Validation of Commercial tool Antibodies

The Antibody Society Webcast series – Antibody Validation #6

Maybe Routine, but non-trivial – Validation in Practice #1

Simon L. Goodman

Science and Technology Program Manager

The Antibody Society

Antibody Validation: a 9-part series

1. Andreas Pluckthun : The different antibody formats
2. Glenn Begley : Antibodies and the reproducibility crisis in biological science
   Cecilia Williams : The Erß story – is your antibody like this?
3. Jan Voskuil : Beware the supplier OEM
   Andy Chalmers : Finding antibodies in the Antibody Databases
4. Anita Bardowski : Which antibody are you looking for? The RRID
   Jan Voskuil : Points to note on the supplier datasheets
5. Giovanna Roncador: : Correct positive and negative controls in validation
6. **Aldrin Gomes** : **Standard technology: "even" Western blots are non-trivial**
   Jim Trimmer : **IHC issues in brain sciences**
7. Travis Hardcastle : Cell KO technology
   Alejandra Solache : Validating Antibodies with KO technology
8. Mike Taussig : Validating antibodies using array technologies
   Fridjhof Lund-Johansen : Mass spectroscopy for mass validation
9. Andrew Bradbury : Why publish sequences?
   Andreas Pluckthun : What are the coming alternatives ?

Maybe Routine, but non-trivial – Validation in Practice #1

The Antibody Society Webcast series – Antibody Validation #6

Jim Trimmer   University of California, Davis, School of Medicine

Aldrin V. Gomes   University of California, Davis

Western blotting: not as easy as it looks

The Antibody Society Webcast series- Antibody Validation #6

Aldrin V. Gomes, PhD
Department of Neurobiology, Physiology, and Behavior
University of California, Davis
avgomes@ucdavis.edu

ANTIBODY SOCIETY

Common Pitfalls in Western Blotting

- ## The ANTIBODIES

Most Important Concepts-1

- Each antibody needs validation for the specific application where it will be used.

  - An antibody validated for use with e.g. *15-20 μg* of rat heart total protein must be re-validated to use with *30 μg* of rat heart total protein.
  - An antibody validated for use in *rat heart* needs to be validated before it can be used in *mouse heart* or *any other tissue*.

  - Native tissues have cell-specific post-translational modifications that can affect antibody interactions.

Most Important Concept-2

- Antibodies that have been validated by other techniques, such as immunohistochemistry, must still be specifically validated for Western blotting.
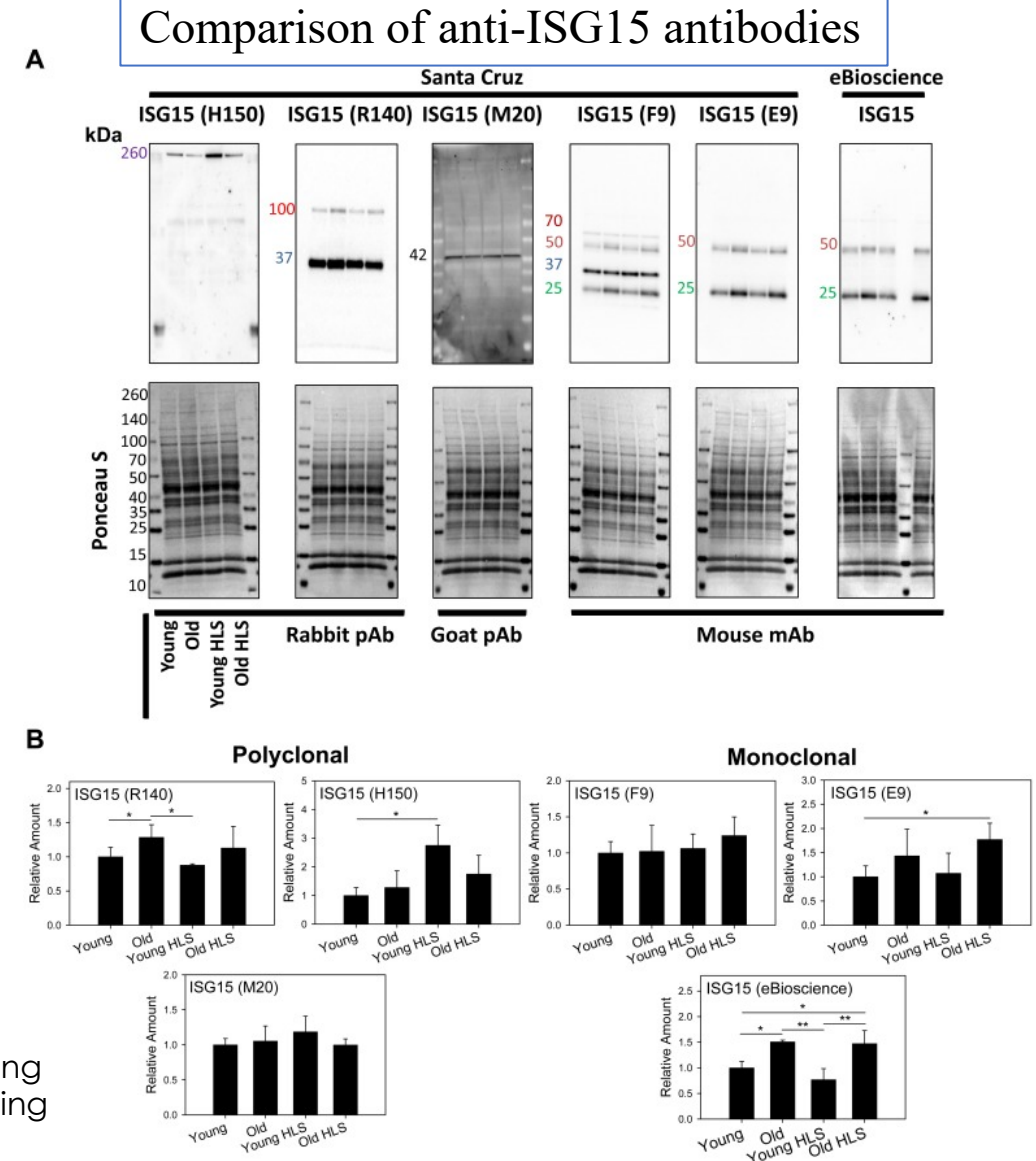
Common Pitfalls in Western Blotting

Using *unvalidated* antibodies can result in unexpected and/or misleading results

- Six different "ISG15 antibodies" resulted in five different results when trying to determine the amount of ISG15 in young and old rat hearts

Gilda JE, Ghosh R, Cheah JX, West TM, Bodine SC, Gomes, A.V. (2015) Western Blotting Inaccuracies with Unverified Antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). PLOS ONE 10(8): e0135392.
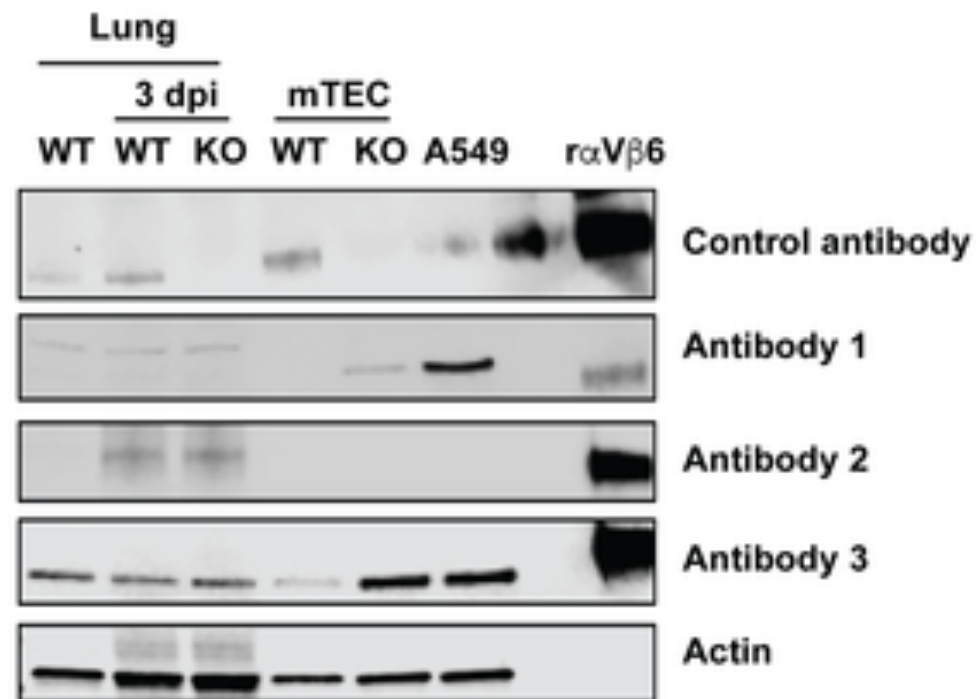https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0135392

Comparison of anti-ISG15 antibodies

Common Pitfalls in Western Blotting

Using *unvalidated* antibodies can result in unexpected and/or misleading results

Specificity of β6 integrin antibodies in western blot

Meliopoulos VA, Schultz-Cherry S (2018) Although it's painful: The importance of stringent antibody validation. PLOS Pathogens 14(1):
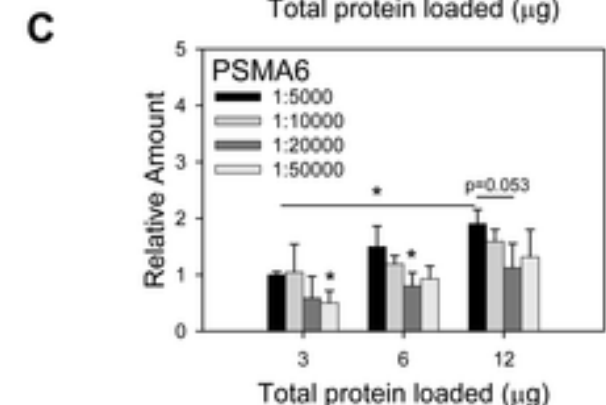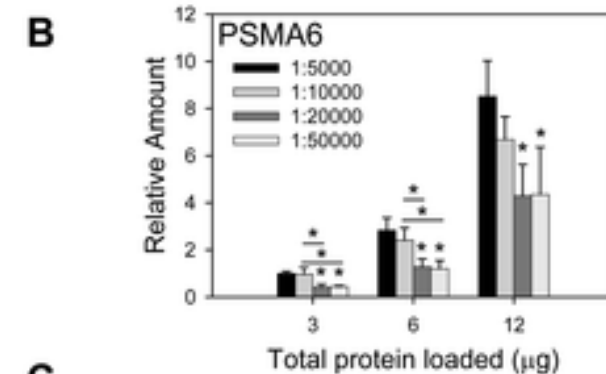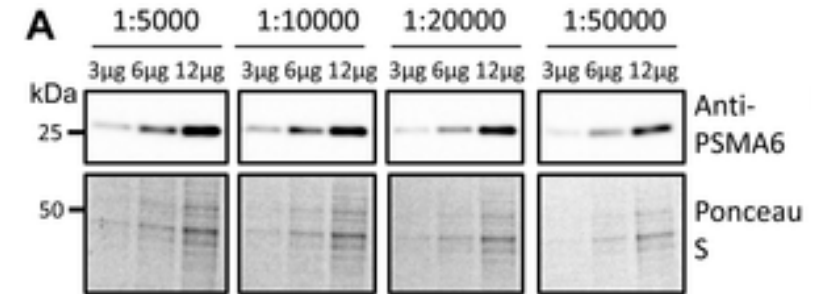https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1006701

Common Pitfalls in Western Blotting

Effect of antibody concentration on linearity of target detected by WB

Using *validated* antibodies INCORRECTLY can result in unexpected and/or misleading results

• Antibody concentration must be optimized for optimal target signal

Gilda JE, Ghosh R, Cheah JX, West TM, Bodine SC, et al. (2015) Western Blotting Inaccuracies with Unverified Antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). PLOS ONE 10(8): e0135392. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0135392

PLOS|ONE

Common Pitfalls in Western Blotting

- Lack of positive and negative controls when using new "batches" of previously validated antibodies.

- Problems with reproducibility due to lot-to-lot variability: can affect both polyclonal and monoclonal antibodies.

- Polyclonal antibodies not appropriately affinity purified: are a heterogeneous mixture. May recognize multiple epitopes on the target, but will also include non-selective antibodies.

Common Pitfalls in Western Blotting

Sample Preparation is often overlooked as a source of irreproducibility

- Cytosolic fractions vs. total cellular extracts can result in substantially different results, both for target protein and housekeeping proteins
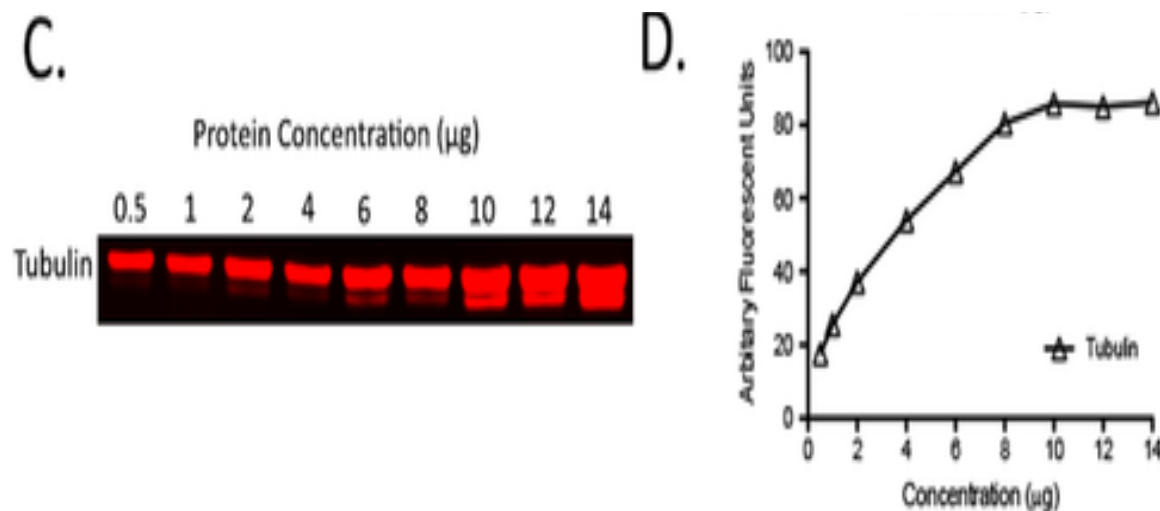
Common Pitfalls in Western Blotting

- The LOADING AND NORMALIZATION

Common Pitfalls in Western Blotting

Housekeeping proteins can be incorrectly used as normalization controls

Housekeeping proteins for WB normalization: one must validate they are not saturated under WB conditions.

Linear range of β-tubulin detection is typically below 10 µg of total protein

Eaton SL, Roche SL, Llavero Hurtado M, Oldknow KJ, Farquharson C, et al. (2013) Total Protein Analysis as a Reliable Loading Control for Quantitative Fluorescent Western Blotting. PLOS ONE 8(8): https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0072457

Common Pitfalls in Western Blotting

Housekeeping proteins can be incorrectly used as normalization controls

"Housekeeping Protein" (HKP) Expression Levels *change* due to:
- Tissue age and Type
- Developmental Changes
- Post-transcriptional Regulation
- Cell Types
- Experimental Conditions

HKPs are usually highly expressed, whereas target proteins are often expressed only in low abundance
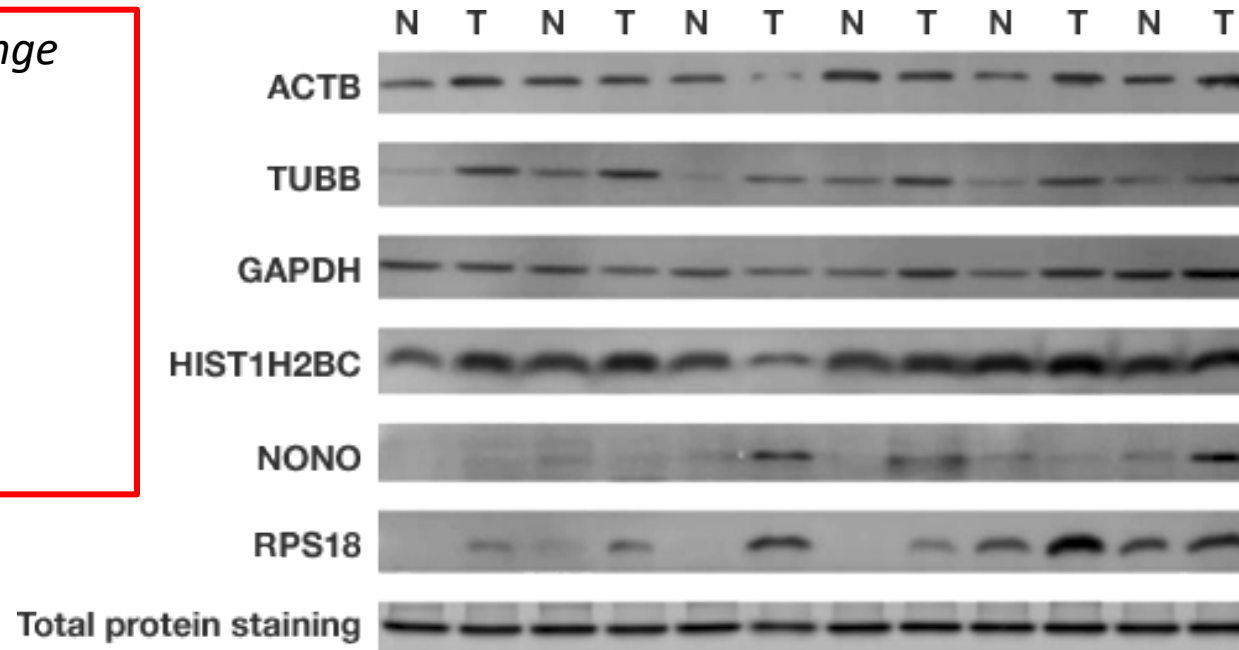

Differences in five candidate housekeeping proteins and total protein staining between tumor and non-cancerous tissues in the validation sample set. Common HKPs are **upregulated** in colorectal adenocarcinoma and hepatocellular carcinoma, making the total protein a better "housekeeper". Hu X et al. (2016). Oncotarget 7, 66, 679–66, 688.

Common Pitfalls in Western Blotting

Total Protein Staining Is A Better Way to Normalize Western Blots

Linearity comparison of stain-free total protein measurement and immunodetection of three housekeeping proteins in 10–50 µg of HeLa cell lysate.
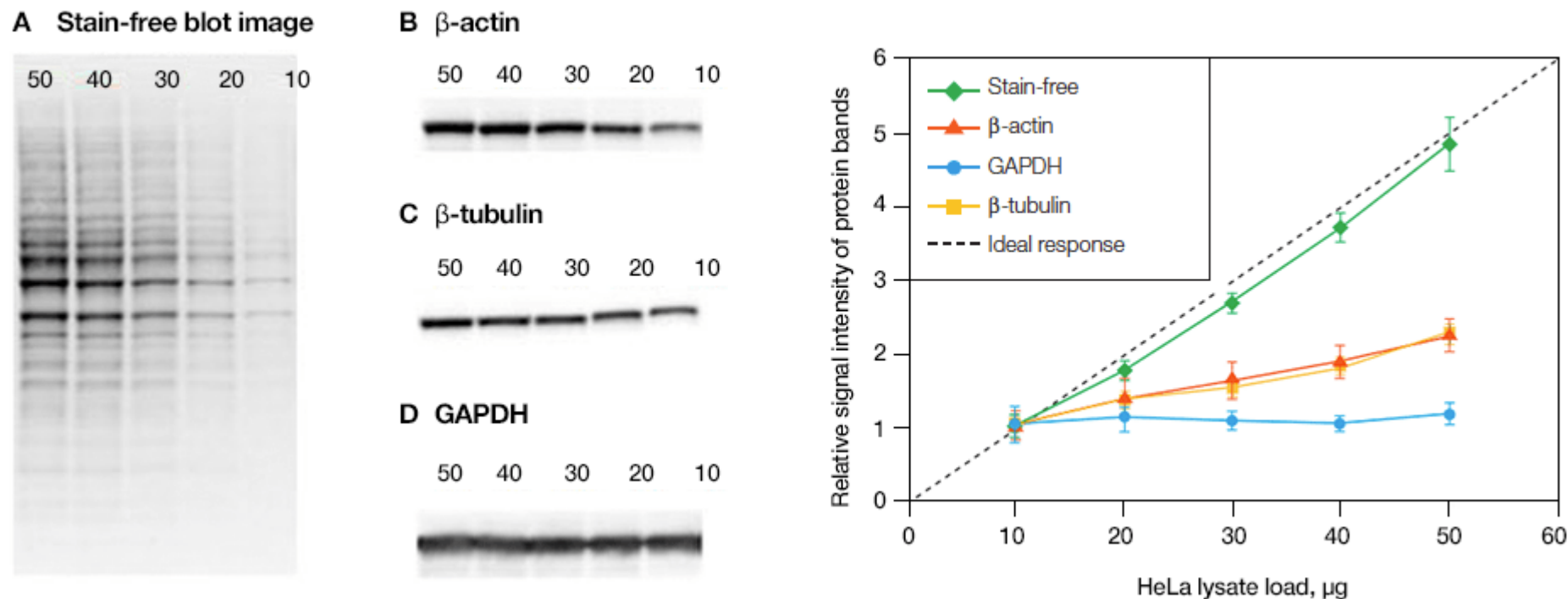
(a), stain-free blot and the chemi blots for (b), β-actin; (c), β-tubulin and (d), GAPDH. Lane labels = total protein load (µg). Although the actin and tubulin signals appear linear, the densitometric ratio (e) was far below the predicted "quantitative response" of loading. The stain-free signal correlated to the expected result. Taylor SC Posch A (2014). Biomed Res Int. 36, 1590

Common Pitfalls in Western Blotting

- The DATA ANALYSIS
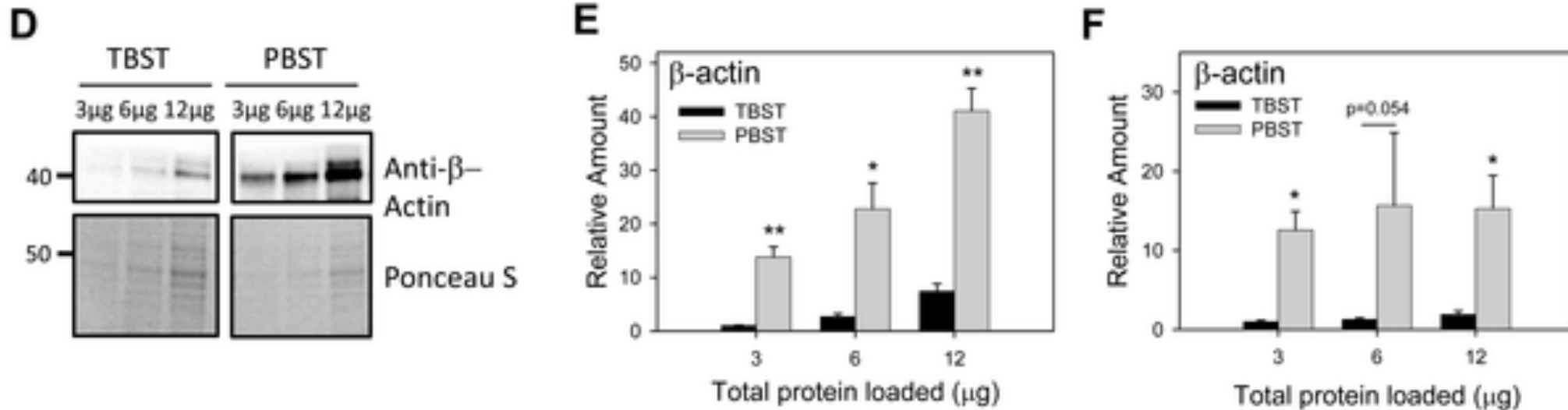
Common Pitfalls in Western Blotting

Lack of both technical and biological replicate samples in your experimental design

- Technical replicates: help identify variations in the technique itself
- Biological replicates: from independent samples, capture random biological variation

- Use software programs compatible with your imaging system and designed for your specific assay.

- Minimize image processing; avoid converting and transferring files between software programs.

Common Pitfalls in Western Blotting

- <u>Low reproducibility</u> due to lack of information: about how specific antibodies were used; the supplier; catalog number; and lot number in publications.

- Effect of buffer reagent on Western blotting linearity

In the example below: incubation with Tris buffered Saline + Tween 20 (**TBST**) vs Phosphate buffered saline + Tween 20 (**PBST**) gave significantly different results.
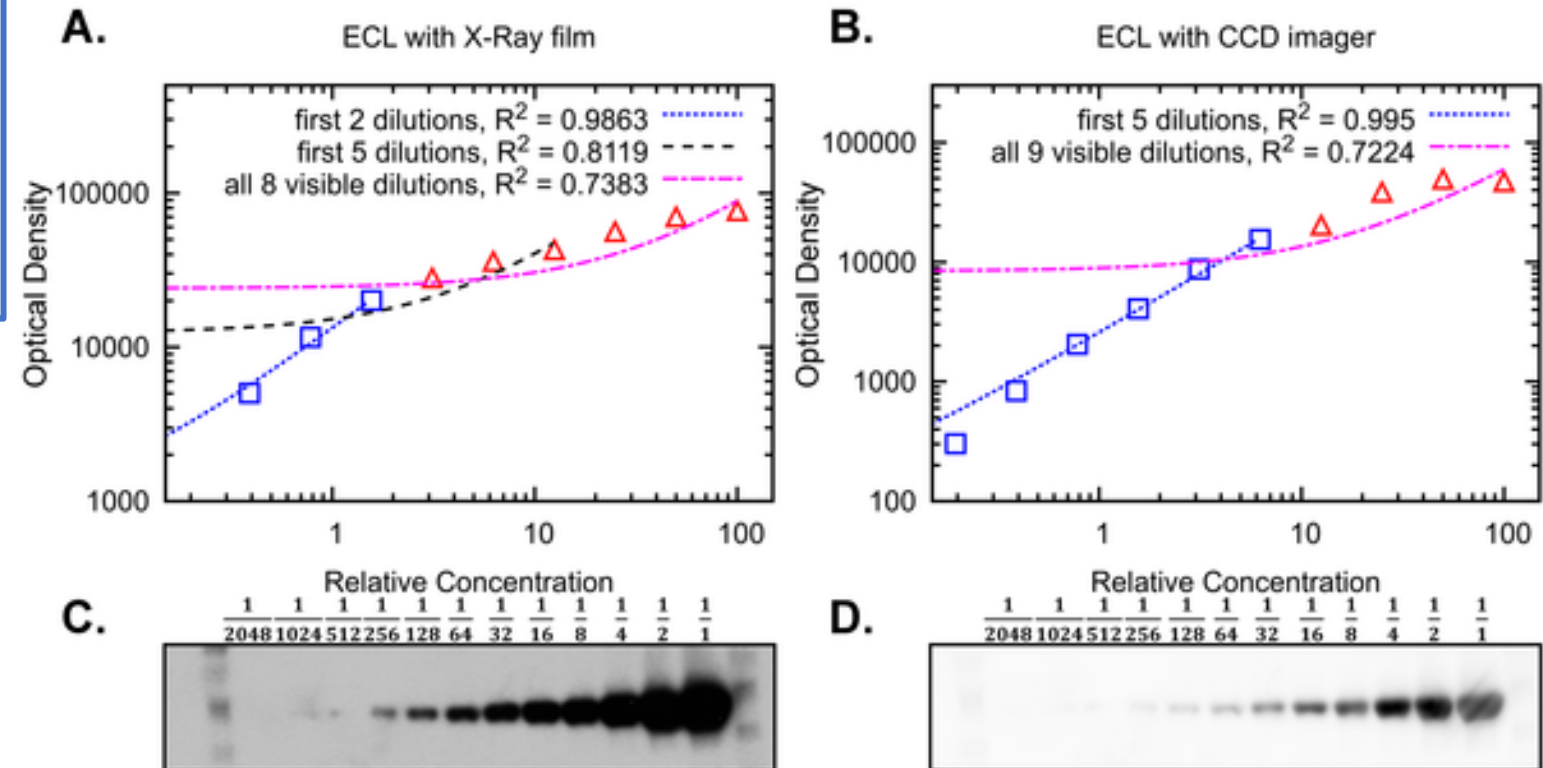
Gilda JE, Ghosh R, Cheah JX, West TM, Bodine SC, et al. (2015) Western Blotting Inaccuracies with Unverified Antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). PLOS ONE 10(8): https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0135392

Common Pitfalls in Western Blotting

Low signal linearity when X-ray film is used to develop HRP bound secondary antibodies

Signal linearity obtained by different Western blot detection systems

Dilution experiments BSA: representative experiments

A. ECL with X-Ray film

first 2 dilutions, $R^2 = 0.9863$
first 5 dilutions, $R^2 = 0.8119$
all 8 visible dilutions, $R^2 = 0.7383$

B. ECL with CCD imager

first 5 dilutions, $R^2 = 0.995$
all 9 visible dilutions, $R^2 = 0.7224$

Summary: Western blot - not as easy as it looks

Antibody validation for each specific Blot condition is critical

Accurate sample preparation-reporting is needed: high levels of chaotropic and other specialized reagents in samples can result in less efficacy and specificity of the Blot

Housekeeping proteins to normalize Western blotting is accurate only when the *HKP* is validated for linearity in the same concentration range of total protein as the target protein. However, few labs validate the housekeeping protein linearity

Western blotting: not as easy as it looks

The Antibody Society Webcast series- Antibody Validation #6

Aldrin V. Gomes, PhD
Department of Neurobiology, Physiology, and Behavior
University of California, Davis
avgomes@ucdavis.edu

Validating Antibodies for IHC: a Complex Technology

The Antibody Society Webcast series – Antibody Validation #6

Jim Trimmer
Department of Physiology & Membrane Biology
University of California, Davis School of Medicine
jtrimmer@ucdavis.edu