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Immunoblotting using precast gels

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Protocol status: Working
We use this protocol and it's working

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Abstract

Immunoblotting is a key technique to visualize changes in protein levels upon treatments. This technique can be challenging and established procedures are required to ensure reproducibility. Here we present our optimized protocol for immunoblotting of protein samples using precast gels and semiwet transfer. This protocol can be used to analyze samples from cell extracts and from in vitro reactions.

Protocol materials

Polysorbate 20 **Thermo Fisher Scientific Catalog #**233360010 In 2 steps

MES-SDS Buffer (20X) **Boston Bioproducts Catalog #**BP-177 Step 7

Tris Base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**648310 Step 14

Gel Knife **Thermo Fisher Catalog #**EI9010 Step 19

Amersham™ Protran® Western blotting membranes nitrocellulose **Merck Catalog #**GE10600041 Step 16

Ponceau S solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**Ponceau S solution Step 26

Glycine **Fisher Scientific Catalog #**BP381-500 In 2 steps

PowerPac™ HC Power Supply **Bio-Rad Laboratories Catalog #**1645052 In 2 steps

Grade 3MM Chr Blotting Paper, sheet, 46 × 57 cm **Cytiva Catalog #**3030-917 Step 17

Carnation Instant Non Fat Dry Milk **Amazon.com Catalog #**12428935 Step 29

Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**62862 Step 39

Fisher BioReagents™ Bovine Serum Albumin Heat Shock Treated **Fisher Scientific Catalog #**BP1600-100 Step 31

Western blot boxes, 3 1/2 x 2 9/16 x 1in. 8.9 x 6.5 x 2.5cm **Fisher Scientific Catalog #**NC1126730 Step 30

PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa **Thermo Fisher Catalog #**26620 Step 10

NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #**NP0007 Step 1

NuPAGE Sample Reducing Agent (10X) **Thermo Fisher Scientific Catalog #**NP0009 Step 1

XCell SureLock® Mini-Cell and XCell IITM Blot Module Kit **Invitrogen - Thermo Fisher Catalog #**EI0002 Step 9

NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well **Thermo Fisher Catalog #**NP0322BOX Step 8

SNAP id® 2.0 Blot Roller **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SNAP2RL Step 21

Immobilon-P PVDF Membrane, 0.45um, roll **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**IPVH00010 Step 16

Sponge Pad for Blotting **Thermo Fisher Catalog #**EI9052 Step 18

Methanol, 99.9%, for analysis **Thermo Fisher Scientific Catalog #**176840025 Step 15

Safety warnings

All steps must be performed using personal protective equipment including gloves and eye protection.

Sample preparation

1 Mix NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #**NP0007 and NuPAGE Sample Reducing Agent (10X) **Thermo Fisher Scientific Catalog #**NP0009 in a 7:3 ratio (for example: 70 µL LDS sample buffer and 30 µL of Reducing Agent).

2 In a 1.5 mL microcentrifuge tube add the following reagents in order: LDS sample buffer/Reducing Agent mix, IPMS lysis buffer, protein sample. For example.

	Sample volume	LDS sample buffer/Reducing Agent
	10 or 15 microliters	5 microliters
	20	7
	30	10

3 Poke hole in top of 1.5 mL microcentrifuge tube with a hypodermic needle.

4 Boil samples at 95 °C for 00:05:00 using a ThermoMixer F1.5. 5m

Equipment

Eppendorf ThermoMixer F1.5 NAME

ThermoMixer TYPE

Eppendorf BRAND

5384000020 SKU

https://www.eppendorf.com/us-en/Products/Temperature-Control-and-Mixing/Instruments/Eppendorf-ThermoMixerF-p-PF-133437 LINK

5 Centrifuge for 00:00:10 in a Mini Centrifuge at Room temperature 10s

Equipment

Fisherbrand Mini-Centrifuge	NAME
Centrifuge	TYPE
Fisherbrand	BRAND
12-006-901	SKU
https://www.fishersci.com/shop/products/fisherbrand-standard-mini-centrifuge/12006901#?keyword=	LINK

6 Samples can be stored at -20 °C until the day of running SDS-PAGE electrophoresis.

SDS-PAGE Electrophoresis

7 Prepare 500 mL 1X MES-SDS Buffer ([M] 50 millimolar (mM) MES, [M] 50 millimolar (mM) Tris Base, [M] 0.1 Mass / % volume SDS, [M] 1 Mass / % volume EDTA, pH 7.3) by diluting MES-SDS Buffer (20X) **Boston Bioproducts Catalog #**BP-177 stock 1:20 in Milli-Q water.

8 Unpack a NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well **Thermo Fisher Catalog #**NP0322BOX and remove protective tape and comb.

9 Assemble gel running tank XCell SureLock® Mini-Cell and XCell IITM Blot Module Kit **Invitrogen - Thermo Fisher Catalog #**EI0002 by placing gel in the tank in front of the buffer core and a second gel or a buffer dam behind the

buffer core. Lock the gel tension wedge in place. Add 1X MES-SDS buffer between the gels and outside the buffer core.

10 Load 8 µL protein molecular weight marker

PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa **Thermo Fisher Catalog #**26620

. If needed load 2 µL at the end of the gel.

11 Load samples to a maximum volume of 20 µL .

12 Place gel tank lid and connect to power supply

PowerPac™ HC Power Supply **Bio-Rad Laboratories Catalog #**1645052 matching colors (for positive and negative).

13 Run gel at 150 V for 02:00:00 at Room temperature or as needed depending on the protein molecular weight and desired separation. 2h

Transfer

14 Prepare 12X transfer buffer:

- 58 g Tris base Tris Base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**648310
- 190 glycine Glycine **Fisher Scientific Catalog #**BP381-500
- Milli-Q water up to 2 L

15 Prepare 2.5 L of 1X transfer buffer ([M] 48 millimolar (mM) Tris, [M] 39 millimolar (mM) Glycine, [M] 20 % (v/v) Methanol) by mixing 212 mL 12X Transfer buffer with 1788 mL milli-Q water and 500 mL Methanol

Methanol, 99.9%, for analysis **Thermo Fisher Scientific Catalog #**176840025 .

16 Cut 1m

Immobilon-P PVDF Membrane, 0.45um, roll **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**IPVH00010

or

Amersham™ Protran® Western blotting membranes nitrocellulose **Merck Catalog #**GE10600041

membrane to the required gel size (usually 9x7 cm) and activate PVDF membrane by incubating in methanol for at least 00:01:00 .

Note

Do not put Nitrocellulose membrane in methanol

17 Cut Grade 3MM Chr Blotting Paper, sheet, 46 × 57 cm **Cytiva Catalog #**3030-917 to the required gel size (usually 9x7 cm).

18 Prepare Sponge Pad for Blotting **Thermo Fisher Catalog #**EI9052 . If dirty, boil in warm tap water in the microwave for 00:05:00 . Soak sponge pads in 1X transfer buffer in a plastic tray. 5m

19 Remove the gel from the cassette by separating the cassette plates with a Gel Knife **Thermo Fisher Catalog #**EI9010 . Using the gel knife, cut off the bottom part and wells of the gel as needed.

20 Place gel in a plastic tray with 1X transfer buffer.

21 Make transfer sandwich inside the cathode core of the blot module in the following order:

- 2 sponge Pad
- 1 filter paper + gel (grab this from transfer buffer tray), Remove air bubbles by carefully rolling a
  SNAP id® 2.0 Blot Roller **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SNAP2RL
  on top of the gel.
- 1 presoaked PVDF or Nitrocellulose membrane. Remove air bubbles using the Blot Roller.
- 1 filter paper. Remove air bubbles using the Blot Roller.
- Sponges up to fill cavity (6-7 total)

22 Close blot module with the anode core and place this in the buffer chamber. Lock the blot module using the gel tension wedge.

23 Place lid and connect to power supply
PowerPac™ HC Power Supply **Bio-Rad Laboratories Catalog #**1645052 matching colors (for positive and negative).

24 Run gel at 35 V for 01:30:00 at Room temperature . 1h 30m

25 Unlock blot module by releasing the tension wedge and remove from buffer chamber. Remove membrane from the sandwich and cut excess membrane using scissors.

26 Incubate membrane in 5m
Ponceau S solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**Ponceau S solution
in a plastic tray for 00:05:00 with shaking in a rocking shaker.

Equipment

Rocking Shaker

NAME	Rocking Shaker
TYPE	Shaker
BRAND	Ohaus
SKU	30391966
LINK	https://us.ohaus.com/en-us/products/equipment/open-air-shakers/rocking-waving-shakers/shaker-rocking-2-tier-shrk07al2-us

27 Wash with milli-Q water 3 times, quick washes. Image in Chemidoc MP if needed.

Equipment

NAME	TYPE	BRAND	SKU
ChemiDoc™ MP Imaging System	Imaging System	Bio-rad	12003154

Blocking

1h

28 Make 1X TBS-T (Tris buffer saline-Tween 20: [M] 20 millimolar (mM) Tris-HCl pH=7.4, [M] 100 millimolar (mM) NaCl, [M] 0.1 % (v/v) Tween-20).

For 1L:

- 20 mL [M] 1 Molarity (M) Tris pH=7.4
- 20 mL [M] 5 Molarity (M) NaCl
- 1 mL Tween-20
  Polysorbate 20 **Thermo Fisher Scientific Catalog #**233360010
- Milli-Q water up to 1 L

29 Make Blocking solution: 1X TBS-T 5% skimmed milk:

- 12.5 g powder skimmed milk
  Carnation Instant Non Fat Dry Milk **Amazon.com Catalog #**12428935 .
- Fill up to 250 mL with 1X TBS-T.

30 Add Blocking solution to a Western Blot box

1h

Western blot boxes, 3 1/2 x 2 9/16 x 1in. 8.9 x 6.5 x 2.5cm **Fisher Scientific Catalog #**NC1126730

and place the membrane in this solution. Incubate in a rocking shaker for at least 01:00:00 at Room temperature

Primary antibody

1h

31 Prepare antibody in 1X blocking solution (for HRP detection) or in TBST

[M] 5 Mass / % volume BSA

Fisher BioReagents™ Bovine Serum Albumin Heat Shock Treated **Fisher Scientific Catalog #**BP1600-100

(for infrared detection) at the desired concentration.

32 Discard blocking solution from Western blot box and replace by diluted antibody. Incubate at 4 °C Overnight in rocking shaker. 20h

33 Next day recover antibody in tube and wash membrane 3 times for 00:05:00 with 1X TBS-T 5m

Secondary antibody

1h

34 Prepare HRP or fluorescently labelled secondary antibody in blocking solution or TBST respectively.

35 Add diluted secondary antibody to Western blot box a incubate at Room temperature for 01:00:00 in rocking shaker. 1h

36 Wash membrane 4 times for 00:05:00 with 1X TBS-T 5m

37 Image in Chemidoc MP for HRP detection or LI-COR Odyssey FC imager for infrared detection.

Equipment

ChemiDoc™ MP Imaging System	NAME
Imaging System	TYPE
Bio-rad	BRAND
12003154	SKU

Equipment

Odyssey CLx	NAME
Imaging System	TYPE
LI-COR	BRAND
Odyssey CLx	SKU
https://www.licor.com/bio/odyssey-clx/	LINK

38 Membrane can be stored up to a week at 4 °C until performing stripping.

Stripping

10m

39 Prepare Stripping buffer:

- weight 15 g glycine Glycine **Fisher Scientific Catalog #**BP381-500

- Dissolve in 800 mL milli-Q water
- Adjust pH to 2.2
- add 1 g SDS

  Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**62862
- 10 mL Tween 20 Polysorbate 20 **Thermo Fisher Scientific Catalog #**233360010
- Bring volume up to 1 L with milli-Q water

40 Incubate membrane 00:10:00 with stripping buffer in rocking shaker at Room temperature . 10m

41 Repeat step 37

42 Wash twice with TBST

43 go to step #28 and probe with a different antibody.