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

Ralitsa R. Madsen[1]

[1]University of Edinburgh

Jun 26, 2019

Working dx.doi.org/10.17504/protocols.io.4r4gv8w

Ralitsa R. Madsen
University of Edinburgh, University of Cambridge

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.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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.

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

| NAME | CATALOG # | VENDOR |
|---|---|---|
| cOmplete™, EDTA-free Protease Inhibitor Cocktail | 05056489001 | Sigma Aldrich |
| 20X MES Buffer | NP0002 | Thermo Fisher Scientific |
| NuPAGE Antioxidant | NP0005 | Thermo Fisher Scientific |
| Molecular Biology Grade Water | 10154604 | Fisher Scientific |
| BSA | A7906 | Sigma Aldrich |
| NUPAGE LDS sample buffer (4x) | NP0007 | Thermo Fisher Scientific |
| 1x NUPAGE MOPS SDS running buffer (20x) | NP0001 | Thermo Fisher Scientific |
| DC™ Protein Assay Kit | 500-0112 | BIO-RAD |
| cOmplete ULTRA Tablets Mini EasyPack PhosStop | 4906845001 | Sigma Aldrich |
| Precision Plus Protein™ Dual Color Standards | 1610374 | BIO-RAD |
| NuPAGE Sample Reducing Agent (10X) | NP0009 | Thermo Fisher Scientific |
| NuPAGE 4-12%BT midi 12 2 well PAGE gels | WG1401BOX | Thermo Fisher Scientific |
| iBlot™ 2 Transfer Stacks nitrocellulose regular size | IB23001 | Thermo Fisher Scientific |
| Immobilon Western Chemiluminescent HRP Substrate | WBKLS0500 | Sigma Aldrich |
| EZBlue™ Gel Staining Reagent | G1041 | Sigma Aldrich |
| Fisherbrand™ Cell Scrapers | 11587692 | Fisher Scientific |
| XCell4 SureLock™ Midi-Cell | WR0100 | Thermo Fisher Scientific |

MATERIALS TEXT

TBS/T for washes; [M]20 Milimolar (mM) Trizma base, [M]150 Milimolar (mM) NaCl, pH = 7.6 (tip: prepare a larger volume of 10X stock solution and dilute each time; use deionised $H_2O$).



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

SAFETY WARNINGS

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

BEFORE STARTING

**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, amd 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 phosphoproteincs and regular WB of cytoplasmic proteins)*

[M] **20 Milimolar (mM)** HEPES (pH = 7.4)
[M] **150 Milimolar (mM)** NaCl
[M] **1.5 Milimolar (mM)** $MgCl_2$
[M] **10 Volume Percent** Glycerol
[M] **1 Volume Percent** TritonX-100
[M] **1 Milimolar (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 Milimolar (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 Milimolar (mM)** * $Na_3VO_4$ (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 Milimolar (mM)** HEPES (pH = 7.4)
[M] **150 Milimolar (mM)** NaCl
[M] **1.5 Milimolar (mM)** $MgCl_2$
[M] **10 Volume Percent** Glycerol
[M] **1 Volume Percent** TritonX-100
[M] **1 Milimolar (mM)** EGTA (dissolves at 37 °C and pH 8, stirring required)
[M] **100 Milimolar (mM)** NaF
[M] **10 Milimolar (mM)** $Na_4P_2O_7$

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 Milimolar (mM)** * $Na_3VO_4$ (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 Milimolar (mM)** Tris-HCl (pH = 7.5)
[M] **150 Milimolar (mM)** NaCl
[M] **1 Milimolar (mM)** $Na_2EDTA$



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

[M] **1 Mass/Volume Percent** SDS

[M] **1 Mass/Volume Percent** Sodium deoxycholate

[M] **2.5 Milimolar (mM)** $Na_4P_2O_7$

[M] **1 Milimolar (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 Milimolar (mM)** * $Na_3VO_4$ (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.

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 30m

Incubate for minimum **00:30:00** on ice.

7 05s

Vortex each sample for **00:00:05** .

8 5m

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.

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



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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.

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 $H_2O$.

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 $H_2O$ 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.



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

15m

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

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 µl (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 µg/µl) | V for loading of 4 gels with 5 µg per well (prepare for 20 µg in 80 µl, load 20 µl 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.*

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

20 Heat samples at **70 °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).

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

22 Load each sample using gel loading tips; load **10 µl** 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** ).



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

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 stik 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: [M] **3 Mass/Volume Percent** 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 $H_2O$ for filter paper wetting, tissue paper, scalpel, forceps, transfer packs, iBlot2 machine.

25 When the gel has finished running, dissassemble the casette, 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 $H_2O$ 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 $H_2O$. 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 fride (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 



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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.

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.

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited