Aug 09, 2024

Immunoblotting using precast gels

DOI
https://dx.doi.org/10.17504/protocols.io.14egn6j8pl5d/v1

Francisco Bustos[1,2]

[1]Pediatrics and Rare Diseases Group, Sanford Research, Sioux Falls, SD, USA;
[2]Department of Pediatrics, Sanford School of Medicine, University of South Dakota, Sioux Falls, SD, USA

Francisco Bustos
Sanford Research

DOI: https://dx.doi.org/10.17504/protocols.io.14egn6j8pl5d/v1

Protocol Citation: Francisco Bustos 2024. Immunoblotting using precast gels. **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn6j8pl5d/v1

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

Created: August 07, 2024

Last Modified: August 09, 2024

Protocol Integer ID: 104903

Keywords: using precast gels immunoblotting, precast gels immunoblotting, protocol for immunoblotting, protein sample, immunoblotting, using precast gel, precast gel, changes in protein level, protein level, samples from cell extract, protein, cell extract

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

- PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa **Thermo Fisher** **Catalog #**26620
- PowerPac™ HC Power Supply **Bio-Rad Laboratories** **Catalog #**1645052
- NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific** **Catalog #**NP0007
- NuPAGE Sample Reducing Agent (10X) **Thermo Fisher Scientific** **Catalog #**NP0009
- Gel Knife **Thermo Fisher** **Catalog #**EI9010
- XCell SureLock® Mini-Cell and XCell IITM Blot Module Kit **Invitrogen - Thermo Fisher** **Catalog #**EI0002
- SNAP id® 2.0 Blot Roller **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**SNAP2RL
- Grade 3MM Chr Blotting Paper, sheet, 46 × 57 cm **Cytiva** **Catalog #**3030-917
- Carnation Instant Non Fat Dry Milk **Amazon.com** **Catalog #**12428935
- Immobilon-P PVDF Membrane, 0.45um, roll **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**IPVH00010
- Amersham™ Protran® Western blotting membranes nitrocellulose **Merck** **Catalog #**GE10600041
- Sponge Pad for Blotting **Thermo Fisher** **Catalog #**EI9052
- Ponceau S solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Ponceau S solution
- Fisher BioReagents™ Bovine Serum Albumin Heat Shock Treated **Fisher Scientific** **Catalog #**BP1600-100
- Glycine **Fisher Scientific** **Catalog #**BP381-500
- Sodium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**62862
- Polysorbate 20 **Thermo Fisher Scientific** **Catalog #**233360010
- Methanol, 99.9%, for analysis **Thermo Fisher Scientific** **Catalog #**176840025
- MES-SDS Buffer (20X) **Boston Bioproducts** **Catalog #**BP-177
- NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well **Thermo Fisher** **Catalog #**NP0322BOX
- Tris Base **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**648310
- Western blot boxes, 3 1/2 × 2 9/16 × 1in. 8.9 × 6.5 × 2.5cm **Fisher Scientific** **Catalog #**NC1126730

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 9×7 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 9×7 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 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. 5m

Equipment

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

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

Equipment

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

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 × 2 9/16 × 1in. 8.9 × 6.5 × 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.