Antibody Screening by Immunoblot

YCharOS Standard Operating Procedure

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Table of contents

1. Introduction

1.1 Objectives

This protocol describes how to screen commercial antibodies by immunoblot using wildtype (WT) and knockout (KO) cell lysates from expressing cell line background.

1.2 Protocol overview

Cell lines used for antibody screening are selected based on their adequate expression using DepMap [1] portal and/or PaxDB [2] database(s). A KO line is then generated. Both WT and KO cells are lysed in a denaturing lysis buffer (RIPA). Proteins are separated on a polyacrylamide gel and transferred to a nitrocellulose membrane. These membranes are incubated with commercial primary antibodies and then with HRP secondary antibodies. A specific band is detected in the parental cell lysis and should disappear in the KO lysate.

1.3 Technical and safety considerations

The following information should be read before starting the procedure:

- ◊ Cell lines must be handled using a Class II biosafety laminar flow hood to create a sterile environment for cell culture experiments and to protect the worker from possible biohazards. McGill University Environmental Health and Safety (EHS) office regulations must be followed.
- ◊ Personal Protective Equipment (PPE), such as lab coat, face mask and disposable gloves, must be worn while handling cell lines.
- ◊ Avoid exposing the cells to prolonged periods outside the incubator (cells should be kept most of the time at the 37°C with 5% CO_2).
- ◊ When freezing or thawing cells, avoid keeping cells in freezing media outside the freezer for prolonged periods.
- ◊ Antibodies should be aliquoted and kept at -20°C.
- ◊ Cell lysates can be prepared, aliquoted and stored long term at - 80°C or short term at - 20°C.

1.4 Abbreviation list

A	Ampere
APS	Ammonium Persulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
cm	Centimeter

CO_2	carbon dioxide
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DOC	sodium deoxycholate
dpi	dots per inch
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EHS	McGill University Environmental Health and Safety
FBS	Fetal bovine serum
g	Gram
HCl	Hydrochloric Acid (Hydrogen Chloride)
HRP	horseradish peroxidase
kDa	Kilodalton
KO	Knockout
L	Liter
LSB	Laemmli Concentrate Sample Buffer
M	Molar
ml	Milliliter
mm	Millimeter
mM	Millimolar
MW	molecular weight marker
NaCl	sodium chloride
NaH_2PO_4	Sodium Phosphate Monobasic Anhydrous
NaOH	Sodium Hydroxide
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PPE	Personal Protective Equipment
RIPA	Radioimmunoprecipitation assay buffer
rpm	revolutions per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
TBST	TRIS buffer solution with 0.1% Tween 20
TEMED	Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
V	Volt
WT	Wild Type
µm	Micrometer

2. Materials

2.1. Labware

Item	Supplier, Catalogue number
0.22µm bottle top filter	VWR, 73520-994
0.22µm syringe filter	VWR, CA28145-477
0.5ml screw cap microtube	Sarstedt, 72.730.006
1.5ml microtube	
100 mm Cell Culture Dish	VWR, 10062-880
10ml plastic serological pipets	VWR, 89130-910
150 mm Cell Culture Dish	Fisher, 08-772-6
15ml conical tube	Sarstedt, 62.554.002 and VWR, 89039-664
2ml microtube	
3.5 mL, Open-Top Thickwall Polycarbonate Tube	Beckman Coulter, 349622
50ml conical tube	VWR, 89039-658 and 82050-346
5ml plastic serological pipets	VWR, 89130-908
Autoradiography films	Harvard bioscience, DV-E3018
Ballpoint Pen	Staples, Pentel BK90-B
Benchtop cooler	Fisher, 355501
Cell Counting Slides for TC10™/TC20™ Cell Counter	Bio-rad, 1450015
Cell scraper	Sarstedt, 83.1832
Flat plastic bag	N/A
Glass Low-Form Griffin Beakers	
Gloves	Fisher, 19-048-134
Hemacytometer	
Individually wrapped 10ml plastic serological pipet	VWR, 89130-898
Individually wrapped 1ml plastic serological pipet	VWR, 76097-922
Individually wrapped 25ml plastic serological pipet	VWR, 89130-900
Individually wrapped 5ml plastic serological pipet	VWR, 89130-896
Lab coat	VWR, CA10815-732
Markers	Fisher, 13-379-4
Microtiter plate	VWR, 82050-771
Pipet controller	Diamed, TECPIPAID
Plastic container	N/A
Polypropylene Griffin low-form plastic beaker	Fisher, 1201-4000
Scalpel	

Scissors	
Self-sticking labelling tape	Fisher, 159015R
Single-Channel Variable Volume Pipettor 0.2–2 µL	VWR, 89079-960
Single-Channel Variable Volume Pipettor 100–1000 µL	VWR, 89079-974
Single-Channel Variable Volume Pipettor 2–20 µL	VWR, 89079-964
Single-Channel Variable Volume Pipettor 20–200 µL	VWR, 89079-970
Syringe	
T175 Cell Culture Treated Flasks	12-556-010
Thumb forceps	
Tips	Sarstedt, 70.3050.205

2.2. Reagents

Item	**Supplier, Catalogue number**
0.53mM EDTA	Wisent, 325-060-EL
2-Mercaptoethanol, 99%, pure	Fisher, AC125472500
Acrylamide	Bioshop, ACR010.502
Ammonium Persulfate	Fisher, BP179-100
Aprotinin	Sigma, A1153
Benzamidine, Hydrochloride	VWR, CA80057-856
Bromophenol blue	Sigma, BX1410
BSA powder	Wisent, 800-095-CG
DMEM	Fisher, SH30081.01
DMSO	Sigma, D8418-100ML
DOC	Sigma, D6750
Donkey anti-Goat HRP	Santa Cruz, sc-2020
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher, E478-500
Fetal Bovine Serum (FBS)	Wisent, 080450
Glycine	Wisent, 800-045-IK
Goat anti-Mouse HRP	Fisher, 65-6120
Goat anti-Rabbit HRP	Fisher, 62-6520
Goat anti-Rat HRP	Jackson ImmunoResearch, 112-035-003
Hydrochloric Acid, 36.5 to 38.0% (HCl)	Fisher, A144-212
L-Glutamine	Wisent, 609-065-EL
Leupeptin	Sigma, L8511
Methanol	VWR, camx0485-5

n,n,n',n'-tetramethylethylenediamine (TEMED)	Fisher, BP150-100
Nitrocellulose membrane	
Penicillin/ Streptomycin	Wisent, 450-201-EL
Phenylmethanesulfonyl fluoride (PMSF)	Sigma, P7626-5G
Pierce™ BCA Protein Assay Kit	Fisher, 23225
Pierce™ ECL Western Blotting Substrate	Fisher, PI32106
Ponceau S powder	Fisher, BP103-10
Prestained molecular weight marker	FroggaBio, PM007-0500K
Skim milk powder	Bioshop, SKI400.1
Sodium Chloride (NaCl)	VWR, 97063-366
Sodium dodecyl sulfate (SDS) ≥99.0%	VWR, CA-EM7910
Sodium Hydroxide (NaOH)	Fisher, S318-1
Sodium Phosphate Monobasic Anhydrous (NaH_2PO_4)	Fisher, BP329-1
Sucrose	Fisher, S5-500
SuperSignal™ West Femto Maximum Sensitivity Substrate	Fisher, PI34096
Trichloro acetic acid	Fisher, SA433-500
TRIS base	VWR, 97062-420
Triton X-100	Fisher, BP151-500
Trypsin/ EDTA	Wisent, 325-542-EL
Tween 20	VWR, CA97062-332
Whatman paper	VWR, 28298-020

2.3. Equipment

Item	**Supplier, Catalogue number**
-20 Freezer	
Balance	Ohaus, SP6001
Bright field microscope	N/A
ChemiDoc Imaging System	Bio-rad, 12003153
Class II Type A/B3 Biological Safety Cabinet, Model 1286	Thermo, 19351
CO_2 cylinder	
CO2 Incubator	Fisher, 3110
Comb 12 well 1.5MM	Hoefer, SE511-12-1.5
Cryo-boxes	
Cytiva SG100 Gradient Maker	Fisher, 36-100-3970

Cytiva TE Blotting Unit, Cassettes (Cytiva TE44H)	Fisher, 36-100-3959
Developing machine	
Divider plate, Notched	Hoefer, SE 102 D
DLUX DL cooled Vertical electrophoresis box	
Dry Bath/ Block Heater	
Fridge	
Fume hood	
Gel caster	Hoefer, SE600
Glass plates	Hoefer, SE 102 D
Heat sealer	
Hoefer™ SE 600 Series Complete Vertical Electrophoresis System	Fisher, 03-500-101
Hoefer™ TE Blotting Unit: Safety Lids	Fisher, Catalog No.36-100-3954
Hoefer™ TE56 Transfer chamber	Fisher, 03-500-298
Hot Plate Stirrer	Fisher, SP88857204
Ice maker	
Liquid nitrogen storage, Rack and Box Systems	Fisher, CY509108
Lower buffer chamber	Hoefer, SE6150
Megafuge Refrigerated Centrifuge	N/A
Nutating Mixer	Fisher, 88861041
pH Meter	N/A
Plate reader	
Platform Rocker	Fisher, M79735Q
Power supply	VWR, 95017-278
Scanner	
Sonicator	
Sorvall Legend Micro 21R Centrifuge, Refrigerated	Fisher, 75002447
Sorvall™ Legend™ Micro 21 Microcentrifuge	Fisher, 75002437
Spacers	Hoefer, SE611921.5
TC20™ Automated Cell Counter	Bio-rad, 72.730.006
Thermo Scientific™ Forma™ 89000 Freezer	Fisher, 89-600-86D
Ultracentrifuge	Beckman Coulter, Optima Max Ultracentrifuge
Upper buffer chamber	Fisher, SE6054
Water bath	N/A

3. Cell culture

3.1. Media preparation

3.1.1 Complete media

◊ Please refer to the cell line's datasheet to find the appropriate type of medium to be used.

To 500ml DMEM (Dulbecco's Modified Eagle's Medium), add:

- 50ml FBS (10%)
- 5ml of 200mM L-Glutamine (2mM)
- 5ml of 100x Penicillin/ Streptomycin (100U/ml)
- Store at 4°C
- Warm the media at 37°C before use

3.1.2 Freezing media

To 35ml DMEM, add:

- 10ml of FBS (20%)
- 5ml of DMSO (10%)

3.2. Method

◊ Cell lines must be handled using a Class II biosafety laminar flow hood. All media, solutions and materials must be sterile.

The following method is used to expand adherent cells:

a) Grow cells at 37°C with 5% CO_2, in 20ml appropriate medium, in a T175 cell culture flask to 80% confluence.
b) Wash cells twice with 5 ml of 0.53 mM EDTA.
c) Add 5ml of Trypsin/EDTA and place the flask in the incubator for ~2 minutes until the cells detach.
d) Collect the cells in trypsin and transfer to a 50 ml conical tube containing 5 ml of serum.
e) Spin the cells at low speed (~1200rpm) for 5 minutes and discard the medium.

- To expand the cells (every 3-4 days): suspend the pellet in 20 ml of fresh complete medium and transfer 1-2 ml to each flask containing 20 ml of medium.
  If a specific number of cells must be seeded, count using an automated cell counter or a hemacytometer.

  When plating for cell lysis, use cell culture dishes (convenient for cell scraping).

- To freeze the cells: suspend in 1 ml of freezing medium and freeze in a screw cap microtube (1 ml for $1x10^6$ cells/ vial).

4. Cell lysate preparation

4.1. Solutions and buffers

4.1.1 Protease inhibitors

Aprotinin/ Leupeptin

- Dissolve 10mg of Aprotinin powder in 1ml of MiliQ water
- Dissolve 10mg of Leupeptin powder in 1ml of MiliQ water
- Pool together and aliquot to 20µl (*5mg/ml*)
- Store at -20°C

Benzamidine

To 100ml MiliQ water,

- Add 1g of Benzamidine powder (*64 mM*)
- Filter using a 0.22µm syringe filter
- Store at 4°C

PMSF

To 100ml of 100% Ethanol,

- Add 1g of PMSF (*10mM* or *500x*)
- Dissolve and store at room temperature (RT)

4.1.2 RIPA lysis buffer

1M TRIS-HCL

To 800ml of MiliQ water, add:

- 121.14g of Tris
- Titrate to pH 7.4 with HCl
- Complete volume to 1L with MiliQ water
- Autoclave and store at RT

4M NaCl

- To 500ml MiliQ water, add 116.88g of NaCl
- Dissolve and store at RT

0.5M EDTA

In a glass beaker, to 800ml MiliQ water, add:

- 146g of EDTA powder
- Titrate to pH 8 using NaOH

- Heat on a hot plate stirrer to dissolve
- Autoclave and store at RT

10% SDS

- In a glass beaker, add 50g of SDS powder to 500ml of MiliQ water
- Heat on a hot plate stirrer to dissolve and store at room temperature

10% Triton X-100

- To 450ml MiliQ water, add 50ml of Triton X-100
- Dissolve and store at 4°C

RIPA Buffer

◊ RIPA buffer can be prepared in advance, aliquoted and stored at -20°C. However, triton 1% and protease inhibitors must be freshly added before use.

To a beaker containing 400ml of MiliQ water, add:

- 25ml of 1M TRIS-HCl, pH 7.4 (*50 mM*)
- 18.75 ml of 4M NaCl (*150 mM*)
- 1ml of 0.5M EDTA (*1 mM*)
- 2.5g of DOC (*0.5%*)
- 5 ml of 10% SDS (*0.1%*)
- Titrate to pH 7.4
- Complete volume to 500 ml in MiliQ water
- Aliquot to 9ml in 15ml conical tubes and store at -20°C
- *Before use,* add 1% Triton X-100 (1ml of 10% Triton X-100 to 9ml RIPA buffer) and the following protease inhibitor mix:

- 2µl of Aprotinin/ Leupeptin (*1 µg/ml*)

- 100µl of benzamidine (*0.64 mM*)

- 20µl of PMSF (20 *µM*)

4.1.3 PBS 5x

To a beaker containing 800ml of ddH_2O, add:

- 45g of NaCL (*770mM*)
- 34.5g of NaH_2PO_4 monobasic (*288mM*)
- Titrate to pH 7.4
- Complete volume to 1 L in ddH_2O

- Autoclave and store at room temperature

4.2. Method

a) Place the cell culture dish on ice and wash the cells 3x with ice cold 1x PBS.
b) Discard the PBS and add ice-cold RIPA lysis buffer (1 ml per 150 mm dish).
c) Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube.
(To keep the cell scraper cold, place it in cold water on ice before starting cell lysis)
d) Sonicate the cells at 40% amplitude for 15 sec.
e) Maintain constant agitation for 30 min at 4°C.
f) Centrifuge at ~110,000xg for 15 min at 4°C using an ultracentrifuge.
g) Gently remove the tubes from the rotor and place on ice, transfer the supernatant to a fresh tube kept on ice, and discard the pellet.
h) Measure protein concentration using a BCA protein assay kit.

◊ The concentration of total lysates to load on the gel will vary depending on the expression of the target by the used cell line.

5. Gel electrophoresis and transfer

5.1. Solutions and buffers

5.1.1 Laemmli 5x Concentrate Sample Buffer (LSB5x)

SDS 30%

To 100ml of MiliQ water in a glass beaker, add

- 30g of SDS powder
- Heat on hot plate stirrer to dissolve
- Store at room temperature
◊ Before use, gently warm at 37°C in a water bath

2M Sucrose

To 500ml MiliQ water,

- Add 342g of sucrose powder
- Store at 4°C

2M TRIS-HCl pH 6.8

To 500ml MiliQ water,

- Add 121.14g of TRIS base
- Titrate to pH 6.8 with HCl

- Store at room temperature

LSB5x

For 50ml, add:

- 25ml of 30% SDS
- 14.38ml of 2M Sucrose
- 8.12ml of 2M TRIS-HCl pH 6.8
- 2.5ml of 99% β-mercaptoethanol
- A touch of bromophenol blue
- Aliquot and store at room temperature

5.1.2 Separating buffer 4x

To a beaker containing 800ml of ddH_2O, add:

- 182g of TRIS Base (*1.5M*)
- 4g of SDS (*0.4%*)
- Titrate to pH 8.7 with HCl
- Complete volume to 1L with ddH_2O
- Filter using a 0.22µm bottle top filter
- Store at room temperature

5.1.3 Stacking buffer 4x

To a beaker containing 800ml of ddH_2O, add:

- 60.5g of TRIS base (*0.5M*)
- 4g of SDS (*0.4%*)
- Titrate pH to 6.8 with HCl
- Complete volume to 1L with ddH_2O
- Filter using a 0.22µm bottle top filter
- Store at room temperature

5.1.4 APS 10%

To a beaker containing 50ml of ddH_2O, add:

- 5g of Ammonium Persulfate powder
- Protect from light and store at 4°C

5.1.5 Running buffer 10x

To a beaker containing 3L of ddH_2O, add:

- 120.8g of TRIS base
- 576g of Glycine
- 40g of SDS

- Complete volume to 4L with ddH_2O
- Store at room temperature

5.1.6 Transfer Buffer

◊ Prepare fresh before use.

To 4L of ddH_2O, add:

- 15.15g of TRIS base
- 73g of Glycine
- 1L of Methanol

5.1.7 Ponceau S

To 485ml of ddH_2O, add:

- 1g of Ponceau S powder
- 15ml of Trichloro acetic acid
- Protect from light and keep at room temperature.

5.2. Method

5.2.1 Samples and gel preparation

a) Adjust protein concentrations to load equal amounts of protein in all lanes: 10-200µg of lysate per lane (depending on the target).
b) Add the right volume of 5x LSB for an identical final concentration of LSB in all samples (usually LSB1x or 2x final).
c) Prepare the molecular weight marker sample similarly.
d) Gently heat the samples at 65°C for 10 minutes in a heat block (dry bath).
e) Prepare the appropriate gradient acrylamide gel for your target protein.

Large 5–16% TRIS-HCl gradient gels are used for targets below 200 KDa

Large 3-12 % TRIS-HCl gradient gels are used for targets larger than 200 KDa

To make one large separating gel (30ml total; 15ml of each gradient mixture), add:

Percent Acrylamide	**3% - 12%**		**5% - 16%**	
ddH_2O	9.65ml	5.15ml	8.75ml	3.25ml
30% Acrylamide	1.5ml	6ml	2.5ml	8ml
4x Separating buffer	3.75ml	3.75ml	3.75ml	3.75ml

10% APS	112.5µl	112.5µl	112.5µl	112.5µl
TEMED	5.5 µl	5.5 µl	5.5 µl	5.5 µl

To make one large stacking gel, add:

Percent Acrylamide	**Always 3%**
ddH_2O	6.65ml
30% Acrylamide	1.15ml
4x Stacking buffer	2.5ml
10% APS	62.5µl
TEMED	7.5 µl

f) Load samples on the gel as following: prestained molecular weight marker (MW), lysate 1 (WT), lysate 2 (KO).
g) Run the gel until the front dye reaches 3 mm from the bottom.
(A large gel running at 45V takes approximately 18 hours or 4h at 260V)

5.2.2 Transfer to nitrocellulose membrane

a) Prepare transfer buffer.
b) Disassemble and discard the stacking part of the gel. Prepare your transfer sandwich in a large container filled with running buffer or water.
c) Once the sandwich is ready, close your cassette and insert it in the transfer box (can hold up to four cassettes). Fill with 4L of running buffer + 1L of 100% methanol and run at 1A (ampere) for 2 hours and 30 minutes.
d) After the transfer is done, gently remove the nitrocellulose membrane and stain for 2 minutes in Ponceau S solution (make sure the surface is all covered with Ponceau).
e) Dip three times in clean water to remove excess dye solution. Let the membrane dry on Whatman paper then mark with a pen.
f) Place in a flat plastic bag and scan as a tiff 300 dpi using a scanner.
g) Cut the membrane using a scalpel or scissors.
h) Proceed with blocking and antibody incubation or preserve the dry membrane in a flat plastic bag for future use.

6. Western blotting

6.1. Solutions and buffers

6.1.1 TRIS buffer solution with 0.1% Tween 20 (TBST)

For 10x TBS: to a beaker containing 800ml of ddH_2O, add:

- 24.23g of TRIS base (*200mM*)
- 87.66g of NaCl (*1500mM*)
- Titrate to pH 7.4 with HCl
- Complete volume to 1L with ddH_2O
- Before use, dilute to 1x TBS in ddH2O

For 1x TBST: add 1 ml of Tween20 to 1L of 1x TBS (0.1% Tween20)

6.1.2 Milk 5%

- Add 5g of non-fat milk powder to 100ml of 1x TBST (prepare fresh before use)

6.1.3 BSA 5%

- Add 5g of BSA powder to 100ml of 1x TBST (prepare fresh before use)

6.2. Method

6.2.1 Blocking

a) Place blot strips in a plastic container and add 5% Milk blocking buffer for 1 hour at room temperature.
b) Rinse the membrane with 1x TBS to remove excess milk.

6.2.2 Antibody incubation

a) Prepare the primary antibody dilution in 5% BSA buffer.
b) Place each bot strip in a resealable flat plastic bag, add the primary antibody dilution and seal the bag.
c) Incubate overnight at 4°C with agitation.
d) Cut open the bag and remove the primary antibody. Place the blot strip in a plastic container and wash three times for 10 minutes in 1x TBST.
e) Prepare the secondary antibody dilution in 5% Milk blocking buffer.
f) Incubate the blot with secondary antibody in a sealed plastic bag for 1 hour at room temperature.
g) Wash three times with constant rocking for 10 minutes in 1x TBST.
h) Transfer the membrane on a clean plastic bag and incubate with Pierce™ ECL Western Blotting Substrate for 1 minute.

i) Expose western blots in a chemiluminescence imager or in a dark room using a developing machine.

7. References

1. *DepMap, Broad*. 2019.
2. Wang, M., et al., *Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines.* Proteomics, 2015. **15**(18): p. 3163-8 DOI: 10.1002/pmic.201400441.