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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

	0 In <u>2 steps</u>
MES-SDS Buffer (20X) Boston Bioproducts Catalog #BP-17	7 Step 7
	348310 Step 14
<b>⊠</b> Gel Knife <b>Thermo Fisher Catalog #</b> El9010 Step 19	
<b>⊠</b> Amersham™ Protran® Western blotting membranes nitrocellulo	ose Merck Catalog #GE10600041 Step 16
	Catalog #Ponceau S solution Step 26
PowerPac™ HC Power Supply Bio-Rad Laboratories Catalog	g #1645052 In <u>2 steps</u>
	<b>alog #</b> 3030-917 Step 17
★ Carnation Instant Non Fat Dry Milk Amazon.com Catalog #12	428935 Step 29
Sodium dodecyl sulfate Merck MilliporeSigma (Sigma-Aldric	<b>ch) Catalog #</b> 62862 Step 39
<b>⊠</b> Fisher BioReagents™ Bovine Serum Albumin Heat Shock Trea	ted 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 Fish	ner Scientific Catalog #NC1126730 Step 30
	Da <b>Thermo Fisher Catalog #</b> 26620 Step 10
	atalog #NP0007 Step 1
	tific Catalog #NP0009 Step 1
XCell SureLock® Mini-Cell and XCell IITM Blot Module Kit Invit	trogen - Thermo Fisher Catalog #EI0002 S
NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well     ■     Tris Protein Gels, 1.0 mm, 12-well     Tris Protein	Thermo Fisher Catalog #NP0322BOX Step 8
SNAP id® 2.0 Blot Roller Merck MilliporeSigma (Sigma-Aldri	ch) Catalog #SNAP2RL Step 21
	igma (Sigma-Aldrich) Catalog #IPVH00010
Step 16	
Sponge Pad for Blotting <b>Thermo Fisher Catalog #</b> El9052 St	tep 18
Methanol, 99.9%, for analysis Thermo Fisher Scientific Catal	log #176840025 Step 15

# Safety warnings

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



## Sample preparation

- 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: Δ 70 μL LDS sample buffer and Δ 30 μL of Reducing Agent).
- 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.

Eppendorf ThermoMixer F1.5

ThermoMixer

Eppendorf

Eppendorf

Eppendorf

SRAND

5384000020

SKU

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

5m



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

Fisherbrand Mini-Centrifuge

Centrifuge

TYPE

Fisherbrand

12-006-901

https://www.fishersci.com/shop/products/fisherbrand-standard-minicentrifuge/12006901#?keyword=

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

# \* 1

## SDS-PAGE Electrophoresis

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 #El0002

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 4 8 µL protein molecular weight marker
  - PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa **Thermo Fisher Catalog #**26620
- Place gel tank lid and connect to power supply

  PowerPac™ HC Power Supply Bio-Rad Laboratories Catalog #1645052 matching colors (for positive and negative).
- Run gel at 150 V for 02:00:00 at Room temperature or as needed depending on the protein molecular weight and desired separation.

### Transfer

- 14 Prepare 12X transfer buffer:
  - 58 g Tris base X Tris Base Merck MilliporeSigma (Sigma-Aldrich) Catalog #648310
  - 190 glycine Scientific Catalog #BP381-500
  - Milli-Q water up to 🚨 2 L
- 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

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

or

1m

2h



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).
- Prepare Sponge Pad for Blotting Thermo Fisher Catalog #El9052 . 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.
- Remove the gel from the cassette by separating the cassette plates with a
  Gel Knife Thermo Fisher Catalog #El9010

  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



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



5m



23 Place lid and connect to power supply

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

Run gel at 35 V for 01:30:00 at 8 Room temperature .

1h 30m

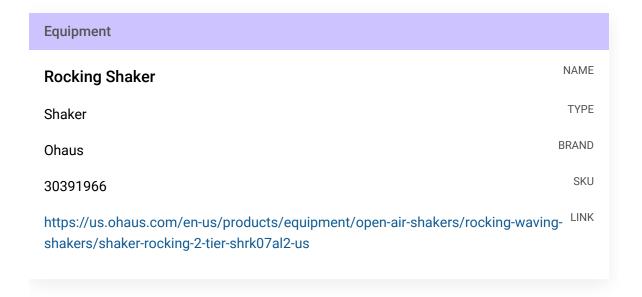
Unlock blot module by releasing the tension wedge and remove from buffer chamber. Remove membrane from the sandwich and cut excess membrane using scissors.

26 Incubate membrane in

5m

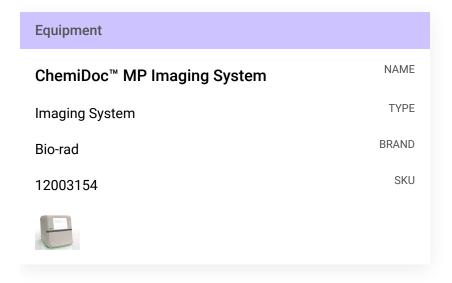
Ponceau S solution Merck MilliporeSigma (Sigma-Aldrich) Catalog #Ponceau S solution

in a plastic tray for 00:05:00 with shaking in a rocking shaker.



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





# **Blocking**

28 Make 1X TBS-T (Tris buffer saline-Tween 20: [M] 20 millimolar (mM) Tris-HCl pH=7.4,

[м] 100 millimolar (mM) NaCl, [м] 0.1 % (v/v) Tween-20).

#### For 1L:

- △ 20 mL [M] 1 Molarity (M) Tris pH=7.4
- 4 20 mL [M] 5 Molarity (M) NaCl
- Д 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

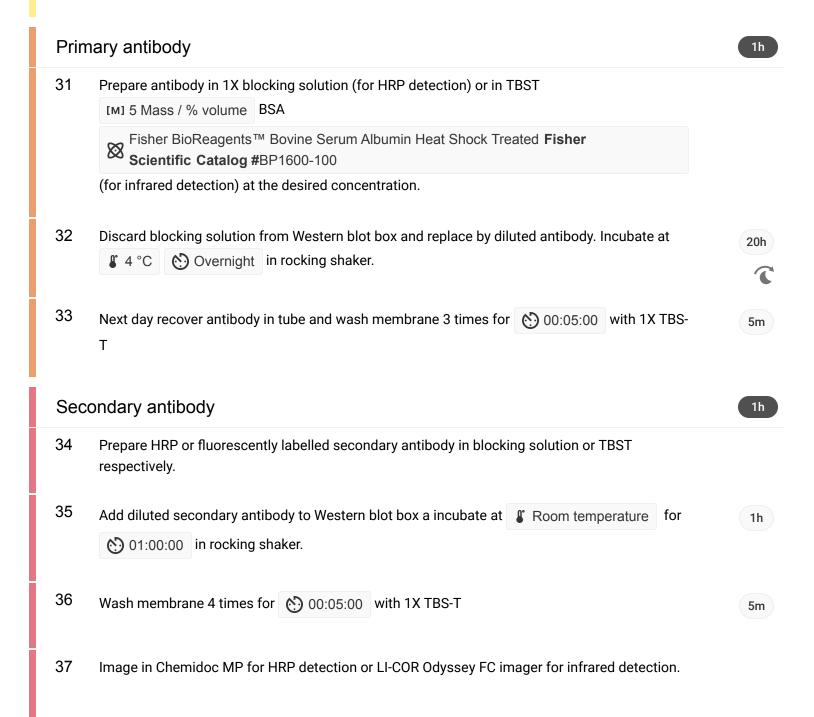
1h

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

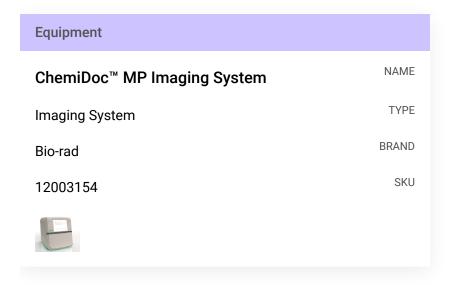
and place the membrane in this solution. Incubate in a rocking shaker for at least

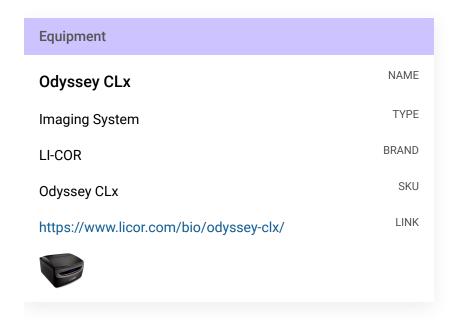
01:00:00 at 8 Room temperature











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



# Stripping

10m

39 Prepare Stripping buffer: 2

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



- Dissolve in <u>A</u> 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.