Western Analysis

Laboratory procedure that allows you to:

1. Verify the expression of a protein

2. Determine the relative amount of a protein present in different samples

3. Analyze protein-protein interactions
Two Main Types of Westerns

1. Denaturing (Most Commonly Used)
   - SDS-PAGE

2. Non-Denaturing
   - Native PAGE
SDS-PAGE Western Blot Method

1. Cells in Culture
2. Cell Removal
3. Human Cells Containing Protein
4. Cell Lysis by Detergents and Sonication
5. Heat Denaturation of Proteins
6. Detergents Bind Proteins
7. SDS or LDS
8. Load Proteins on Gel
9. Apply Electric Current
10. Proteins Separate by Size
Transfer or Blot Protein from Gel to Nitrocellulose and/or PVDF Membrane

Block Membrane with Non-Specific Proteins

Incubate Membrane with $1^\circ$ Antibody

$1^\circ$ Antibody Binds Antigen (i.e. Protein of Interest)

$1^\circ$ Antibody is a Rabbit Anti-Human $\beta$-Actin Antibody

Non-Specific Proteins Bind to Unbound Regions of Membrane
Add IRDye®-Conjugated 2° Antibody

Light Detected by Li-Cor Imager

2° Antibody is a Goat Anti-Rabbit-IR-Conjugated Antibody

Li-Cor Laser
**β-Actin Antibody**

- **Applications**
  - Western blotting
  - IP
  - IHC

- **Species Cross-Reactivity**
  - Human, Rat, Mouse, Canine, Monkey, Horse, Sheep, Chicken

- **Molecular Wt.**
  - 43 kDa

- **Source**
  - Rabbit

**Background:** Actin, a ubiquitous protein in eukaryotes, is the major component of the cytoskeleton. At least six isoforms are known in mammals. Nonmuscle β- and γ-actin, also known as cytoplasmic actin, are predominantly expressed in nonmuscle cells, controlling cell structure and motility (1). α-cardiac and α-skeletal actin are expressed in striated cardiac and skeletal muscles, respectively. Two smooth muscle actins α- and γ-actin are found primarily in vascular smooth muscle and enteric smooth muscle, respectively. These actin isoforms regulate contractile potentials for the muscle cell (2). Actin exist mainly as a fibrous polymer, F-actin. In response to cytoskeletal reorganizing signals during processes such as cytokinesis, endocytosis, or stress, the GTP binds to the actin monomer and polymerizes into F-actin, resulting in an increase in the monomeric, globular form, G-actin (3). The Arp2/3 complex stabilizes F-actin fragments and promotes formation of new actin filaments (4). It has been reported that actin is hyperphosphorylated in primary breast tumors (5).

**Specificity/Sensitivity:** β-Actin Antibody detects endogenous levels of β-actin. This antibody may cross-react with the γ-actin (cytoplasmic) isoform. It does not cross-react with α-skeletal, α-cardiac, or α-vascular smooth, or γ-enteric smooth muscle isoforms.

**Source/Purification:** Polyclonal antibodies are produced by immunizing rabbits with a synthetic peptide (KLH-coupled) corresponding to amino-terminal residues of human β-actin. Antibodies are purified by protein A and peptide-affinity chromatography.

**Background References:**

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**Storage:** Store at -20°C. Do not freeze-thaw.

**Recommended Antibody Dilutions:**
- Western blotting: 1:1000
- Immunoprecipitation: 1:50
- Immunohistochemistry (Paraffin): 1:25

**HCl Protocol:** Citrate/TBST

**Companion Products:**
- Phospho-Collin (Go) Antibody #3311
- Collin Antibody #3312
- Anti-rabbit IgG, HRP-linked Antibody #7074
- Anti-rabbit IgG, HRP-linked Antibody #7074
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #4727
- LumGLO® Reagent and Peroxide #7003

**IMPORTANT:** For Western blots, inactivate membrane with dried antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

**Applications Key:**
- W: Western blotting
- IP: Immunoprecipitation
- HCl: Immunohistochemistry
- F: Flow cytometry
- E: ELISA
- D: Dot Blot
- X: Xenopus
- C: Chicken

Species-endoed in parentheses are predicted to react based on 100% sequence homology.
We will be using 4-12% Bis Tris Gels today because our protein of interest is 45 kDa in size.

For larger proteins (>100 kDa) this type of gel would not be appropriate because the resolution of large proteins on a 4-12% Bis Tris gel is poor.
QUESTION

If we wanted to detect another human protein that was 80 kDa on this blot at the same time as beta actin could we?

Why would it be wise to use a primary antibody against this protein that was generated in a rabbit?
Some Drawbacks of Western Blotting

1. Many steps where errors can occur

2. Large amount of sample needed (5-50 µg)

3. Accurate quantitation is very difficult

4. Time consuming protocol
Western Blot Protocol

1. Sample Preparation

A) Add 10 μg of protein (already prepared for you) to 4 μl of 4X LDS Loading Buffer plus 2.5 μl of 10X Reducing Agent. Then add 14.5 μl purified water for a total volume of 25 μl.

B) Heat sample mixture at 70°C for 10 minutes.
2. **Electrophoresis**
   A) While protein samples are heating, assemble electrophoresis unit.

[Diagram: Unlocked and Locked Position of XCell SureLock Mini-Cell, Invitrogen]

**Demonstration** XCell SureLock Mini-Cell, Invitrogen

B) **Load Gel**
   - Molecular weight marker and protein samples

[Diagram: Loading sample into gel, Invitrogen.com]
C) Add 500 μl Antioxidant to top chamber to maintain proteins in a reduced state and ensure optimal band sharpness.

D) Run gel at 180V for 45 minutes
3. **Transfer**
   
   A) Soak marked (for orientation) nitrocellulose (or PVDF) membrane in transfer buffer containing 10% Methanol at least 10 minutes prior to transfer.
   
   B) When gel run is complete, turn off power source, remove gel from pre-cast plates, place transfer buffer-soaked filter paper sheet on top of gel, remove gel from plate, and place on top of membrane blotting pads that have been removed of bubbles.
   
   C) Place membrane on top of gel and cover with another transfer buffer-soaked filter paper sheet and blotting pads to fill the transfer chamber.
   
   D) Add 500 µl Antioxidant and run transfer at 30V for 1 hour.
4. **Blocking**  
   A) Remove membrane from transfer chamber and incubate in 1X PBS for 2 minutes.

   B) Incubate membrane in 5% BSA at room temperature for 30 minutes with slow shaking.

5. **Primary Antibody Incubation**  
   A) Prepare a 1:1000 dilution of primary antibody (Rabbit Anti-Human β-Actin) in 5% BSA, 1X PBS, 0.2% Tween 20.

   B) Incubate membrane in primary antibody solution overnight at 4°C with gentle rocking.
6. **Membrane Washing**
   A) Wash membrane 3 x 5 minutes each in PBS, 0.1% Tween 20 with gentle shaking at room temperature.

7. **Secondary Antibody Incubation**
   A) Prepare a 1:20,000 dilution of secondary antibody (Goat Anti-Rabbit-IRDye) in 5% BSA, 1X PBS, 0.2% Tween 20. Keep away from light.

   B) Incubate membrane in secondary antibody solution for 30 minutes at room temperature with gentle shaking in the dark.

**QUESTION**

Why are we using Goat Anti-Rabbit IRDye as our secondary antibody?

8. **Repeat Membrane Washing** – See step 6
9. **Visualization of Protein of Interest**

   A) Wash membrane in 1X PBS to remove Tween 20.

   B) Develop western on LiCor Odyssey Imager in ARC.

   C) Identify protein of interest.
IRDye® Protein Marker

45 kDa

β-Actin