2
11	Empty	
12	Empty	
13	Empty	
14	Empty	
15	Empty	





Use 4µL of the All Blue marker and 2µL of the Dual Colour marker. Bring the gels into the gel holder and fill it in with Running buffer (it can be used).

Step 2

Let the samples run at 90V until they reach the separation (lower) gel (15min approx.), then, switch to 180V for approximately 45 minutes. Run the samples until the running front runs out of the gel.

Step 3

Prepare the blotting:

- Activate the PVDF membrane in methanol
- Stop the run and disassemble the 2 glasses. Be sure that the gel is on the short plate
- Assemble the sandwich in new Transfer buffer. On the black part, put the sponge, a Whatmann paper, the gel, the membrane, a Whatmann paper and another sponge. Close the sandwich and put it in the transfer machine
- Use a cooling device and fill the gel box with Transfer buffer (it can be used)
- Run it at 45V for 1h and 50 minutes

Step 4

During the blotting, prepare the blocking solution and the primary antibodies in antibody solution:

- Blocking solution: 2.5% BSA in TBST (prepare 10mL/antibody)
- Primary antibody solution: 5% BSA, 0.1% sodium azide in TBST (10mL/antibody)
- Primary antibodies: dilute the antibodies in primary antibody solution. Check the data sheet of the antibodies for the best concentration of use
    - Vinculin → 1:1000 dilution
    - TurboGFP → 1:1000 dilution
    - HA tag → 1:1000 dilution

Step 5

Once the transfer is done, stop the machine and take out the membrane with care. Cut the membrane as indicated in the scheme above (Figure in section "Step 1") and put each part in the corresponding antibody box.

Step 6

Block the membranes shaking for 20 minutes in blocking solution at RT. WE REUSE THE BLOCKING SOLUTION!

Step 7

Incubate the membranes with the primary antibody overnight at the shaker (4°C).

Step 8

Wash the membranes 3 x shortly with TBST and 3 x 10 minutes with TBST shaking at RT.

Step 9

Incubate the membrane with the secondary antibody in blocking solution shaking for 2h at RT.

- Secondary antibodies: use goat anti-rabbit secondary antibody coupled to HRP (4µL/10mL in 2.5% BSA)

Step 10

Wash membranes 3 x shortly with TBST and 3 x 10 minutes with TBST shaking at RT.

Step 11

Detect the proteins with PURECL - chemiluminescence substrate Vilber and the CCD camera system.

Appendix – buffer recipes

Radioimmunoprecipitation assay (RIPA) buffer

Add to a 500mL bottle of sterile PBS from PAN:

- 5mL IGEPAL/NP40
- 2,5g sodiumdeoxycholate
- 0,5g SDS

Lämmli buffer

To prepare 10mL Lämmli 5x sample buffer add:

- 5mL glicerol
- 0.5mL beta-mercaptoetanol
- 0.83g SDS
- 3.125mL 1M Tris (pH 6.8)
- 1.375mL water

Add bromophenol blue until you see a strong blue colour.

Acrylamide gels

Pipet first the water, gel buffer and acrylamide together. Afterwards, add the 10% APS and TEMED (according to the pipetting scheme).

Mini- Gel 8%	4	6	8	14Gele
Seperating Gel				
ddH2O	9.7	14.55	19.4	33.95ml
LGB	5.0	7.5	10	17.5ml
30% Acrylamid/Bis	5.30	7.95	10.6	18.55ml
10% APS	200	300	400	700µl
TEMED	10	15	20	35µl
	20.210	30.315	40.420	70.735

	4	6	8	14Gele
Stacking Gel				
ddH2O	3.87	5.81	7.75	13.56ml
UGB	1.57	2.35	3.13	5.48ml
30% Acrylamid/Bis	0.82	1.22	1.63	2.85ml
10% APS	50.00	75.00	100.00	175.00µl
TEMED	6.25	9.38	12.50	21.88µl
	6.255	9.382	12.510	21.892

Mini- Gel 10%	4	6	8	14Gele
Seperating Gel				
ddH2O	8.3	12.45	16.6	29.05ml
LGB	5	7.5	10	17.5ml
30% Acrylamid/Bis	6.7	10.05	13.4	23.45ml
10% APS	200	300	400	700µl
TEMED	10	15	20	35µl
	20.210	30.315	40.420	70.735

Mini- Gel 12%	4	6	8	14Gele
Seperating Gel				
ddH2O	7	10.5	14	24.5ml
LGB	5	7.5	10	17.5ml
30% Acrylamid/Bis	8	12	16	28ml
10% APS	200	300	400	700µl
TEMED	10	15	20	35µl
	20.210	30.315	40.420	70.735

Mini- Gel 14%	4	6	8	14Gele
Seperating Gel				
ddH2O	5.6	8.4	11.2	19.6ml
LGB	5	7.5	10	17.5ml
30% Acrylamid/Bis	9.4	14.1	18.8	32.9ml
10% APS	200	300	400	700µl
TEMED	10	15	20	35µl
	20.210	30.315	40.420	70.735

Prepare the lower gel solution and let it polymerize. Then, prepare the upper gel solution.

Store the gels in new running buffer at 4°C.

Lower Gel Buffer (LGB) - 600 ml
Solve 109.03 g Tris (=1.5 M) in ~ 400 ml H2O.
Add concentrated HCl to pH 8.8.
Add water to get a final volume of 576 ml.
Aliquot to 3x 192 ml and autoclave.

Add 8 ml of 10% SDS solution (= 0.4%) per bottle.
Store at 4°C.

Upper Gel Buffer (UGB) - 300 ml
Solve 18.17 g Tris (=0.5 M) in ~ 200 ml H2O.
Add concentrated HCl to pH 6.8.
Add water to get a final volume of 288 ml.
Aliquot to 3x 96 ml and autoclave.
Add 4 ml of 10% SDS solution (= 0.4%) per bottle.
Store at 4°C.

Running buffer

To prepare 5L of 10x Running buffer dissolve in water:

- 720,6g glycine
- 151,4g Tris
- 50g SDS

Dilute 200mL of 10x Running buffer in 1.8L of water to obtain 1x Running buffer.

Transfer buffer

To prepare 1L of 10x Transfer buffer add:

- 500mM TrisBase (60.5g)
- 1000mM Glycin (75g)
- 0.1% SDS (1g)

Adjust pH to 8.3 with concentrated HCl.

Dilute 200mL of 10x Transfer buffer, 200mL of methanol in 1.6L of water to obtain 1x Transfer buffer.

TBST

To prepare 1L of 10x TBS add to water:

- 80g NaCl
- 2g KCl
- 80g TrisBase

Adjust pH to 7.4 with 37% HCl.

Mix 200mL of TBS 10x with 1.8L water to obtain TBS 1x.

For TBST add 0.1% Tween20 to TBS (1x).