ROCKLAND

Validating Antibodies for Western Blotting

Carl Ascoli, Ph.D.

Validating an antibody is not the same as characterizing an antibody. A data image of an antibody used in an immunoassay with or without controls could be described as characterizing an antibody. But validating an antibody means that you have determined the antibody's sensitivity for the target, its specificity for off target binding, and its reproducibility—especially to demonstrate that the antibody's properties are consistent from lot-to-lot.

Legitimate methods for validating antibodies are immunoassay specific, meaning that the methods used for Western blotting would not necessarily apply to other applications such as ELISA, immunoprecipitation or immunohistochemistry.

Recent proposals, including the 'conceptual pillars' for application-specific antibody validation, proposed by Mathias Uhlen and colleagues, during the International Working Group on Antibody Validation (IWGAV) and most recently the Workshop on Antibody Validation organized by the Global Biological Standards Institute (GBSI), describe a structural framework for antibody validation. These specific details for assessing the performance of individual antibodies in validation methods add to the conceptual framework.

Purified proteins—native or recombinant—can be used to assess reactivity with a specific antibody

This method has limited value but is a legitimate method for antibody validation if other options are not available. The immunogen or a protein produced by immunoprecipitation (IP) may also be used. Size markers and positive and negative controls are required for each Western blot.

Options that add confidence to the assessment:

1. Add varying amounts of protein loaded to different lanes of the gel to assess antibody reactivity.
2. If recombinant, a second antibody against the fusion tag, such as anti-GFP or anti-FLAG could be used to co-localize with the target protein.
3. If recombinant, a second protein with the same promoter and fusion tag could be used to demonstrate that the antibody does not identify the tag portion which would demonstrate a lack of specificity.
4. If recombinant, a null or mock unpurified lysate could be used to demonstrate that the antibody does not identify non-specific bands which would demonstrate a lack of specificity.
5. Combine a. and d. Spike a null lysate with varying amounts of purified protein and load into different lanes of the gel to show band intensity increases when the amount of protein is increased and that the antibody does not react non-specifically.
6. Multiple antibodies against the target could be used to co-localize with the target protein.
7. The target protein could be analyzed by mass spectrometry for proper identification.

Lysates from cells overexpressing the target protein

Either cell-free expression systems like rabbit reticulocyte lysates or stably- or transiently-transfected cell lysates can be used to assess reactivity with a specific antibody. This method has greater value than using purified protein but is not as valuable as detecting native protein in cells or tissues. Size markers and positive and negative controls are required for each Western blot.

Options that add confidence to the assessment:

1. Options 2-6 specified for purified proteins may also apply to lysates with minor adjustments incorporated into the test material as needed.
2. Induce varying degrees of protein expression to produce different amounts of protein loaded to different lanes of the gel to show band intensity increases when expression levels increase, e.g. vary amount of ligand that controls expression by binding to promoter.
3. Spike a null lysate with varying amounts of purified protein and load into different lanes of the gel to show band intensity increases when the amount of protein is increased.
4. Use lysates that express the target protein at a level anywhere from 0.2X to 5X of native expression levels as determined by the literature or by measuring native RNA or protein.
5. Two-dimensional Western blotting is used to identify the target protein by both size and isoelectric point.
6. The ability of the antibody to bind processed forms, orthologs and isoforms is assessed.

Results that diminish confidence in the assessment:

7. A significant number of major or minor bands are observed in lysates that do not correspond with known forms of the target protein.
8. Additional band(s) are observed near the molecular weight of the target protein.

Lysates from native cells expressing the target protein

Either cultured cells or tissues that represent normal, disease

state or drug induced/stimulated systems can be used to assess reactivity with a specific antibody. This method has the greatest value for assessing the performance of antibodies for research. Size markers and positive and negative controls are required for each Western blot.

Options that add confidence to the assessment:

1. Options 5–6 specified for lysates from cells overexpressing the target protein would apply when testing lysates from cells expressing native protein.
2. Multi-lysate panels could be produced from cells known to express or not express the target of interest based on genomic or proteomic studies. Antibody binding would correlate with known expression levels.
3. The levels of target protein can be modulated by adding drugs, growth factors or by altering the growth conditions of the cells which would result in different amounts of protein. These samples should show corresponding changes in band intensity when loaded to different lanes of the gel.
4. Genetic manipulations could be used to affect the expression of the protein such as knock out genes, siRNA, shRNA, CRISPR/Cas 9 and other approaches of RNA Interference and gene manipulation. Controls showing proper expression levels are required for comparison.

Results that diminish confidence in the assessment:

5. A significant number of major or minor bands are observed in lysates that do not correspond with known forms of the target protein.
6. Additional band(s) are observed near the molecular weight of the target protein.

Special considerations for antibodies that recognize post-translational modifications (PTMs) are helpful when the target protein has been specifically modified with a PTM that can be regulated (e.g., phosphorylation, acetylation, or glycosylation).

PTM specific antibodies are often produced using modified and unmodified peptides, so peptide conjugates are considered to be legitimate tools for validation, e.g. validate using peptide BSA conjugates when peptide KLH conjugates are used for immunization. Size markers and positive and negative controls are required for each Western blot.

Options that add confidence to the assessment:

1. Perform a peptide competition assay where the PTM specific antibody is pre-incubated with modified peptide and/or unmodified peptide. Pre-incubation with the modified peptide should eliminate PTM specific antibody binding and diminish or eliminate staining of the target protein. Pre-incubation with the unmodified peptide should have no effect on PTM specific antibody binding.
2. Use a peptide array to confirm the reactivity of the PTM specific antibody for the modification in context with surrounding amino acids. Typically the array will include other protein modifications and similar amino acid substitutions to confirm specificity. Peptide arrays are often used to assess the specificity of epigenetic modifications of histones.
3. Similar to option 3 specified for lysates from native cells expressing the target protein, use an inducing agent to activate the PTM and enhance staining of the target protein. Conversely, staining of the target protein should be absent or diminished when an inhibitory agent is added.
4. Perform *in vitro* dephosphorylation/deacetylation of a phosphorylated/acetylated recombinant protein by enzymatically treating the protein with a phosphatase/deactylase. The staining of the target protein should be absent or diminished upon phosphatase/deactylase treatment.
5. Perform *in vitro* phosphorylation/acetylation of an unphosphorylated/unacetylated recombinant protein by enzymatically treating the protein with a kinase/acetylase. The staining of the target protein should be present or enhanced upon kinase/acetylase treatment.
6. Alter the recombinant protein by introducing a point mutation, such as a transversion to change serine to alanine, e.g. a codon change from TCA to GCA. This will block phosphorylation of the target protein. The staining of the target protein should be absent upon introduction of the mutation.

Results that diminish confidence in the assessment:

7. A significant number of major or minor bands are observed in lysates that do not correspond with known forms of the target protein.
8. Additional band(s) are observed near the molecular weight of the target protein.