Western Blot Protocol

Complete Protocol from Cell Lysis to Band Visualization

Overview

Western blotting (immunoblotting) detects specific proteins in cell or tissue lysates through protein separation by size, transfer to membrane, and antibody-based detection. The complete procedure takes approximately 2-3 days.

SAFETY

RIPA buffer contains SDS and detergents. β-mercaptoethanol is toxic with strong odor. Acrylamide is a neurotoxin. Always wear gloves, lab coat, and safety glasses. Work in fume hood when handling β-ME. Consult Safety Data Sheets before use.

Day 0: Preparation

- Prepare inhibitor stocks
- Make base RIPA buffer
- Prepare working buffers

Day 1: Lysis & Transfer

- Cell lysis & BCA assay
- Cast and run gels
- Overnight protein transfer

Day 2: Antibodies & Detection

- Block membrane
- Primary & secondary antibodies
- Detection and imaging

Sample Requirements

Sample Type	RIPA Buffer Volume	Expected Yield
6-well plate (1 well, 80-100% confluent)	60-80 µL	100-300 µg
60 mm dish (80-100% confluent)	100-200 µL	200-500 µg
100 mm dish (80-100% confluent)	300-500 µL	500-1500 µg

Required Materials

Equipment

- Refrigerated microcentrifuge (4°C)
- Cell scrapers
- 37°C incubator (for BCA)
- Plate reader (for BCA assay)
- Gel casting apparatus
- Electrophoresis power supply and tank
- 95°C heat block
- Transfer apparatus and power supply
- Rocker/shaker platform
- Dark room or gel imaging system

RIPA Buffer Components

- Tris-HCl pH 7.6 (1 M stock)
- NaCl (5 M stock)
- Sodium Deoxycholate powder
- SDS powder (10% stock available)
- NP-40 (Igepal CA-630)
- cOmplete™ EDTA-free Protease Inhibitor tablets
- PhosSTOP™ Phosphatase Inhibitor tablets
- DTT (optional)

Gel Electrophoresis Reagents

- 40% Acrylamide mix
- 1.5M Tris-HCl pH 8.8
- 0.5M Tris-HCl pH 6.8
- 10% SDS
- 10% APS (fresh weekly)
- TEMED
- n-Butanol
- 10X Tris/Glycine/SDS running buffer
- 5X Loading dye
- β-mercaptoethanol
- Precision Plus Protein Ladder

Antibody Reagents

- 10X TBS-T buffer
- Ponceau S stain
- Evaporated milk powder
- BSA crystals
- Primary antibodies (-20°C)
- Secondary antibodies (-20°C)
- HRP Peroxide Solution
- HRP Luminol Reagent

Day 0: Preparation | Day 1: Lysis & Gel | Day 1 Evening: Transfer | Day 2: Antibodies | Day 2-3: Detection

Day 0: Preparation

Prepare Protease/Phosphatase Inhibitor Stocks

Mark Done

25X Protease Inhibitor Stock

1. Make 4-8 mL at a time
2. Add 1 cOmplete™ tablet per 2 mL autoclaved water
   - For 4 mL: 2 tablets + 4 mL water
   - For 8 mL: 4 tablets + 8 mL water
3. Vortex vigorously until fully dissolved
4. Aliquot 200-400 µL into 1.5 mL tubes
5. Label: "25x Protease Inhibitor" + date
6. Store at -20°C
7. **Use one aliquot per experiment, discard after thawing**

20X Phosphatase Inhibitor Stock

1. Make 4-8 mL at a time
2. Add 1 PhosSTOP™ tablet per 2 mL autoclaved water
   - For 4 mL: 2 tablets + 4 mL water
   - For 8 mL: 4 tablets + 8 mL water
3. Vortex vigorously until fully dissolved
4. Aliquot 200-400 µL into 1.5 mL tubes
5. Label: "20x Phosphatase Inhibitor" + date
6. Store at -20°C
7. **Use one aliquot per experiment, discard after thawing**

> **CRITICAL: Inhibitor aliquots are stable at -20°C for 1 year. Always use fresh aliquots - do not refreeze after thawing!**

Prepare Base RIPA Buffer (100 mL)

Mark Done

Component	Final Concentration	Amount for 100 mL
Tris-HCl pH 7.6	25 mM	2.5 mL of 1 M stock
NaCl	150 mM	3 mL of 5 M stock
Sodium Deoxycholate	1% (w/v)	1 g
SDS	0.1% (w/v)	0.1 g
NP-40	1% (v/v)	1 mL

Component	Final Concentration	Amount for 100 mL
Na_3VO_4 (Sodium orthovanadate)	1 mM	100 µL of 1 M stock
ddH_2O	-	to 100 mL

1. Add ~80 mL ddH_2O to beaker with stir bar
2. Add Tris-HCl and NaCl stock solutions
3. Add sodium deoxycholate powder, stir until dissolved
4. Add SDS powder, stir until dissolved (will foam - this is normal)
5. Add NP-40, mix well
6. Add Na_3VO_4 (phosphatase inhibitor), mix well
7. Adjust pH to 7.6 if needed
8. Bring to 100 mL final volume with ddH_2O
9. Store at 4°C for up to 6 months
10. **Do NOT add protease inhibitors until day of use**

Alternative: Strong RIPA Buffer (for difficult-to-lyse samples)

Use this stronger formulation when working with tough cells, tissues, or nuclear proteins. Contains additional chelating agents and built-in phosphatase inhibitors.

Component	Final Concentration	Amount for 100 mL
Tris-HCl pH 7.4	50 mM	5 mL of 1 M stock
NaCl	150 mM	3 mL of 5 M stock
Sodium Deoxycholate	1% (w/v)	1 g
SDS	0.1% (w/v)	0.1 g
Triton X-100	1% (v/v)	1 mL
EDTA	0.5 mM	50 µL of 1 M stock
Na_3VO_4 (Sodium orthovanadate)	1 mM	100 µL of 1 M stock
NaF (Sodium fluoride)	50 mM	5 mL of 1 M stock
ddH_2O	-	to 100 mL

Key differences from standard RIPA:

- Higher Tris concentration (50 mM vs 25 mM) for better buffering
- Triton X-100 instead of NP-40 (stronger detergent)
- EDTA added (chelates divalent cations, protects from metalloproteases)
- Na_3VO_4 and NaF included (phosphatase inhibitors built-in)
- Best for: tough cells, tissue samples, nuclear proteins, phosphoproteins

Note: With built-in Na_3VO_4 and NaF, you may reduce added phosphatase inhibitor on day of use, but still add protease inhibitors as usual.

Reference: doi:10.17504/protocols.io.dky4xv

Day 1: Cell Lysis, BCA, and Gel Running

Prepare Working RIPA Buffer

Mark Done

For 1 mL working RIPA:

Base RIPA buffer (from 4°C)	**900 µL**
25X Protease Inhibitor	**40 µL**
20X Phosphatase Inhibitor	**50 µL**
1 M DTT (optional)	**1 µL**
Total	**~1 mL**

Keep on ice, use within 2-4 hours

Lyse Cells

Mark Done

RIPA Volumes by Plate Size:

Plate/Flask Size	RIPA Volume
6-well (1 well)	60-80 µL
60 mm dish	100-200 µL
100 mm dish	300-500 µL
T-75 flask	750-1000 µL

1. **Pre-chill centrifuge to 4°C**

2. Place culture dish on ice

3. Remove media and wash 2x with ice-cold PBS

4. Remove all PBS (residual PBS dilutes buffer)

5. Add appropriate volume of RIPA buffer

6. Scrape cells thoroughly in multiple directions

7. Transfer lysate to pre-labeled 1.5 mL tube on ice

8. Incubate for lysis:
   - Option A (Preferred): Rotating wheel at 4°C for 30 min
   - Option B: On ice for 30 min, vortex every 10 minutes

9. Centrifuge at 14,000 × g for 20 min at 4°C

10. Transfer supernatant to fresh pre-chilled tube on ice

11. Discard pellet (cell debris, nuclei)

> **NOTE: Can store lysate at -80°C at this point or proceed immediately to BCA assay**

BCA Protein Quantification

Mark Done

1. Dilute samples 1:100 in PBS (4 µL sample + 396 µL PBS)

2. Prepare BSA standards according to kit instructions

3. Add 100 µL standards/samples to wells in triplicate

4. Prepare BCA working reagent (Reagent A + B at 50:1)

5. Add 100 µL working reagent to each well

6. Incubate at 37°C for 30 minutes

7. Read absorbance at 562 nm

8. Calculate concentrations from standard curve

9. Divide reading by 10 to get actual µg/µL (accounts for 1:100 dilution)

Example Calculation:

BCA reading: 200 µg/mL → Actual concentration: 200 ÷ 10 = **20 µg/µL**

Target 20 µg protein per lane in 20 µL total volume:
Sample needed: 20 µg ÷ 2 µg/µL = 10 µL sample + 10 µL H_2O

Prepare Samples for Loading

Mark Done

> **Prepare 5X loading dye with β-mercaptoethanol ahead of time:**
> • 1 mL 5X loading dye (from 4°C fridge) + 50 µL β-mercaptoethanol

- 5X dye will smell like rotten eggs if βME added correctly
- Store at room temperature or 4°C, use within 1 month

1. **Calculate volumes needed per sample**

Example: Loading 20 µg protein per lane

- If sample = 2 µg/µL: need 10 µL sample
- Add 10 µL Millipore H_2O (to reach 20 µL)
- Final: 20 µL sample + 5 µL loading dye = 25 µL per lane

2. **Mix sample volume and Millipore H_2O volume in labeled Eppendorf tubes**
   - Label tubes clearly with sample names
   - Pipette calculated sample volume
   - Add Millipore H_2O to bring to 20 µL total
3. **Add 5 µL 5X loading dye with βME** to each tube
4. **Vortex samples** briefly to mix thoroughly
5. **Spin down** samples briefly in microcentrifuge (2-3 seconds)
6. **Cap samples** securely and place in **95°C heat block for 5 minutes**
   - This denatures proteins for SDS-PAGE
   - Ensure caps are tightly closed to prevent evaporation
7. **Allow samples to cool completely** before removing caps
   - Prevents sample loss from pressure buildup
   - Takes 2-3 minutes on ice or at room temperature
8. **Spin down** samples again briefly to collect condensation
9. Samples are now ready to load on gel (keep on ice if not loading immediately)

SAFETY: β-mercaptoethanol is toxic and smells strongly. Work in fume hood when possible. Wear gloves!

Cast and Run Gel

Mark Done

Choose gel percentage based on protein size:

Protein Size	Gel %	Examples
10-40 kDa	15%	Small peptides
20-80 kDa	12%	Most proteins
40-120 kDa	10%	β-actin (45 kDa)

Protein Size	Gel %	Examples
60-200 kDa	8%	Vinculin (116 kDa)

Running conditions:

1. Load samples (25 µL total: 20 µL sample/water + 5 µL loading dye)
2. Load 5 µL ladder + 5 µL loading dye in lane 1
3. Run at 60V for ~30 minutes (stacking gel)
4. Increase to 110V for 2-4 hours (separating gel)
5. Stop when blue dye reaches bottom

> **CRITICAL: Add TEMED last when making gels - it initiates polymerization!**

Day 1 Evening: Protein Transfer

Prepare Transfer Sandwich

Mark Done

> **Timing: 16 hours overnight (no more than 16 hours)**

1. Fill container with cold transfer buffer (4°C)
2. Activate PVDF membrane:
   - Cut membrane to size of gel
   - DO NOT TOUCH MEMBRANE WITH BARE HANDS
   - Submerge in methanol, shake 2 min (until translucent)
   - Transfer to transfer buffer, equilibrate 2-5 min
3. Remove gel from cassette and cut away stacking gel
4. Assemble sandwich in transfer buffer (all submerged):

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Black Sponge → Filter → Gel → Membrane → Filter → Black Sponge
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5. **ROLL OUT AIR BUBBLES** after each layer
6. Close clamshell firmly
7. Place in transfer box with cold buffer
8. Red side faces toward positive electrode
9. Run at **25V for 16 hours** (overnight)

CRITICAL: No air bubbles between gel and membrane! Air bubbles prevent transfer. Do not exceed 16 hours.

Day 2: Blocking and Antibodies

Visualize and Cut Membrane

Mark Done

1. Remove membrane from transfer apparatus
2. Stain with Ponceau S for 5 minutes with gentle rocking
3. Remove excess Ponceau S stain by rinsing with DI water
4. Verify ladder transferred and check for even transfer
5. Take photo for reference
6. Cut horizontal strips for each protein of interest
7. Cut notch in upper right corner (track orientation)
8. Wash away Ponceau with TBS-T (5 min × 2)

Block Membrane

Mark Done

4% Milk Blocking Solution (50 mL):

- 2 g evaporated milk powder
- 50 mL TBS-T
- Mix well until dissolved
- Incubate 1 hour at RT on rocker

Primary Antibody Incubation

Mark Done

4% BSA Solution (50 mL):

- 2 g BSA crystals (from 4°C fridge, western shelf)
- 50 mL TBS-T
- Vortex until dissolved

Common Lab Dilutions:

Antibody	Dilution	Incubation
OCT1	1:3000	16 hr, 4°C
β-actin	1:10,000	16 hr, 4°C
Vinculin	1:10,000	16 hr, 4°C
Most others	1:1000	1-2 hr RT or 16 hr 4°C

1. Dilute primary antibody in 4% BSA
2. Rinse membranes briefly with TBS-T (5 min)
3. Add primary antibody solution to strips
4. Incubate with gentle rocking (1-2 hr RT OR 16 hr at 4°C)

> **NOTE: Can save and reuse primary antibody if needed. Store at 4°C with 0.02% sodium azide.**

Day 2-3: Detection and Imaging

Secondary Antibody

Mark Done

1. Wash after primary antibody with TBS-T (5 min × 2)
2. Prepare 4% milk solution (2 g milk + 50 mL TBS-T)
3. Dilute secondary antibody in 4% milk (typically 1:5000)
4. Add secondary antibody solution to strips
5. Incubate 1 hour at RT on rocker
6. Wash with TBS-T (5 min × 3)

Common Secondary Dilutions:

- Anti-rabbit HRP: 1:5000 (most proteins)
- Anti-rabbit HRP: 1:10,000 (vinculin, β-actin)
- Anti-rabbit HRP: 1:3000 (OCT1)
- Anti-mouse HRP: 1:5000 (adjust as needed)

ECL Detection and Imaging

Mark Done

1. Prepare ECL reagent: Mix HRP Peroxide + HRP Luminol 1:1 (fresh!)

2. Lightly dry strips on Kimtech wipes

3. Arrange on plastic sheet (protein side up)

4. Add ECL reagent evenly over strips (~1-2 mL per strip)

5. Incubate 1-2 minutes at RT

6. Drain off excess reagent

7. Image with gel imaging system:
   - Short exposure (10-30 sec): High abundance proteins
   - Medium exposure (1-5 min): Most proteins
   - Long exposure (10+ min): Low abundance proteins

8. Save images with sample info and exposure time

Which ECL reagent to use:

• OCT1: Use refrigerated reagent (4°C)

• β-actin/Vinculin: Use room temperature reagent

• Low abundance: Use enhanced/SuperSignal reagents

Verify results: Correct MW? Single band? Signal in all samples? Even loading?

Troubleshooting

Protein Extraction Issues

Problem	Possible Cause	Solution
Low protein yield	Insufficient lysis time Too much buffer	Increase to 30-45 min Reduce RIPA volume
High background	Incomplete clearing	Centrifuge longer, higher speed
Protein degradation	No protease inhibitors	Add fresh inhibitors, work on ice

Transfer Issues

Problem	Possible Cause	Solution
No transfer	Wrong polarity Air bubbles	Check red to red, black to black Roll out all bubbles
Incomplete transfer	Transfer time too short	Use full 16 hours at 25V

Problem	Possible Cause	Solution
Uneven transfer	Air bubbles Dried regions	Roll thoroughly Keep everything wet

Detection Issues

Problem	Possible Cause	Solution
High background	Insufficient blocking Too much secondary	Block longer Lower secondary concentration
No signal	Wrong dilution Expired antibody	Optimize dilution Use fresh antibody
Weak signal	Antibody too dilute Short exposure	Increase antibody concentration Increase exposure time

Quick Reference

RIPA Working Buffer (per 1 mL)

- 900 µL Base RIPA
- 40 µL 25X Protease Inhibitor
- 50 µL 20X Phosphatase Inhibitor
- 1 µL 1M DTT (optional)

Sample Loading (per lane)

- 20 µL sample/water mix (10-20 µg protein)
- 5 µL 5X loading dye + βME
- = **25 µL total**

Gel Running

1. 60V for 30 min (stacking)
2. 110V for 2-4 hours (separating)

Transfer

- 25V for 16 hours (no more!)

Primary Antibody (Standard)

- 1:1000 in 4% BSA
- 16 hours at 4°C (or 1-2 hr at RT)

Secondary Antibody (Standard)

- 1:5000 in 4% milk
- 1 hour at RT

Washes

- After primary: 5 min × 2 with TBS-T
- After secondary: 5 min × 3 with TBS-T

Common Housekeeping Proteins

- β-actin: 45 kDa
- Vinculin: 116-124 kDa
- GAPDH: 37 kDa
- α-tubulin: 50 kDa

Buffer and Gel Recipes

8% Resolving Gel (for two mini gels)

Autoclaved H_2O	**5.5 mL**
40% Acrylamide	**2 mL**

1.5 M Tris-HCl pH 8.8	**2.5 mL**
10% SDS	**100 µL**
10% APS	**100 µL**
TEMED	**6 µL (add last!)**

10% Resolving Gel (for two mini gels)

Autoclaved H_2O	**5 mL**
40% Acrylamide	**2.5 mL**
1.5 M Tris-HCl pH 8.8	**2.5 mL**
10% SDS	**100 µL**
10% APS	**100 µL**
TEMED	**6 µL (add last!)**

12% Resolving Gel (for two mini gels)

Autoclaved H_2O	**4.5 mL**
40% Acrylamide	**3 mL**
1.5 M Tris-HCl pH 8.8	**2.5 mL**
10% SDS	**100 µL**
10% APS	**100 µL**
TEMED	**6 µL (add last!)**

Stacking Gel (for two mini gels)

Autoclaved H_2O	**7.4 mL**
40% Acrylamide	**1.3 mL**

0.5 M Tris-HCl pH 6.8	**1.3 mL**
10% SDS	**100 µL**
10% APS	**100 µL**
TEMED	**10 µL (add last!)**

1X Running Buffer (1 L)

- 100 mL 10X Tris/Glycine/SDS buffer
- 900 mL Millipore H_2O

1X Transfer Buffer (2 L)

- 200 mL 10X Transfer buffer
- 200 mL ethanol (or 400 mL methanol)
- 1600 mL Millipore H_2O
- Store at 4°C (must be cold for transfer)

1X TBS-T Wash Buffer (1 L)

- 100 mL 10X TBS-T
- 900 mL Millipore H_2O
- You'll need ~500 mL per membrane