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Immunoblotting

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This protocol describes collection of protein from cultured cells and immunoblotting, including immunoblotting of large proteins using a proprietary tris-acetate buffer system.

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Immunoblot, Western blot, Tris-glycine, Tris-acetate, siRNA, cGAMP, HT-DNA, ASAPCRN

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Solutions to prepare:

DMEM solution:

A	B
FBS	10%
Penicillin	100 U/ml
Streptomycin	100 mg/ml
L-glutamine	2 mM

RIPA buffer:

NaCl	150 mM
Tris	10 mM
EDTA	0.5 mM
NP40	0.50%

Supplemented immediately before use with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche)

TBS:

A	B
Tris-Cl	50 mM
NaCl adjust pH to 7.5	150 mM

TBST: TBS with 0.1% TWEEN-20 (Sigma-Aldrich)

3x Laemmli buffer:

A	B
Tris-HCl	188 mM
SDS	3%
Glycerol	30%
Bromophenol blue	0.01%
β-mercaptoethanol	15%

Tris-glycine running buffer:

A	B
Tris base	25 mM
Glycine	192 mM
SDS in milliQ water	0.10%

Tris-glycine transfer buffer:

A	B
Tris	25 mM
Glycine	192 mM
Methanol in milliQ water	20%

Chill to 4˚C

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Cell culture and treatments 3d

1 Culture the HeLa-M cells at **37 °C** in 5% CO_2 and DMEM containing 10% FBS, **100 U/ml** penicillin, **100 mg/mL** streptomycin, and **2 Milimolar (mM)** L-glutamine (all from Gibco).

2 For any given experiment, plate the cells at such density so as to be approximately 90% confluent at the time of lysis.

3 For experiments using siRNA, transfect 60 pmols of the indicated siRNA using **6 µL** Lipofectamine RNAiMax (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol. Lyse the cells **72:00:00** after siRNA transfection. 3d

4 For experiments using cGAMP, transfect **8 µg/L** of cGAMP using **6 µL** Lipofectamine RNAiMax (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol.

5 For experiments using herring testes (HT)-DNA, transfect **1 µg** HT-DNA using **3 µL** Lipofectamine 2000 (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol.

Cell lysis and sample preparation 40m

6 Supplement RIPA buffer with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche) and chill **On ice** .

7 

Aspirate media from cells and rinse cells with PBS **On ice** . Aspirate PBS thoroughly.

8 

Pipette RIPA lysis buffer onto cells and scrape cells using a cell lifter (Corning).

9 

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Pipette lysis buffer containing cell mass into Eppendorf tube.

10  30m

Incubate Eppendorf tube **On ice** for **00:30:00** .

11 

Every 10 minutes, pipette lysis mixture up and down 10 times with a P-200 pipette tip (a total of 3 cycles).

NOTE: Take care not to introduce bubbles.

12  10m

Centrifuge at **15000 x g** for **00:10:00** at **4 °C** and collect the post-nuclear supernatant in a new Eppendorf tube.

NOTE: Samples can be snap frozen in liquid nitrogen at this step and stored at -70˚C.

13 Determine protein concentration in sample using Pierce BCA assay (ThermoFisher).

14 

Prepare samples at desired concentration and add 3x Laemmli buffer.

Gel electrophoresis and immunoblotting (Tris-glycine buffer system) 4h 50m

15  5m

Incubate samples at **95 °C** for **00:05:00** .

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16 During this incubation, prepare gel apparatus with Mini PROTEAN TGX 4-20% trisglycine gels (Bio-Rad) and running buffer.

17 Load samples into gel and run until dye front reaches bottom (90-120 V).

18 Remove gel and set up transfer cassette with nitrocellulose membrane.

19 Transfer at 120 V for **01:00:00** at **4 °C** in tris-glycine transfer buffer. 1h

20 Remove nitrocellulose membrane and stain for total protein with ponceau stain.

21 

Wash with milliQ water.

22 Block membrane with 5% milk in TBST for **01:00:00** at **22 °C** . 1h

23  1h

Incubate membrane with primary antibodies in 2.5% milk in TBST **Overnight** at **4 °C** .

NOTE: Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

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24

Wash membrane with TBST. Repeat a total of 3 times.

24.1 5m

Wash membrane for **00:05:00** with TBST (1/3).

24.2 5m

Wash membrane for **00:05:00** with TBST (2/3).

24.3 5m

Wash membrane for **00:05:00** with TBST (3/3).

25 1h

Incubate membrane with secondary antibodies conjugated to IRdye 800CW or IRdye 680CW (1:10,000, Licor) in 2.5% milk in TBST for **01:00:00** at **22 °C** .

26

Wash membrane with TBST. Repeat a total of 3 times.

26.1 5m

Wash membrane for **00:05:00** with TBST (1/3).

26.2 5m

Wash membrane for **00:05:00** with TBST (2/3).

26.3 5m

Wash membrane for **00:05:00** with TBST (3/3).

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27

Wash membrane with TBS. Repeat a total of 3 times.

27.1 5m

Wash membrane for **00:05:00** with TBS (1/3).

27.2 5m

Wash membrane for **00:05:00** with TBS (2/3).

27.3 5m

Wash membrane for **00:05:00** with TBS (3/3).

28

Image membranes using a Licor Odyssey Infrared Imager.

Gel electrophoresis and immunoblotting (Tris-acetate buffer system) 20h 55m

29 For VPS13C immunoblotting, lyse samples and collect the post-nuclear supernatant as above.

30 10m

Mix post-nuclear supernatant with NuPAGE LDS Sample Buffer and Reducing Agent (Thermofisher) and incubate for **00:10:00** at **70 °C** .

31

During this incubation, prepare gel apparatus with NuPage Tris-Acetate 3-8% gels and NuPage Running Buffer (Thermofisher).

32 Remove gel and set up transfer cassette with nitrocellulose membrane.

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33 Transfer at 0.05 mA for **16:00:00** at **4 °C** in NuPage transfer buffer (Thermofisher). 16h

34 Remove nitrocellulose membrane and stain for total protein with ponceau stain.

35

Wash with milliQ water.

36 Block membrane with 5% milk in TBST for **01:00:00** at **22 °C** . 1h

37 2h

Incubate membrane with primary antibodies in 2.5% milk in TBST for **02:00:00** at **22 °C** .

NOTE: Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

38

Wash membrane for with TBST. Repeat a total of 3 times.

38.1 5m

Wash membrane for **00:05:00** with TBST (1/3).

38.2 5m

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Wash membrane for **00:05:00** with TBST (2/3).

38.3 5m

Wash membrane for **00:05:00** with TBST (3/3).

39 1h

Incubate membrane with secondary antibodies conjugated to IRdye 800CW or IRdye 680CW (1:10,000, Licor) in 2.5% milk in TBST for **01:00:00** at **22 °C** .

40

Wash membrane with TBST. Repeat a total of 3 times.

40.1 5m

Wash membrane for **00:05:00** with TBST (1/3).

40.2 5m

Wash membrane for **00:05:00** with TBST (2/3).

40.3 5m

Wash membrane for **00:05:00** with TBST (3/3).

41

Wash membrane with TBS. Repeat a total of 3 times.

41.1 5m

Wash membrane for **00:05:00** with TBS (1/3).

41.2 5m

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Wash membrane for **00:05:00** with TBS (2/3).

41.3 5m

Wash membrane for **00:05:00** with TBS (3/3).

42

Image membranes using a Licor Odyssey Infrared Imager.

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