RIPA Buffer:

50 mM Tris-HCl pH 6.8

500 mM NaCl

1% (v/v) NP40

0.5% (w/v) Na-deoxycholate

0.1% (w/v) SDS

0.05% (w/v) NaN_3

Keep in the refrigerator (4-8°C)

At max 30 min prior to cell lysis, supplement the RIPA buffer with 1 mM PMSF (stock solution 100 mM), 1 x Complete™ (stock solution 25x, Roche Applied Science), 1 mM NaF (stock solution 200 mM) and 1 mM Na_3VO_4 (stock solution 200 mM) and keep the lysis buffer on ice.

3x SDS- Samplebuffer:

0,5M Tris- HCl pH 6,8 (3,94g in 50mL)	37,5mL
SDS	6,0g
Glycerol	30,0mL
Bromphenolue	15,0mg

 ad 100mL with Aqua dest.

Keep at RT

Prior to use add 15% Mercaptoethanol!!!

The volume of the lysis buffer depends on the size of the surface that is being used to grow cells. For example, for 6 cm dish I use 80 µl lysis buffer, for 10 cm dish I would use 150 to 200 µl. I use this

small volume for VSMC because they are not rich in protein. For other cell types I assume it would be fine to use a slightly higher volume of the lysis buffer.

Cell lysis and protein sample prep:

1. Aspirate the growth medium
2. Wash cells once with ice cold PBS and aspirate
3. Put cells on ice
4. Add lysis buffer and make sure it distributes evenly across the whole surface area
5. Incubate on ice for 20 min
6. Use cell scrapers and scrape the surface vigorously to „break“ the cells. Do this on ice.
7. Hold the dish tipped on one side and carefully by using cell scrapers collect the whole cell material into the lower part of the dish, aspirate the whole material with the pipet and transfer it into a pre-cooled labeled Eppendorf tube.
8. Store the sample at -80°C for max 7 days or continue with the protein extraction
9. Centrifuging the samples at 4°C for 20 min at 11000 g
10. Transferring supernatants into new eppies (1.5 ml)
11. 5 µl of each sample supernatant was diluted with 45 µl ddH2O for the Bradford assay
12. To the rest of the sample 3x SDS Sample Buffer was added to achieve 1x SDS Sample Buffer
13. Samples were incubated around 10 Min at 95°C, spun down and stored at -20°

Bradford assay for protein quantification:

1. Dilute the 5x RotiQuant (Carl Roth, Germany) with dest. water to achieve 1x dilution
2. Pipette 190 µl/well of the RotiQuant 1x into a transparent 96-well plate
3. Pipette in triplicates the serial albumin dilutions (for the standard curve) – 10 µl/well for a total volume 200 µl/well
   Stock solution BSA: 1mg/mL → aliquot and freeze at -20

Final concentration in Bradford solution [µg/mL]	Calibration curve concentration [µg/mL]	for 1000µL (10 Aliquots á 100µL)
2,5	50	50 + 950 (Stock + H_2O)
5	100	100 + 900
7,5	150	150 + 850
10	200	200 + 800
15	300	300 + 700
20	400	400 + 600
25	500	500 + 500

4. Pipette in triplicates 10 µl/well of the 1:10 protein sample dilution from the step 11

5. Shake the plate for 3 sec and let stay for 2 sec
6. Measure absorbance at 595 nm in Tecan Sunrice plate reader
7. Calculate the concentration in each protein sample by using the calibration curve (total dilution of protein samples is 10 (step 11) x 20 (step 4) = 200 x!!!)

SDS-PAGE

1. Pour the 6% PAA separation gel and let it stack, cover it with stacking gel, put the 1.5 mm spacer with 10 pockets and let it stack

Receipe 2x:				
	Separation gel			Stacking gel
H2O (ml)	8.4			6.7
1,5 M Tris HCl pH 8.8 (ml)	4		1,25 M Tris HCl pH 6.8 (ml)	1
30% PAA (ml)	3.2			2
10% SDS (µl)	160			100
TEMED (µl)	16			10
10% APS (µl)	160			100
total (ml)	15			7.5

2. Keep the gel in a wet cloth at +4°C for a maximum of 24 h
3. For the Novus Biologicals AB against TRPV1, 40 µg of protein per slot is required (according to the product data sheet), and for the Abcam AB against TRPV1 80 µg of protein per slot is recommended in the product data sheet, but I used also 40 µg, because the required volume would not fit the slot
4. Electrophoresis perforemd at 60 V for ca. 30 min and then at 150 V for ca. 80 min

<u>Electrophoresis buffer (10x) :</u>

250 mM Tris-Base

1.92 M Glycine

35 mM SDS

− prior to use (1x solution) 100 ml 10x solution

ad 1000 ml Aqua dest.

5. Proteins were transfered onto a PVDF membrane (prior incubated shortly in methanol) at 110 mM for 90 min

<u>Blotting buffer (5x) :</u>

125 mM Tris-Base

970 mM Glycine

− prior to use (1x solution) 200 ml 5x solution

200 ml Methanol

ad 1000 ml Aqua dest.

6. Blocking the membrane in 5% milk powder in 1x TBS-T buffer for 1 h at RT or overnight at 4-8°C

TBS-T, pH 8.0:

25 mM Tris-base

190 mM

0.1% Tween 20

7. Washing the membrane 3x in TBST, each wash 3-5 min

8. Incubating the antibody in primary antibody, diluted as recommended in the product data sheet in TBST, for 2 h at RT or overnight at 4-8°C

9. Washing as in step 7

10. Incubating the membrane in the HRP-conjugated secondary antibody diluted in the TBST as recommended in the product data sheet for 1 h at RT

11. Washing as in step 7

12. Incubating the membrane shortly in ECL solution and detection with a LAS-3000™

luminescent image analyzer

ECL:

4.5 ml Aqua dest.

0.5 ml Tris-base, 1 M pH 8.5

22.5 µl Luminol (stock solution: 0.44 g Luminol, ad 10 ml DMSO)

11.12 µl p-Coumaric acid (stock solution: 0.15 g p-Coumaric acid, ad 10 ml DMSO)

1.5 µl H_2O_2 (30%)

Antibody	**Product ID**	**Company**
Vanilloid R1/TRPV1 Antibody	NBP1-97417	Novus biologicals
Anti-VR1 antibody [BS397] – C-terminal	ab203103	Abcam
α/β-tubulin antibody	#2148	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked Antibody	#7074	Cell Signaling Technology
Anti-mouse IgG, HRP-linked Antibody	#7076	Cell Signaling Technology