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Cellular protein extraction and Western blotting using dry transfer (iBlot system)

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ABSTRACT

A generic protein extraction and Western blotting protocol, allowing both for total cell lysis or cytoplasmic protein extraction only. The cytoplasmic extraction version has been used when Western blotting for PI3K and MAPK/ERK signalling components; total cell lysis with sonication has been used when Western blotting for histones.

GUIDELINES

The protocol assumes that the cells have been collected as follows prior to lysis:

- 1) Washed once with cold DPBS.
- 2) Snap-frozen on dry ice or liquid nitrogen, followed by long-term storage at

-80 °C .

MATERIALS

MATERIALS

- cOmplete™, EDTA-free Protease Inhibitor Cocktail Sigma
Aldrich Catalog #05056489001
- 20X MES Buffer Thermo Fisher
Scientific Catalog #NP0002
- NuPAGE Antioxidant Thermo Fisher
Scientific Catalog #NP0005
- Molecular Biology Grade Water Fisher
Scientific Catalog #10154604
- BSA Sigma
Aldrich Catalog #A7906
- NUPAGE LDS sample buffer (4x) Thermo Fisher
Scientific Catalog #NP0007
- 1x NUPAGE MOPS SDS running buffer (20x) Thermo Fisher
Scientific Catalog #NP0001

OPEN ACCESS



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MANUSCRIPT CITATION:
This protocol is an amalgamate of previous protocols provided by Dr Gemma Brierley (Institute of Metabolic Science, University of Cambridge) and Dr Tijana Mitic (CVS, University of Edinburgh), with additional modifications based on my own PhD/postdoc experience.

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We use this protocol and it's working

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25116


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 DC™ Protein Assay Kit BIO-RAD Catalog #500-0112


 cOmplete ULTRA Tablets Mini EasyPack PhosStop Sigma Aldrich Catalog #4906845001

 Precision Plus Protein™ Dual Color Standards BIO-RAD Catalog #1610374

 NuPAGE Sample Reducing Agent (10X) Thermo Fisher Scientific Catalog #NP0009

 NuPAGE 4-12%BT midi 12 2 well PAGE gels Thermo Fisher Scientific Catalog #WG1401BOX

 iBlot™ 2 Transfer Stacks nitrocellulose regular size Thermo Fisher Scientific Catalog #IB23001

 Immobilon Western Chemiluminescent HRP Substrate Sigma Aldrich Catalog #WBKLS0500


 EZBlue™ Gel Staining Reagent Sigma Aldrich Catalog #G1041

 Fisherbrand™ Cell Scrapers Fisher Scientific Catalog #11587692

 XCell4 SureLock™ Midi-Cell Thermo Fisher Scientific Catalog #WR0100


TBS/T for washes; [M] 20 millimolar (mM) Trizma base, [M] 150 millimolar (mM) NaCl, pH = 7.6 (tip: prepare a larger volume of 10X stock solution and dilute each time; use deionised H₂O).

SAFETY WARNINGS

 Some reagents are toxic if inhaled or similar and should be handled according to the accompanying materials safety sheet.

BEFORE START INSTRUCTIONS

Prepare the required lysis buffers depending on application

The buffers can be prepared in bulk and stored at  4 °C (we have previously stored buffers A and B for 2-3 years, and they continued to work). Always check for precipitates in the stock before preparing a working aliquot.

Take out smaller aliquots when required and supplement with the indicated inhibitors. It might be a good idea to sterile-filter the working aliquot before use.

* Add fresh before use.

Type A buffer: whole-cell lysis; relatively mild (used for immunoprecipitation of

phosphoproteins and regular WB of cytoplasmic proteins)

[M] 20 millimolar (mM) HEPES (pH = 7.4)

[M] 150 millimolar (mM) NaCl

[M] 1.5 millimolar (mM) MgCl₂

[M] 10 % volume Glycerol

[M] 1 % volume TritonX-100

[M] 1 millimolar (mM) EGTA (dissolves at 37 °C and pH 8, stirring required)

Store at 4 °C - when needed, prepare a 10 ml working aliquot and supplement with the following immediately before use:

[M] 1 millimolar (mM) * PMSF (prepared as 100X stock dissolved in isopropanol and store as smaller aliquots at -20 °C ; short half-life once in aqueous solution)

[M] 2 millimolar (mM) * Na₃VO₄ (has to be dissolved in a particular way, for more details follow [this link](#); prepare as 100X stock solution and store as smaller aliquots at -20 °C)

* 1X EDTA-free protease inhibitor tablet (to 10 ml buffer)

* 1X PhosStop tablet (to 10 ml buffer)

Type B buffer: whole-cell lysis; relatively mild (used for nitrocellulose-based reverse phase protein arrays and regular WB of cytoplasmic proteins; for original reference, see Macleod et al. 2017 doi: 10.1007/978-1-4939-7201-2)

[M] 50 millimolar (mM) HEPES (pH = 7.4)

[M] 150 millimolar (mM) NaCl

[M] 1.5 millimolar (mM) MgCl₂

[M] 10 % volume Glycerol

[M] 1 % volume TritonX-100

[M] 1 millimolar (mM) EGTA (dissolves at 37 °C and pH 8, stirring required)

[M] 100 millimolar (mM) NaF

[M] 10 millimolar (mM) Na₄P₂O₇

Adjust pH to 7.4 and store at 4 °C - when needed, prepare a 10 ml working aliquot and supplement with the following immediately before use:

[M] 2 millimolar (mM) * Na₃VO₄ (has to be dissolved in a particular way, for more details follow [this link](#); prepare as 100X stock solution and store as smaller aliquots at -20 °C)

* 1X EDTA-free protease inhibitor tablet (to 10 ml buffer)

* 1X PhosStop tablet (to 10 ml buffer)

Type C buffer: whole-cell lysis; harsh for nuclear lysis (used for WB of histones) - modified RIPA buffer (higher SDS concentration used)

[M] 20 millimolar (mM) Tris-HCl (pH = 7.5)

[M] 150 millimolar (mM) NaCl
[M] 1 millimolar (mM) Na₂EDTA
[M] 1 millimolar (mM) EGTA (dissolves at 37 °C and pH 8, stirring required)
[M] 1 Mass / % volume SDS
[M] 1 Mass / % volume Sodium deoxycholate
[M] 2.5 millimolar (mM) Na₄P₂O₇
[M] 1 millimolar (mM) β-glycerophosphate
1 µg/ml leupeptin

Store at 4 °C - when needed, prepare a 10 ml working aliquot and supplement with the following immediately before use:

[M] 1 millimolar (mM) * Na₃VO₄ (has to be dissolved in a particular way, for more details follow [this link](#); prepare as 100X stock solution and store as smaller aliquots at -20 °C)

* 1X EDTA-free protease inhibitor tablet (to 10 ml buffer)

* 1X PhosStop tablet (to 10 ml buffer)

Cell lysis and protein extraction

- 1 Pre-chill the benchtop microcentrifuge to 4 °C .
Prepare cell scraper and a beaker with PBS - this will be used to rinse the cell scraper in between processing of different samples.
- 2 Aliquot 10 ml of stock lysis buffer and dissolve the required supplements that need to be added fresh (see "Guidelines" for buffer details). Keep on ice.
- 3 Allow the snap-frozen cells to thaw on ice (one plate at a time, process each well for scraping and rinse the scraper in PBS in between individual samples).
- 4 Add 150 µL of ice-cold lysis buffer per well and scrape the cells on ice.

Note

The volume can be reduced to 100 µl depending on expected yield. I use this volume when working with human pluripotent stem cells which give a high protein yield from a 6-well (3-5 mg/ml); this may not be the case with other cell lines and should be tested empirically.

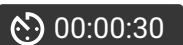
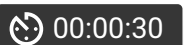
- 5 Transfer cell lysates to pre-labelled and pre-chilled tubes.
- 6 Incubate for minimum 00:30:00 on ice.

30m



7 Vortex each sample for  00:00:05 .

5s

8 If performing nuclear lysis for extraction of histones: sonicate the samples on a Diagenode Bioruptor using 5 pulses of 3  00:00:30 ON,  00:00:30 OFF; setting =high. Following sonication, the lysates should appear clear/runny.

5m

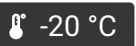


9 Centrifuge the lysate at  4 °C and  12000 x g for  00:10:00 .

9.1 In the mean time, thaw previously prepared BSA standards (0 - 2 mg/ml).

10 Transfer the supernatant to new pre-chilled tubes and discard the pellets.

Note

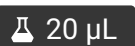
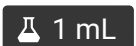
Stopping point: the samples can be stored at  -20 °C at this point and processed for protein concentration measurements at a later time. Freeze-thawing should generally be limited, but up to 3 times has worked well for me in the past (alternatively, make multiple aliquots to avoid freeze-thawing of the entire volume each time).

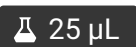
11 Dilute the samples for protein concentration measurements (usually 1:5 but will depend on the exact samples and the expected yield). Use molecular-grade H₂O.


Note

Remember to dilute the lysis buffer the same.

It is easiest to perform these and all subsequent dilutions in PCR strip tubes.

12 Prepare DC assay solution A+S according to the manufacturer's instructions (BioRad): use  20 µL Reagent S to  1 mL Reagent A (prepare a mastermix in excess of what you will need; e.g. for loading of 8 standards and 8 samples in quadruplicate = 25 * 16 * 4 * 1.2 (excess) = 1920 ~ 2 ml (2 ml Reagent A + 40 µl Reagent S)).

13 Add  25 µL of the A+S mix to each well of a 96-well plate for protein concentration measurements (e.g. Sterilin™ Clear Microtiter™ Plates; Thermo Scientific Sterilin 611F96 (for absorbance measurements) #11349163)


14 Add  5 µL of each sample or standard dilution in triplicate; use multichannel pipettor if dealing with multiple samples and all diluted in PCR strip tubes.

Example layout

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 (0 mg/ml)	S1	S1	G7_25	G7_25	G7_25	Lysis buffer dilution	Lysis buffer dilution	Lysis buffer dilution			
B	S2 (0.125 mg/ml)	S2	S2	G7_26	G7_26	G7_26						
C	S3 (0.250 mg/ml)	S3	S3	G7_27	G7_27	G7_27						
D	S4 (0.5 mg/ml)	S4	S4	G7_28	G7_28	G7_28						
E	S5 (0.750 mg/ml)	S5	S5	G7_29	G7_29	G7_29						
F	S6 (1 mg/ml)	S6	S6	G7_30	G7_30	G7_30						
G	S7 (1.5 mg/ml)	S7	S7	G7_31	G7_31	G7_31						
H	S8 (2 mg/ml)	S8	S8	G7_32	G7_32	G7_32						

I prepare my BSA standards in molecular grade H₂O and aliquot into two sets of PCR strip tubes; one set is kept at 4C (working set), and the second set is stored at -20C.

15 Add  200 µL of Reagent B to each well.

16 Wrap plate in foil and put on an orbital shaker at 300 rpm for  00:15:00 .

15m



17 Read 595 nm absorbance on a suitable plate reader.

18 Calculate sample concentration based on the standard curve and adjusted for the applied dilution; remember to subtract the lysis buffer only blank.

19 Calculate the amount of sample needed for loading of the required number of wells, taking into account the required mixing with 4X LDS loading buffer and 10X Reducing Agent (RA). 10 µg

sample per well in 20 μ l (for midi gels) is a good starting point for most applications, but this may require adjusting on a case-by-case basis.

Samples	Protein concentration mg/ml (equivalent to μ g/ μ l)	V for loading of 4 gels with 5 μ g per well (prepare for 20 μ g in 80 μ l, load 20 μ l per well)	4X LDS	10X RA	LB_top_up	Final V
G7_25	1.549019608	12.9	20	8	39.1	80
G7_26	1.276348039	15.7	20	8	36.3	80
G7_27	0.580882353	34.4	20	8	17.6	80
G7_28	1.21752451	16.4	20	8	35.6	80
G7_29	1.862132353	10.7	20	8	41.3	80
G7_30	1.144607843	17.5	20	8	34.5	80
G7_31	0.387254902	51.6	20	8	0.4	80
G7_32	1.585784314	12.6	20	8	39.4	80
G7_33	1.441176471	13.9	20	8	38.1	80

Example table of calculations for loading of 4 wells (3+1 extra to take loss into account). LB, lysis buffer.

Note



Once the dilutions have been prepared, return the original samples to $-20\text{ }^{\circ}\text{C}$ for long-term storage. It is also possible to store diluted samples in LDS/RA at $-20\text{ }^{\circ}\text{C}$, thaw and heat for loading at a later time.

- 20 Heat samples at $70\text{ }^{\circ}\text{C}$ for 00:10:00 (if prepared in PCR strips, use thermocycler). It is better to use a lower temperature to limit potential protein degradation while still achieving denaturation. Once completed, leave the samples to cool off at Room temperature; pulse-spin to pull-down any condensed liquid.
- 21 In the meantime, prepare running buffer according to manufacturers conditions (use 20X MOPS or 20X MES stock buffers - choice will depend on the size of your proteins of interest; use

deionised H₂O for dilution).

Note

For the inner gel chamber, prepare a separate aliquot of running buffer and supplement with NuPAGE Antioxidant ( 500 μ L per  200 mL).

- 22 Load each sample using gel loading tips; load  10 μ L protein ladder at the start and/or end of the gel (or as required). Run the gel at 120V until the dye front has reached the plate bottom (usual run time  02:00:00).



Note

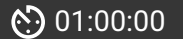

For good results, it is absolutely essential to mix the samples before loading!

Note


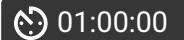


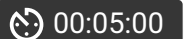
Note that each midi tank can take 4 gels, however, loading of the gels becomes more difficult when they face backwards and may result in imprecision. It is best to stick to 12-well gels or less to minimise sample diffusion which takes place the longer the samples sit without external voltage during loading.

Transfer, blocking and primary antibody incubation

- 23 Prepare blocking buffer:  3 Mass / % volume BSA to 1X TBS/T (Tween-20 at 0.1 %).
- 24 Prepare the required materials for transfer: trays with EZ gel staining reagent, trays with double-deionised H₂O for filter paper wetting, tissue paper, scalpel, forceps, transfer packs, iBlot2 machine.
- 25 When the gel has finished running, disassemble the cassette, cut excess gel material off and transfer the gel using the iBlot2 system according to the manufacturer's instructions (Programme P3 for  00:07:00 is a good starting option, but may require changing based on protein of interest). When assembling the gel transfer stack, wet the filter paper in double-deionised H₂O and tap dry on some tissue paper before putting on top of the gel; this will reduce water-copped corrosion and the formation of "green" streaks on the membrane). 10m
- 26 Once transferred, cut the membrane if necessary and decide on a marking system to be able to track the identify of the membrane if multiple membranes are being transferred (e.g. corner triangles cut with scalpel; number identifying gel number). Transfer the membrane to a box with blocking solution covering the entire gel. Put to rock on a shaker for 45 minutes to 1 hour. 1h

- 27 Stain the transferred gels in EZ Blue solution for  01:00:00 , then destain using deionised H₂O. Both steps should be performed on a shaker. The gels can be imaged (epi-illumination or 700/800 nm fluorescence) several days later if necessary - leave on bench at room temperature once destaining completed.
- 28 Prepare dilutions of primary antibody in blocking buffer according to the manufacturer's instructions.
- 29 Once the membrane has finished blocking, remove the blocking solution (can be reused), and add the primary antibody dilution so that it covers the entire blot surface. Incubate at  4 °C with rotation: typically overnight, but can be left for longer if necessary.

Secondary antibody incubation and ECL detection

- 30 Decant used primary antibody into original tube (for reuse) and return to the fridge (primary antibody should be supplemented with 0.02 % sodium azide for continued fridge storage).
- 31 Wash the membrane 5X for 5 minutes each in 1X TBS/T buffer.

- 32 Dilute the required secondary antibody in blocking buffer. We typically use 1:10,000 dilutions of either one of the following antibodies: anti-rabbit IgG HRP-linked antibody (CST #7074S), anti-mouse IgG HRP-linked antibody (7076S).
- 33 Incubate the membrane in secondary antibody for  01:00:00 at  Room temperature on a shaker. Make sure that the solution covers the entire surface of the membrane.
- 34 Decant secondary antibody and store in fridge for one more reuse if necessary.
- 35 Wash the membrane 5X for 5 minutes each in 1X TBS/T buffer.

- 36 Prepare ECL solution according to the manufacturer's instructions. Use 2 ml ECL mix per 10 cm x 8 cm membrane.
- 37 Place the membrane on a flat surface (e.g. glass plate), making sure that excess washing solution has been drained off, and apply the ECL solution. Ensure that the blot is covered evenly.
- 38 Incubate for  00:05:00 , then proceed with detection using a chemiluminescence imaging system.

Note

Tip: make sure the imaging settings are written down in a readme.txt file, alongside experimental information (samples, transfer condition, antibody dilutions and lot numbers, blocking conditions)

- 39** Once detected, the membrane can be stored in TBS/T in the fridge for up to two weeks if required for repeated incubations.