Cell MENTOR Handbooks

Hallmarks of Antibody Validation

A Guide to the Cell Signaling Technology Antibody Validation Toolbox

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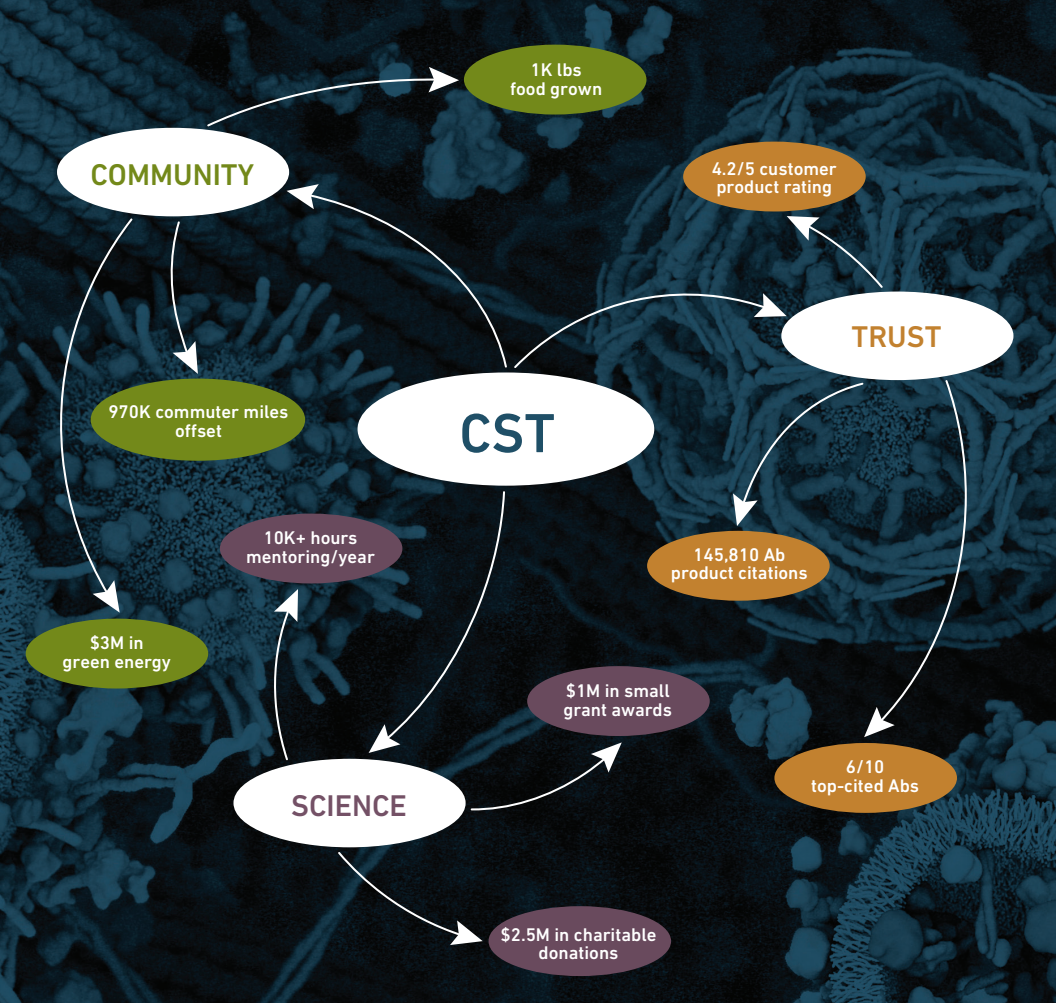

Discovering Pathways Together for 20 Years

Over the last 20 years, our mission at Cell Signaling Technology has been to support the local Community, make a positive impact in biological Science, and provide products that ensure complete Trust in the results. You haven't just been along for the ride. You've helped us define what we stand for and live it every day. You've inspired us to think bigger and tackle greater challenges. Thank you for standing with us.

For the next 20 years, we'll keep delivering the most Consistent, Sensitive, and Trustworthy antibodies possible, because we believe every antibody should create a pathway to success.

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20 THANK YOU 1999-2019

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GET TECHNICAL

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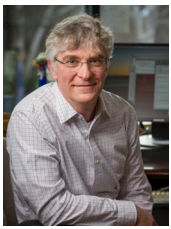
Introduction

Antibodies are essential reagents that support all levels of scientific research. Used in a multitude of applications to identify, quantify, and isolate specific target biomolecules, they have recently become the focus of intense scrutiny for their contribution to the ongoing reproducibility crisis. As awareness grows that poorly characterized antibodies are one of the reasons that many experimental results cannot be replicated, researchers expect increasingly rigorous in-house validation from antibody manufacturers.

At Cell Signaling Technology (CST), we understand that there is no single assay that can determine the validity of an antibody. Confirming that an immunoreagent is sufficiently specific and sensitive depends on the application and protocol being used, the type and quality of sample being analyzed, and the inherent biophysical properties of the antibody itself. To ensure our antibodies will work in your experiment, we adhere to the Hallmarks of Antibody Validation™, six complementary strategies that can be used to determine the functionality, specificity, and sensitivity of an antibody in any given assay. CST adapted the work by Uhlen, et. al., ("A Proposal for Validation of Antibodies." *Nature Methods* (2016)) to build the Hallmarks of Antibody Validation, based on our decades of experience as an antibody manufacturer and our dedication to reproducible science. These comprise:

- Binary strategy
- Ranged strategy
- Orthogonal strategy
- Multiple antibody strategy
- Recombinant strategy
- Complementary strategies

We guarantee that our antibodies are fit for purpose by carefully tailoring the combination of validation strategies applied to each product. This means customizing our validation process according to the biological role of the target, while considering the sensitivity requirements of the downstream assay, the availability of appropriate testing models, and the relevance of each method to target investigation. The aim of this handbook is to provide an overview of the Hallmarks of Antibody Validation, describing how our approach is part of a realizable solution to achieve experimental reproducibility and assuring you that CST is your partner of choice for high-quality antibodies to drive your research.



Roberto Polakiewicz, PhD
Chief Scientific Officer
Cell Signaling Technology

GET TECHNICAL

Binary Strategy

By: Katie Crosby, Director, Immunohistochemistry, Cell Signaling Technology

A binary approach is one of the best ways to evaluate antibody specificity. By testing an antibody in biologically relevant positive and negative expression systems, it is possible to confirm that it recognizes the target antigen in its native environment without crossreacting with other biomolecules present in the sample. Binary models include endogenous cells or tissues where expression of the target protein is known or predicted to be positive (high) or negative (low), genetic knockouts, and the use of treatments to induce or inhibit expression or modification of a protein target.

For binary testing to be effective, data should always be verified using an orthogonal method, such as genetic sequencing to confirm knockout or proteomic profiling to verify expression levels. To provide utmost confidence in antibody performance, additional validation strategies should also be employed. Moreover, each model that is used for binary validation of an antibody should be tested in each application for which the antibody is intended to be used - just because an antibody is specific by western blot does not mean that it will be as specific by immunohistochemistry (IHC); this approach is illustrated in the data below.

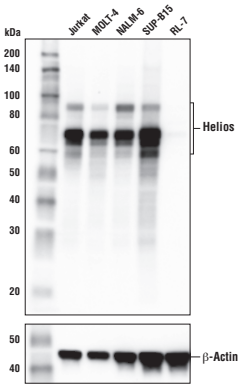
Endogenous controls

Cells and tissues that endogenously express the target of interest are the simplest form of positive control. Used in parallel with samples derived from similar material that does not demonstrate target expression, they provide a rapid yes/no answer regarding specific antigen recognition by an antibody.

To ensure this testing strategy delivers meaningful insight, it is important to select sample material representative of a true positive or negative control. This can be achieved by mining the literature, by utilizing genomic, transcriptomic, or proteomic databases; or comparing the immunostaining produced by the test antibody against that of a fully validated antibody against a distinct antigen on the same target.

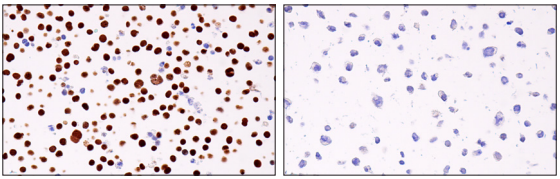
Figures 1 and **2** provide examples of antibody validation using endogenous controls, highlighting the concept that models used to validate an antibody should be tested in each intended application. Here, **Figure 1** shows western blot evaluation of the Helios (E4L5U) rabbit mAb in Jurkat and RL-7 cells, which are positive and negative for Helios expression respectively, while **Figure 2** shows IHC evaluation of the antibody in the same cell models. It can be seen that the data from the two applications are supporting, with loading controls ensuring that sample (analyte) quality does not lead to misinterpretation of results.

Figure 1



WB analysis of extracts from various cell lines using Helios (E4L5U) (upper) or β-Actin (D6A8) Rabbit mAb (lower). As expected, Helios protein is not expressed in RL-7 cells.

Figure 2



IHC analysis of paraffin-embedded Jurkat cell pellet (left, positive) or RL-7 cell pellet (right, negative) using Helios (E4L5U).

Genetic knockouts

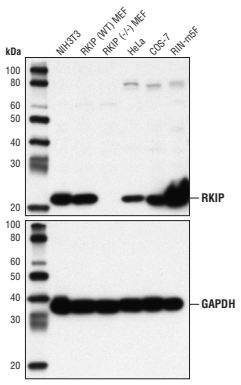
Endogenous binary models are not always an option since some proteins are ubiquitously expressed or data to support expression may be conflicting or unreliable. In these cases, mouse knockout models are widely used controls for binary evaluation of antibody specificity. Typically provided by collaborators, mouse knockout samples comprise wild type and knockout cells and tissues. These exhibit native expression and no expression of the target antigen respectively, and represent an ideal testing paradigm when available.

Another method to achieve genetic knockout is to use CRISPR-mediated gene disruption, a technique which has seen increased utility in recent years following its simplification into an intuitive and highly flexible system. An advantage of this approach is that it provides a positive and negative expression model in the same cell line. However, caution must be applied because disruption of the gene is not always complete, sometimes resulting in truncations or gene fragments which may yield signal. It is important that target knockdown be confirmed using an orthogonal, antibody-independent, strategy. It is additionally important to note that CRISPR knockout in cells, while very informative about an antibody's performance in cell-based assays, does not necessarily translate to tissue-based assays, like IHC, in which multiple cell types are present.

In addition to CRISPR-mediated knockdown, small (or short) interfering RNA (siRNA) is a synthetic RNA duplex that is commonly used to induce short-term silencing of protein coding genes. Yet another option, short or small hairpin RNA (shRNA) is typically contained within a DNA vector and introduced into cells via transfection or viral transduction. Gene silencing results from the expression of the shRNA, with the possibility of prolonged silencing if the transfected or transduced cells are selected for by leveraging the selection marker of a vector, or if there is stable integration of the shRNA into the host genome.

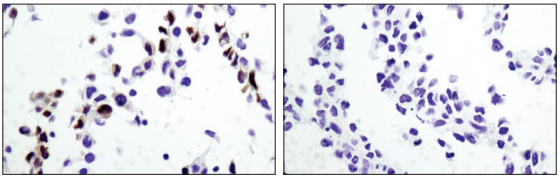
Again, it is critically important that the genetic knockout models used to validate an antibody are tested in each anticipated application independently. **Figures 3** and **4** show western blot and IHC assessment of the RKIP (D42F3) rabbit mAb in wild type and RKIP knockout mouse embryonic fibroblast (MEF) cells, while **Figures 5** and **6** illustrate western blot and immunocytochemical analysis using the 10 Thymidine Kinase 1 (E2H7Z) rabbit mAb to probe wild type or thymidine kinase 1 knockout HCT116 cells. In both instances, data from the two applications are supporting.

Figure 3



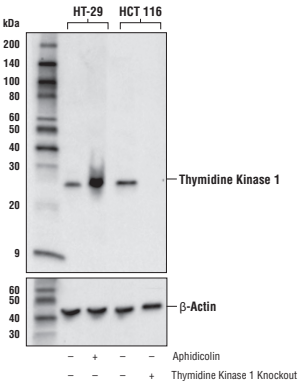
WB analysis of extracts from various cell lines, including RKIP wild-type (WT) and RKIP knock-out (-/-) MEF cells, using RKIP (D42F3) (upper) and GAPDH (D16H11) (lower). The RKIP MEF cells were generously provided by Dr. Marsha Rosner, University of Chicago, Chicago, IL.

Figure 4



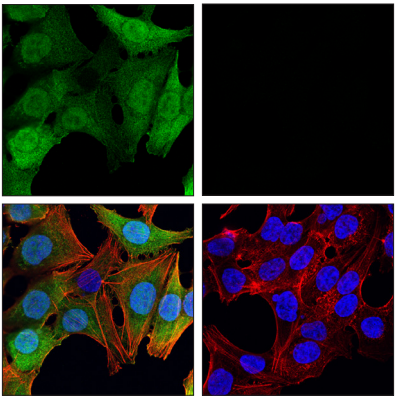
IHC analysis of paraffin-embedded wild-type MEF (left) and RKIP knock-out MEF (right) cell pellets using RKIP (D42F3). Cells provided courtesy of Dr. Marsha Rosner, University of Chicago, Chicago, IL.

Figure 5



WB analysis of extracts from HT-29 cells, mock treated (-) or aphidicolin-treated (10 µg/ml, 24 hr; +) and HCT 116 cells, wild-type (+) or thymidine kinase 1 knockout (-), using Thymidine Kinase 1 (E2H7Z) (upper) or β-Actin (D6A8) (lower).

Figure 6



Confocal IF analysis of HCT 116 cells, wild-type (left, positive) or thymidine kinase 1 knockout (right, negative), using Thymidine Kinase 1 (E2H7Z) (green). Actin filaments were labeled with DyLight™ 554 Phalloidin (red). Samples were mounted in ProLong® Gold Antifade Reagent with DAPI (blue).

Target antigen modification

Another form of binary model involves the use of treatments (agonists or antagonists) to induce or inhibit the expression, localization, or post-translational modification (PTM) of a protein target. Where these treatments result in near-complete positive and negative signal, they can be considered binary validation. Target antigen modification is a preferred approach to validate antibodies against post-translationally modified targets such as phosphorylated or acetylated proteins.

As with any validation technique, suitable loading and expression controls are of critical importance to validation methods that are reliant on modification of the target antigen. These are used to confirm the efficacy of the treatment and to ensure that the samples being evaluated are equivalent in both quality and quantity. It is also important that the treatment is as specific to the target as possible. For example, a specific agonist or inhibitor of a protein or pathway should always be considered before using a more general treatment like phosphatases or deacetylases that nonspecifically remove PTMs from a broad range of targets.

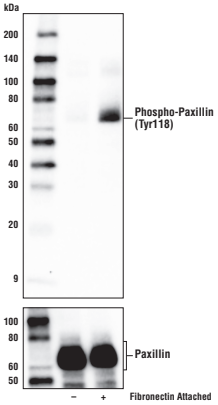
Figures 7 and **8** show validation of a phospho-paxillin (Tyr118) antibody in HeLa cells, using cellular attachment to a fibronectin-coated surface to stimulate phosphorylation on the tyrosine residue, resulting in induced immunocytochemical labeling.

Combined positive and negative controls

An additional binary strategy, most frequently used in IHC, involves validating an antibody in tissue samples that contain both positive and negative cells within the same section. This is illustrated in **Figures 9** and **10**, where the observed staining pattern is consistent with the known expression profile of the targets (ie, positive staining in the correct cells and no staining in the negative cells).

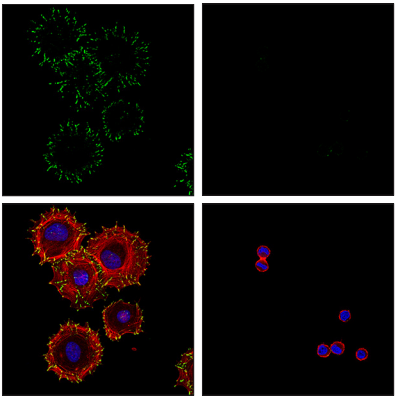
Although binary models provide valuable insight, they are not always readily available or easy to generate quickly for the purpose of validating an antibody reagent. To support binary testing data, or as an alternative means of assessing antibody performance, additional validation methods should always be employed.

Figure 7



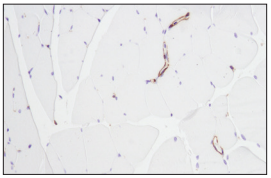
WB analysis of extracts from HeLa cells in suspension, untreated (-) or induced attachment to fibronectin-coated plate surface (10 µg/ml, 1 hr; +), using Phospho-Paxillin (Tyr118) (E9U9F) (upper) and Paxillin (D9G12) (lower).

Figure 8



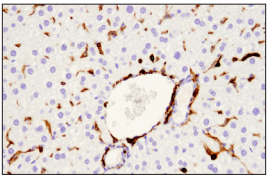
HeLa cells were detached with 0.04% trypsin and 0.03% EDTA, suspended in serum-free medium for one hour, and either processed in suspension (right) or plated on fibronectin-coated slides and allowed to adhere for an additional hour prior to fixation (left). Confocal IF analysis was performed after staining with Phospho-Paxillin (Tyr118) (E9U9F) (green), DyLight™ 554 Phalloidin (red), and ProLong® Gold Antifade Reagent with DAPI (blue).

Figure 9



IHC analysis of paraffin-embedded mouse skeletal muscle using α-Smooth Muscle Actin (D4K9N). Note staining of vascular smooth muscle, as anticipated, and the lack of staining of myocytes that lack smooth muscle actin.

Figure 10



IHC analysis of paraffin-embedded mouse liver using CD45 (D3F8Q). Note lack of staining of the CD45 negative hepatocytes, as expected.

Cell Signaling Technology validation of antibodies recommended for IHC includes:

- Paraffin-embedded cell pellets with known expression levels of the target
- Assessment of specificity and potential off-target binding in the model organism being studied using normal and diseased whole tissues and tissue microarrays, including xenografts and syngeneic tumors if needed
- Verification of modification specificity using peptide blocking or phosphatase treatment
- Optimization of dillutions and protocols, and provision of validated control reagents

Ranged Strategy

While a binary approach is one of the more desirable ways to evaluate antibody specificity, binary models are not always readily available and can be time consuming or expensive to produce for the sole purpose of validating an antibody. Moreover, to assess the sensitivity of an antibody in the application and protocol being used, a complementary hallmark is required.

Ideally suited to this purpose, a ranged strategy includes both endogenous and heterologous models that express high, moderate, and low levels of the target of interest. A ranged approach is critical to understand the optimal working conditions of an antibody; however, its importance to the overall antibody validation process may often be overlooked.

The most important yet subtle difference between ranged strategy and binary strategy is that ranged models rely on differences in target expression or modification that are not black and white. Typically, ranged models are more reflective of actual biology, where expression of the target is high or low in one cell line or tissue relative to another, or is modified only slightly by agonist or antagonist treatment. This means that, while ranged testing results are significant, they are not as striking or as easily interpretable as data generated by binary evaluation.

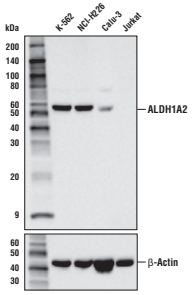
Also included within the ranged strategy hallmark are methods such as siRNA that inhibit gene/protein expression but do not always result in complete ablation. By allowing direct comparison of treated samples to the original material, these techniques add considerable value to antibody validation and are typically easier to establish. As described for binary testing, ranged testing should be coupled to an orthogonal strategy to confirm expression.

Multiple samples

The prototypical example of a ranged model is the use of multiple samples with varying degrees of protein expression or signal. Yet while it is possible to correlate experimental data with previously published or predicted analysis, this is not always the case, illustrating the need to evaluate an antibody with several approaches to build confidence in specificity and sensitivity.

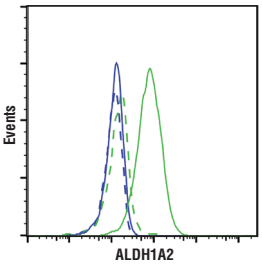
Figures 1 and **2** provide examples of ranged strategy using multiple samples. In **Figure 1**, Western blot data have been generated using the ALDH1A2 (E6O6Q) rabbit mAb to probe lysates from various cell lines, indicating high expression of ALDH1A2 in K-562 cells relative to expression in Jurkat. **Figure 2** shows flow cytometric analysis of K-562 and Jurkat cells using the same ALDH1A2 (E6O6Q) antibody. It can be seen that the data from the two applications are supporting, with loading controls ensuring that sample (analyte) quality does not lead to misinterpretation of results. Critically, the observed expression of ALDH1A2 is consistent with publicly available bioinformatic databases, an orthogonal strategy.

Figure 1



WB analysis of extracts from various cell lines using ALDH1A2 (E6O6Q) (upper) and β-Actin (D6A8) (lower). Expression levels of ALDH1A2 among cell lines are consistent with expectations based on publicly available bioinformatic databases.

Figure 2



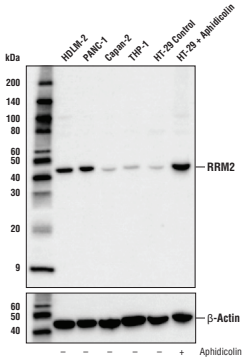
Flow cytometric analysis of Jurkat cells (blue) and K-562 cells (green), using ALDH1A2 (E6O6Q) (solid lines) or a concentration-matched Rabbit (DA1E) Isotype Control (dashed lines). Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) was used as a secondary antibody.

Treated samples

In cases where endogenous expression may be unsuitable, a direct comparison of treated and untreated samples is useful to evaluate antibody specificity. This highlights the importance of understanding the biological role of a target such that the appropriate model(s) can be employed.

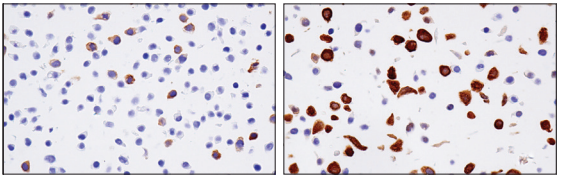
Figures 3, 4, and **5** show data generated using the RRM2 (E7Y9J) rabbit mAb to probe untreated HT-29 cells and HT-29 cells treated with aphidicolin to induce RRM2 expression. It can be seen by western blot, IHC, and immunocytochemical analysis that RRM2 expression is not binary, but instead transitions from low to high following aphidicolin treatment. Testing the model in each application in which the antibody is intended to be used provides assurance of antibody specificity in that application.

Figure 3



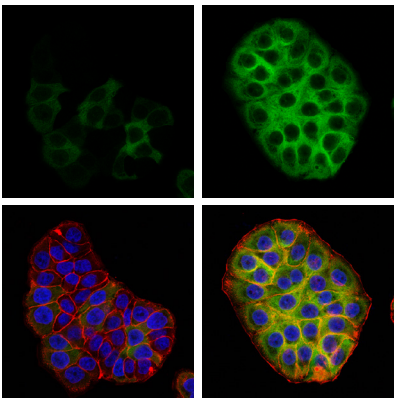
WB analysis of extracts from various cell lines using RRM2 (E7Y9J) (upper) or β-Actin (D6A8) (lower). In lanes 5 and 6, HT-29 cells were treated with vehicle control (-) or Aphidicolin (10 µg/mL, 24 hr; +), showing enrichment of RRM2 expression at the G1/S phase border.

Figure 4



IHC analysis of paraffin-embedded HT-29 cell pellet, untreated (left) or treated with Aphidicolin (right), using RRM2 (E7Y9J).

Figure 5



Confocal IF analysis of HT-29 cells, untreated (left) or treated with Aphidicolin (10 µg/ml, 24 hr; right), using RRM2 (E7Y9J) (green). Actin filaments were labeled with DyLight™ 554 Phalloidin (red). Samples were mounted in ProLong® Gold Antifade Reagent with DAPI (blue).

Multiple models

Although the data shown in **Figures 1-5** clearly illustrate differences in target expression across various samples or following a carefully chosen treatment, in none of these cases is the signal completely abrogated. To verify specificity and validate experimental findings, it is therefore sensible to employ multiple models. Results obtained using these models should be consistent with published biological or orthogonal data and supported by the findings obtained in other applications or techniques.

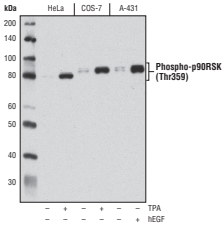
Figures 6 and **7** illustrate the use of multiple models and treatments to validate a phospho-p90RSK (Thr359) rabbit mAb via methods that are consistent with the known biological mechanism of the target. **Figure 6** shows western blot analysis of lysates prepared from HeLa, COS-7, and A-431 cells following serum starvation and treatment with 12-O-Tetradecanoylphorbol-13-acetate (TPA) or human epidermal growth factor (hEGF), clearly indicating increased target expression under defined conditions. In **Figure 7**, the same antibody has been used to perform immunocytochemical analysis of A-431 cells after treatment with hEGF. The same models are used in both applications, with results of independent assays supportive of each other.

Evaluating sensitivity

It is important to note that the sensitivity of an antibody can vary significantly from one context and application to the next. This means that validation that may appear binary in one application is instead ranged in another, as illustrated in **Figures 8** and **9**. **Figure 8** shows western blot analysis of phospho-c-Jun (Ser73) expression in lysates prepared from NIH/3T3 and C6 cells following exposure to UV light. In these cell lines and this application, the resulting data appear binary with a signal only detectable in the treated samples. In **Figure 9**, the same antibody has been used for immunocytochemical analysis of HeLa cells after treatment with anisomycin. Here, the signal appears weak but detectable in the untreated cells.

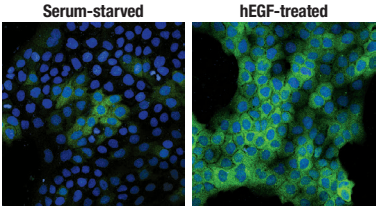
It is vital to note that, although the phospho-c-Jun signal is absent in the untreated lanes of the western blot, this does not mean that the target itself is absent. Instead, the signal may simply be below the threshold of detection in the western blot application. Additionally, while a weak signal is detectable by immunocytochemical analysis of the untreated cell population, this may indicate greater sensitivity in this application or it may reflect nonspecific binding. For these reasons, multiple approaches should always be used to support the observations obtained in a single experiment.

Figure 6



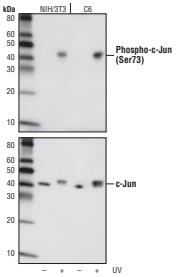
WB analysis of extracts from HeLa, COS-7, and A-431 cells, starved overnight and either untreated (-) or treated (+) with TPA (200 nM, 15 min) or Human Epidermal Growth Factor (hEGF) (100 ng/mL, 15 min), using Phospho-p90RSK (Thr359) (D1E9).

Figure 7



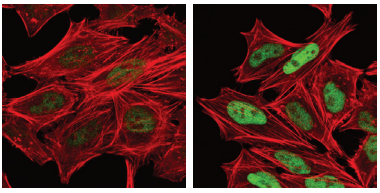
Confocal IF analysis of A-431 cells, serum-starved (left), treated with hEGF (100 ng/mL, 15 min; right), using Phospho-p90RSK (Thr359) (D1E9) (green). Blue pseudocolor = DRAQ5® (fluorescent DNA dye).

Figure 8



WB analysis of extracts from NIH/3T3 or C6 cells, untreated or UV-treated, using Phospho-c-Jun (Ser73) (D47G9) (upper) or c-Jun (60A8) (lower).

Figure 9



Confocal immunofluorescent analysis of HeLa cells, untreated (left) or anisomycin-treated (right), using Phospho-c-Jun (Ser73) (D47G9) (green). Actin filaments have been labeled with DY-554 phalloidin (red).

CST validation of antibodies recommended for flow cytometry includes:

- Use of positive and negative cell lines, KO models, and primary cells
- Comparison of signal to isotype control to estimate nonspecific binding of primary antibodies
- Treatment with pathway-specific inhibitors/activators
- Optimization of protocols and determination of optimal dilutions
- Extensive quality control testing to guarantee stability over time and eliminate lot-to-lot variability

Orthogonal Strategy

An orthogonal strategy for antibody validation involves crossreferencing antibody-based results with data obtained using non-antibody-based methods. This approach is critical to verify existing antibody validation data and to identify any effects or artifacts that are directly related to the antibody in question. Providing an additional level of detail to support results generated by the other strategies outlined within this handbook, orthogonal validation often utilizes data which are available in the public domain.

Depending on the antigenic target, non-antibody-based methods can include mining previously published results, studying expression analysis via 'omics techniques (genomics, transcriptomics, and proteomics), and employing other established antibody-independent methods such as in situ hybridization or RNA sequencing (RNA-seq). Correspondingly, an orthogonal strategy can also be used to ensure that any antibody validation performed in-house uses the most relevant biological models for the target of interest.

In its simplest form, an orthogonal strategy dictates that results obtained in the other hallmarks require corroboration by non-antibody-based detection methods. As just one example, positive and negative expression of the target observed by binary or ranged strategies should always be confirmed using an orthogonal approach, such as genetic sequencing to confirm knockout or transcriptomic analysis of mRNA to confirm expression.

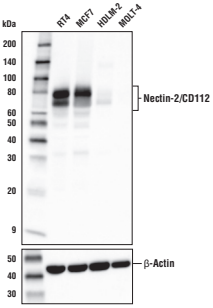
As with all the strategies discussed in this handbook, relying on a single source of information or an individual result is never a good idea. For some targets, there is a significant amount of expression and biological data available (albeit sometimes conflicting) to guide validation strategies. For other targets, it may be necessary to perform appropriate experiments in-house to support antibody testing. Where data from multiple, trusted sources are available, this can, of course, save both time and resources; however, it is important to note that effective orthogonal validation may require additional effort.

Validation using 'omics data

Although established immunostaining techniques such as western blot and IHC provide a quick visual indication of antibody specificity, it is vital that any antibody validation data generated using these methods are supported by orthogonal testing. One way of achieving this is to mine publicly available databases (eg, CCLE, BioGPS, Human Protein Atlas, DepMap Portal, COSMIC) for genomic and transcriptomic profiling information to help understand whether observed immunostaining results are relevant or are instead due to antibody-related artifacts.

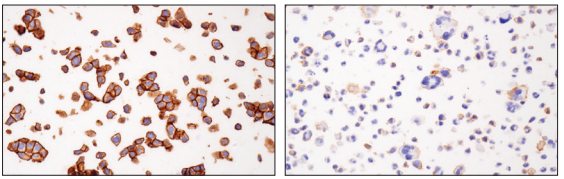
As an example, **Figure 1** shows western blot detection of Nectin-2 in a variety of cell lines using the Nectin-2/CD112 (D8D3F) rabbit mAb. Elevated expression is noticeable in RT4 and MCF7, while only minimal expression can be seen in HDLM-2 and MOLT-4. These results are mirrored by IHC analysis of RT4 and HDLM-2 cell pellets (**Figure 2**), clearly illustrating that data from two different applications correlate exceptionally well with the predicted expression of Nectin-2 based on genomics and transcriptomics data, as shown in **Figure 3**.

Figure 1



WB analysis of extracts from various human cells using Nectin-2/CD112 (D8D3F) (upper) and β-Actin (D6A8) (lower).

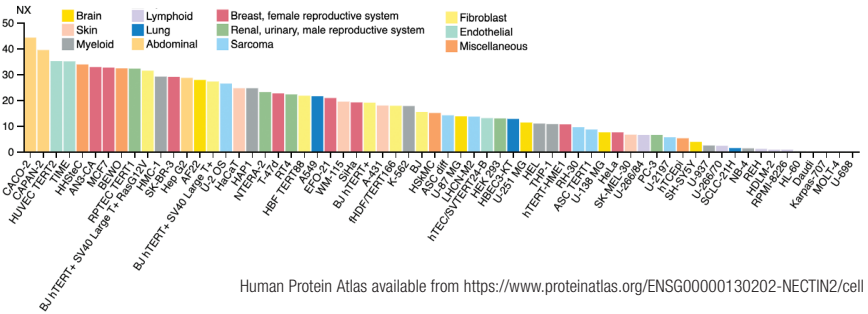
Figure 2



IHC analysis of paraffin-embedded RT4 cell pellet (left, high-expressing) or HDLM-2 cell pellet (right, low-expressing) using Nectin-2/CD112 (D8D3F).

Figure 3

RNA Normalized Expression



Human Protein Atlas available from https://www.proteinatlas.org/ENSG00000130202-NECTIN2/cell

Validation of antibody data generated using imaging techniques

Complementing the various 'omics techniques, orthogonal methods such as *in situ* hybridization, RNA-seq, and RNAscope® allow for the detection of protein expression and/or localization in tissues. These approaches are especially useful for validating antibody data that have been generated using imaging techniques like immunocytochemistry or IHC.

Figure 4 shows IHC analysis of mouse Olfm4 using the Olfm4 (D6Y5A) rabbit mAb. This illustrates positive staining in mouse small intestine and negative staining in colon, a finding which is supported by western blot (**Figure 5**). The observed staining pattern is consistent with numerous published orthogonal strategies, including in situ hybridization.[1,2]

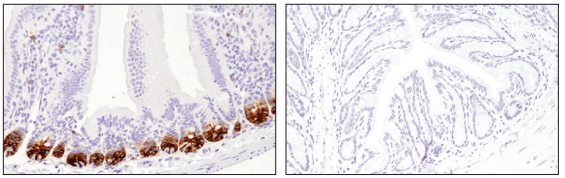
The defining criterion of success for an orthogonal strategy is consistency between the known or predicted biological role and localization of a gene/protein of interest and the resultant antibody staining. This highlights the importance of verifying the specificity and functionality of all reagents in the model and application that will be used in downstream experiments.

Like the other hallmarks described within this handbook, no single validation strategy is sufficient in isolation. Although orthogonal strategies provide evidence that an antibody is behaving as expected, it is critical to combine orthogonal testing with other validation approaches to assure confidence in antibody performance.

References

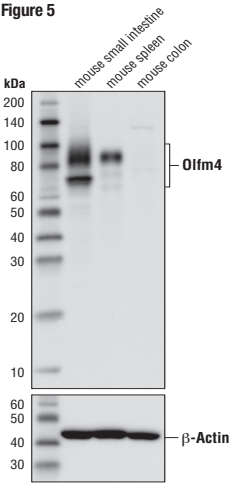
1. Flier, L. G. V. D., Gijn, M. E. V., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D. E., … Clevers, H. (2009). Transcription Factor Achaete Scute-Like 2 Controls Intestinal Stem Cell Fate. Cell, 136(5), 903–912. doi: 10.1016/j.cell.2009.01.031
2. Flier, L. G. V. D., Haegebarth, A., Stange, D. E., Wetering, M. V. D., & Clevers, H. (2009). OLFM4 Is a Robust Marker for Stem Cells in Human Intestine and Marks a Subset of Colorectal Cancer Cells. Gastroenterology, 137(1), 15–17. doi: 10.1053/j.gastro.2009.05.035

Figure 4



IHC analysis of paraffin-embedded normal mouse small intestine (left) and colon (right) using Olfm4 (D6Y5A).

Figure 5



WB analysis of extracts from mouse small intestine (positive), mouse spleen (positive), and mouse colon (negative) using Olfm4 (D6Y5A) (upper) and β-Actin (D6A8) (lower).

CST immunofluorescence antibody validation steps:

- Use of cell lines or tissues with known target expression levels to verify specificity, leveraging knockout or null cell lines when available
- Establishment of minimum intensity/noise thresholds
- Verification of subcellular localization through high-resolution imaging, matching to published reports
- Analysis of expression patterns across complex tissue arrays
- Phosphatase treatment of cells to verify phospho-specificity
- Examination of activation state specification, target expression, and translocation using ligands or inhibitors to modulate pathway activity
- Optimization of fixation and permeabilization conditions; recommendation of alternative protocols if necessary
- Stringent testing to ensure lot-lot consistency

Multiple Antibody Strategy

A multiple antibody strategy is a powerful approach to antibody validation. One of the most common methods to achieve this is to immunoprecipitate (IP) the target with one antibody and subsequently detect it by western blotting with another antibody against the same target. This provides confidence that both antibodies are binding the correct biomolecule.

Another familiar method of multiple antibody validation involves using two or more antibodies against distinct, nonoverlapping epitopes on the same target to produce directly comparable immunostaining data. This is typically demonstrated through techniques such as western blotting, immunocytochemistry or IHC. By probing identical samples with multiple antibodies in parallel, it is possible to gain a relatively quick visual indication of antibody specificity.

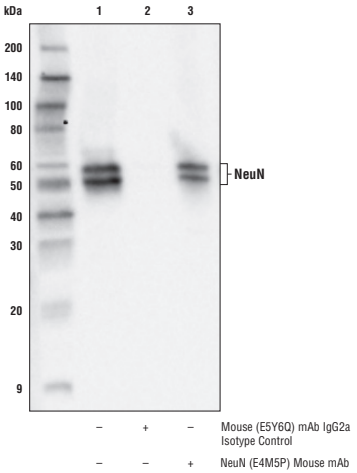
In situations where two antibodies against the same target are not available, alternative strategies can be employed to determine antibody specificity. For example, IP followed by mass spectrometry is a method increasingly used to detect proteins that have been enriched by an antibody that is under evaluation.

As with any of the hallmarks of antibody validation, a multiple antibody strategy should never be the only strategy employed to determine antibody specificity. For example, to eliminate the possibility that while antibody binding is comparable by IHC, both reagents in fact recognize the same incorrect, nontarget biomolecule, multiple antibody testing data should always be supported by additional antibody validation strategies. Moreover, the multiple antibody approach should never be used in a "blinded" manner as a means to screen or select antibodies; all antibodies tested this way should be validated independently using the other strategies outlined within this handbook.

Immunoprecipitation

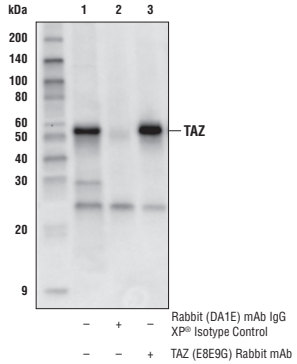
IP is one of the most obvious approaches to multiple antibody validation, providing a clear visual indication that two distinct antibodies bind the same target. The evidence is strongest if the antibodies recognize different regions of the molecule, as illustrated in **Figure 1**. Here, a NeuN (E4M5P) mouse mAb has been used to immunoprecipitate the NeuN protein from rat brain extracts and a NeuN (D4G4O) rabbit mAb has been employed for western blot analysis. A similar dataset is shown in **Figure 2**, using a TAZ (EBE9G) rabbit mAb for IP and a second TAZ (D316D) rabbit mAb for western blotting. In both cases, the inclusion of an isotype control helps to confirm antibody specificity.

Figure 1



IP of NeuN protein from rat brain tissue extracts. Lane 1 is 10% input, lane 2 is Mouse (E5Y6Q) mAb IgG2a Isotype Control, and lane 3 is NeuN (E4M5P). Western blot analysis was performed using NeuN (D4G4O).

Figure 2



IP of TAZ protein from HeLa cell extracts. Lane 1 is 10% input, lane 2 is Rabbit (DA1E) Isotype Control, and lane 3 is TAZ (E8E9G). Western blot analysis was performed using TAZ (D3I6D).

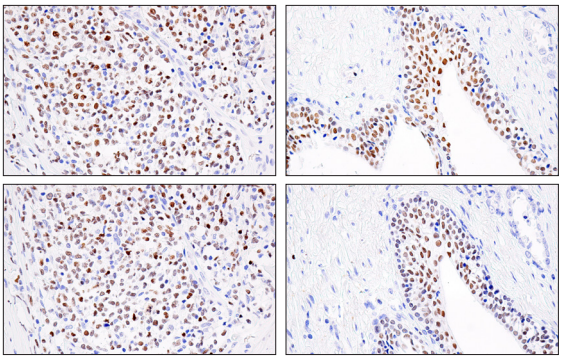
Detection of distinct antigens

The use of multiple antibodies to probe identical samples via immunostaining techniques is a straightforward yet underutilized approach to confirm antibody specificity. If two or more distinct antibody reagents demonstrate an identical staining pattern or antigen localization, this provides confidence that an antibody is staining the target specifically.

It is possible to use a previously validated antibody to confirm observed immunostaining data from an antibody that is undergoing validation. If the results obtained with the test antibody reproduce those of the validated antibody, this is seen as an indication of specificity. For example, **Figure 3** shows IHC analysis of two different tissue types using two Helios rabbit mAbs that recognize distinct epitopes on the human protein. The staining pattern in both tissues is comparable, suggesting that both antibodies are specific to the target. In **Figure 4**, two different MAGE-A4 rabbit mAbs have been used to stain human squamous cell lung carcinoma tissue. Again, both antibodies can be seen to produce similar staining, delivering confidence in their specificity.

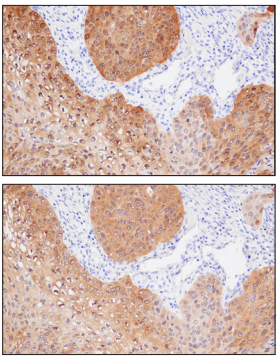
In addition to providing side-by-side data from two antibodies that recognize different epitopes on the same target, it is ideal to find comparable data across several products. For instance, **Figures 5** and **6** show western blot data associated with two unique antibodies against CD200 to be similar, highlighting the consistency of the models used to test the antibodies and the similarity of the results that have been generated. In contrast, if data for two different products against the same target differ vastly, the validity of the results may be called into question.

Figure 3



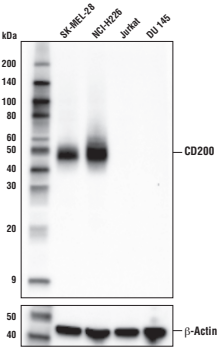
IHC analysis of paraffin-embedded human B-cell non-Hodgkin's lymphoma (left) or prostate carcinoma (right) using Helios (E4L5U) (upper) or Helios Antibody (lower). These two antibodies detect independent, unique epitopes on human Helios. The similar staining patterns obtained with both antibodies help to confirm the specificity of the staining.

Figure 4



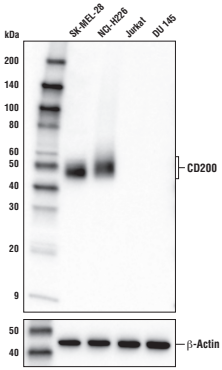
IHC analysis of paraffin-embedded human squamous cell lung carcinoma using MAGE-A4 (E7O1U) (upper) or MAGE-A4 Antibody (lower). These two antibodies detect independent, unique epitopes on human MAGE-A4. The similar staining patterns obtained with both antibodies help to confirm the specificity of the staining.

Figure 5



WB analysis of extracts from various cell lines using CD200 (E5I9V) (upper) or β-Actin (D6A8) (lower).

Figure 6



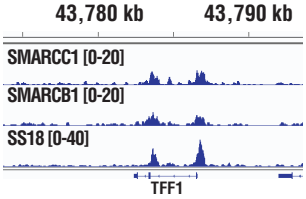
WB analysis of extracts from various cell lines using CD200 (E2K4C) (upper) or β-Actin (D6A8) (lower).

Chromatin immunoprecipitation

Multiple antibodies can also be used to validate results in chromatin immunoprecipitation (ChIP) experiments. Employed to identify proteins such as transcription factors and cofactors that bind to a specific piece of chromatin in vivo, ChIP is used to probe protein-DNA interactions within the natural chromatin context of the cell. By using multiple antibodies against non-overlapping epitopes of the same target protein, or multiple antibodies against different target proteins within the same DNA-binding complex and coupling ChIP with qPCR or NG-seq analysis, researchers can benefit from a highly versatile method of antibody cross-validation.

Figure 7 shows ChIP data from an experiment using three antibodies against three different protein targets within the SW1/SNF complex – SMARCC1, SMARCB1 and SS18. Following ChIP and subsequent analysis by NG-seq, it can be seen that all three antibodies yield very similar results.

Figure 7



ChIP was performed with crosslinked chromatin from MCF7 cells grown in phenol red free medium and 5% charcoal stripped FBS for 4 d, followed by treatment with β-estradiol (10 nM, 45 min) and either SMARCC1/BAF155 (D7F8S), SMARCB1/BAF47 (D8M1X), or SS18 (D6I4Z), using SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads). DNA Libraries were prepared using SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina®. SMARCC1/BAF155, SMARCB1/BAF47, and SS18 are all subunits of SWI/SNF complex. The figure shows binding across pS2/TFF1, a known target gene of SWI/SNF complex.

CST ChIP and ChIP-seq antibody validation steps:

- Antibody specificity is first determined by analyzing immune-enrichment of at least two known positive and one known negative target loci by ChIP-qPCR. The antibody must show a minimum fold enrichment for known positive loci compared to known negative target loci.
- Antibody specificity is further analyzed for histone methylysine and methylarginine antibodies using the proprietary histone peptide arrays.
- Every antibody is titrated to determine optimal performance in the ChIP assay.
- Too little or too much antibody can both be detrimental to the immune-enrichment of target loci.
- Antibody sensitivity for ChIP-seq is then confirmed by analyzing the signal:noise ratio of target enrichment across the genome in antibody:input control comparisons. The antibody must provide an acceptable minimum number of defined enrichment peaks and a minimum signal:noise threshold compared to input chromatin.
- For sequence-specific DNA-binding transcription factors, antibody specificity is determined by performing motif analysis of enriched chromatin fragments.
- Antibody specificity is further determined by comparing enrichment across the genome using multiple antibodies against distinct target protein epitopes.
- Antibody specificity is confirmed using antibodies against different subunits of a multiprotein complex.
- Antibody specificity is further confirmed by comparing enrichment across the genome to published ChIP-seq data (ie, ENCODE) using additional antibodies for a given target protein.

Recombinant Strategy

For antigenic targets where expression of the protein is very low or unknown, the use of recombinant proteins or heterologous expression in a surrogate cell line may be necessary for antibody validation. Although endogenous systems are preferred for their closer representation of *in vivo* conditions, recombinant strategy offers several advantages.

Firstly, recombinant strategy can be used to verify the crossreactivity of an antibody with protein isoforms or conserved family members, providing useful information regarding the antibody's potential for off-target binding based on antigen homology. Recombinant strategy can also be used to test the sensitivity of an antibody through titrating the target protein by expression or dilution.

Additional uses of recombinant strategy include validating and optimizing the ability of an antibody to work in IP and generating a standard positive control for western blot applications. Moreover, by providing the option to express various, subtly different forms of a protein, recombinant strategy allows for verification of antibody specificity against site-specific mutants.

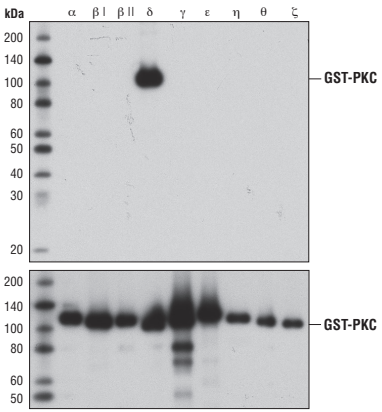
Recombinant proteins

A common use of recombinant proteins is to verify the specificity of an antibody for one or more members of a protein family. To illustrate this, **Figure 1** shows western blot analysis of nine different recombinant PKC isoforms to confirm the specificity of the PKCδ (D10E2) rabbit mAb. In this instance, antibody binding is isoform-specific, with no off-target binding observed for the other recombinant isoforms tested.

In a slightly different approach, **Figure 2** demonstrates the pan-reactivity of a 14-3-3 (pan) antibody. The western blot data clearly show antibody recognition of all six recombinant isoforms tested, providing supporting evidence for isoform reactivity. Importantly, the inclusion of a loading control shows differences in band intensity to be due to protein load rather than a result of varying antibody affinity for different isoforms.

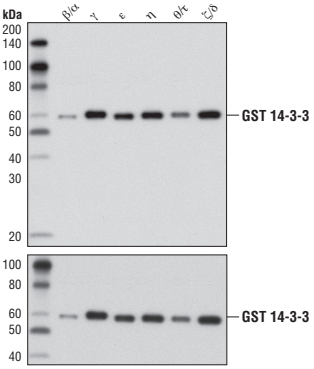
A key point to note here is that, while recombinant protein or peptide arrays can be used to determine antibody specificity and crossreactivity, results from these types of strategies can be misleading due to the artificial nature of these assays. Moreover, several key features of these systems currently lack standardization, highlighting once again the need to back any recombinant strategy with the other hallmarks outlined in this handbook.

Figure 1



WB analysis of bacterially expressed, GST-tagged, purified PKC isoforms, using PKCδ (D10E2) (upper) or GST (91G1) (lower), demonstrating specificity for PKCδ.

Figure 2



WB analysis of purified, recombinant, GST-tagged 14-3-3 isoforms using 14-3-3 (pan) (upper) or GST (91G1), (lower) demonstrating isoform crossreactivity.

Heterologous expression

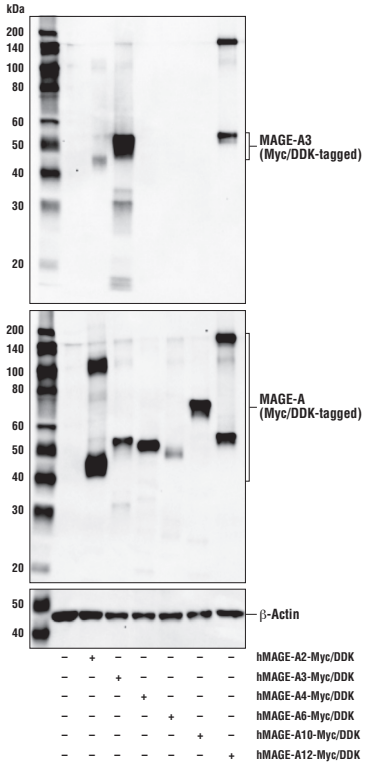
DNA plasmids designed to express the antigenic target(s) of interest in a heterologous system are a popular alternative to using recombinant proteins. Again, this strategy is commonly used to verify antibody specificity and determine crossreactivity with isoforms, homologs, and orthologs. Proteins expressed in this way are readily analyzed by established techniques, such as western blot or immunocytochemical staining.

Heterologous expression is a required step in validating antibodies against epitope tags, proteins that are not endogenously expressed in mammalian cells, or proteins that are expressed under only a limited set of conditions or in rare cell populations. Since these targets cannot be analyzed in endogenous systems, heterologous expression is essential to verify antibody specificity.

Figure 3 shows western blot analysis of a MAGE-A3 antibody using lysates prepared from 293T cells following transfection with constructs expressing various Myc/DDK-tagged MAGE isoforms. MAGE-A3 is a nuclear protein frequently expressed in various tumor cells and is not endogenously expressed by 293T. These data illustrate antibody specificity for MAGE-A3, with a DYKDDDK antibody used to demonstrate successful transfection and a β-actin antibody employed as a loading control.

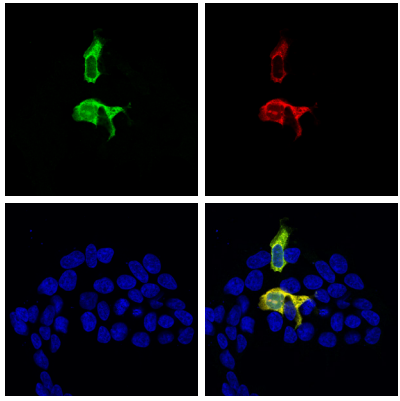
In **Figure 4**, data illustrating the validation of an antibody to the non-natively expressed protein Cas9 are shown in a model consistent with its intended experimental use. Here, myc-tagged Cas9 was transfected into 293T cells and detected using the Cas9 (*S. pyogenes*) (E7M1H) rabbit mAb to perform immunocytochemical staining. The Myc-Tag (9B11) mouse mAb was used to confirm expression of the cDNA.

Figure 3



WB analysis of extracts from 293T cells, mock transfected (-) or transfected (+) with constructs expressing Myc/DDK-tagged full-length human MAGE-A2 (hMAGE-A2-Myc/DDK), Myc/DDK-tagged full-length human MAGE-A3 (hMAGE-A3-Myc/DDK), Myc/DDK-tagged full-length human MAGE-A4 (hMAGE-A4-Myc/DDK), Myc/DDK-tagged full-length human MAGE-A6 (hMAGE-A6-Myc/DDK), Myc/DDK-tagged full-length human MAGE-A10 (hMAGE-A10-Myc/DDK), and Myc/DDK-tagged full-length human MAGE-A12 (hMAGE-A12-Myc/DDK), using MAGE-A3 (E9S4X) (upper), DYKDDDK Tag Antibody (middle), and β-Actin (D6A8) (lower).

Figure 4



Confocal IF analysis of 293T cells transiently transfected with a myc-tagged Cas9 (S. pyogenes) construct, using Cas9 (S. pyogenes) (E7M1H) (green) and Myc-Tag (9B11) (red). Colocalization of green and red signals appear yellow in the composite image (bottom-right). Samples were mounted in ProLong® Gold Antifade Reagent with DAPI (blue).

Detection of site-specific mutants

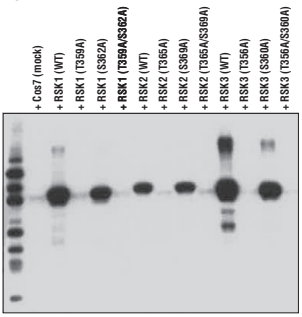
Because a recombinant strategy allows for the expression of an almost infinite number of constructs, it is not limited to expressing wild-type proteins. Confirmation of the specificity of a site-specific antibody can be verified using site-specific mutants of the protein expressed exogenously/recombinantly in bacterial or mammalian cells.

Illustrating this concept, **Figure 5** demonstrates specificity of the phospho-p90RSK (Thr359) (D1E9) rabbit mAb for the Thr359-phosphorylated form of the p90RSK protein. In this instance, the antibody was used to probe lysates prepared from cells expressing the wild-type protein, a Thr359 to Ala (T359A) mutant, a Ser362 to Ala (S362A) mutant, or a dual mutant (T359A/S362A), in addition to equivalent sites on p90RSK family members RSK2 and RSK3.

These results highlight two important features of the antibody: specificity for the threonine site and a capacity to equivalently detect the conserved site on other family members. These kinds of experiments are rarely done or are overlooked by most vendors.

Since recombinant strategy differs significantly from the other hallmarks in that it relies on non-native expression of antigenic targets, it is of paramount importance to combine it with other validation strategies to achieve meaningful insight regarding antibody specificity and sensitivity. Used in isolation, recombinant strategy can be misleading, but in combination with the other hallmarks it represents an extremely powerful approach to antibody validation.

Figure 5



WB analysis of extracts from 293T cells, mock-transfected (mock), or transfected with constructs expressing wild-type (WT) RSK1, RSK2, and RSK3, or the indicated site-specific mutations, using Phospho-p90RSK (Thr359) (D1E9), demonstrating phospho-specific reactivity of the antibody with RSK1 (Thr359), RSK2 (Thr365) and RSK3 (Thr356).

CST validation of antibodies recommended for western blot includes:

- Examination of several cell lines and/or tissues of known expression levels to verify specificity and determine species crossreactivity
- Treatment of cell lines with growth factors, chemical activators, or inhibitors to induce or inhibit target expression to verify specificity; phosphatase treatment to confirm phospho-specificity
- Use of siRNA transfection or knockout cell lines to verify target specificity
- Side-by-side comparison of lots to ensure lot-to-lot consistency
- Use of multiple antibodies raised against nonoverlapping epitopes to confirm specificity
- Optimal dilutions and buffers are predetermined, positive and negative cell extracts are specified, and detailed protocols are optimized, saving valuable time and reagents

Complementary Strategies

Depending on the antigenic target being studied and the application being used, it may be advisable to employ complementary strategies during antibody validation. These approaches can provide vital information regarding antibody specificity or functionality and can be carefully tailored to the biological nature of the target as well as to the exacting requirements of the downstream assay.

Complementary strategies include the use of peptide arrays and/or ELISAs to determine the specificity of an antibody for a post-translational modification (PTM), and various peptide blocking methods to prevent antibody binding to a defined antigen. Protocol optimization is also included within the complementary strategies hallmark, as are various functional assays such as neutralization or protein activation using an antibody as an agonist. All these methods provide additional data to support results generated using the other hallmarks of validation.

Determining PTM specificity

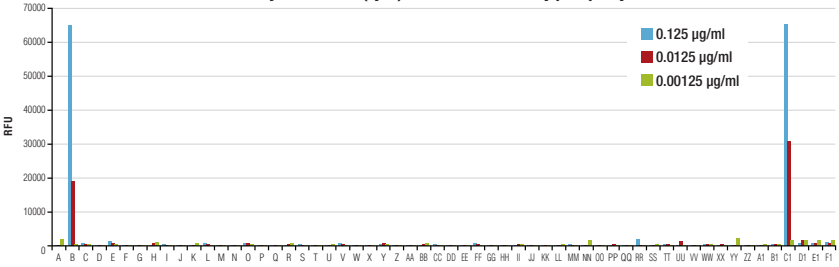
PTMs such as phosphorylation, acetylation, methylation, ubiquitination, and sumoylation are major sources of protein variation. Since the number and types of PTM present on a protein may dictate both structure and function, researchers require highly specific antibodies to detect and quantify these modifications.

Primarily used to validate antibodies against histone modifications, as well as antibodies against other PTMs, peptide arrays and competitive ELISAs are useful tools to determine PTM specificity and to assess the impact of proximal modifications on the specificity and sensitivity of an antibody. By providing the capacity to evaluate multiple PTMs simultaneously, these systems rapidly provide large quantities of valuable multiplex data.

- **Peptide array**
  Arrays of modified and unmodified histone tail peptides can be used to validate antibody specificity. This is illustrated in **Figure 1**, which shows the use of such an array to screen the Mono-Methyl-Histone H3 (Lys4) (D1A9) rabbit mAb against a selection of peptides and modifications. The results demonstrate that not only does the antibody specifically detect acetylated lysine 4 of Histone H3 but that the presence of nearby modifications (eg, phosphorylation at serine 6) has no impact on antibody specificity or functionality.

Figure 1

Mono-Methyl Histone H3 (Lys4) (D1A9) is highly specific for mono-methyl-histone H3 (Lys4) and is not affected by phosphorylation at Thr6.

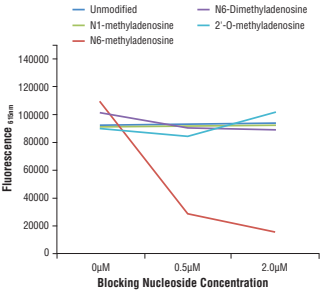


A	H3 (Lys4) non-methyl	**R**	H3 (Lys79) mono-methyl	**FF**	H3 (Arg2) symmetric-di-methyl/ (Lys4) mono-methyl	**OO**	H3 (Lys9) mono-methyl/(Ser10) phospho	**XX**	H3 (Lys9) mono-methyl/(Ser10/ Thr11) phospho
B	H3 (Lys4) mono-methyl	**S**	H3 (Lys79) di-methyl	**GG**	H3 (Arg2) symmetric-di-methyl/ (Lys4) di-methyl	**PP**	H3 (Lys9) di-methyl/(Ser10) phospho	**YY**	H3 (Lys9) di-methyl/(Ser10/ Thr11) phospho
C	H3 (Lys4) di-methyl	**T**	H3 (Lys79) tri-methyl	**HH**	H3 (Arg2) symmetric-di-methyl/ (Lys4) tri-methyl	**QQ**	H3 (Lys9) tri-methyl/(Ser10) phospho	**ZZ**	H3 (Lys9) tri-methyl/(Ser10/ Thr11) phospho
D	H3 (Lys4) tri-methyl	**U**	H4 (Lys20) non-methyl	**I I**	H3 (Arg2) asymmetric-di-methyl/ (Lys4) mono-methyl	**RR**	H3 (Arg26) asymmetric-di-methyl/(Lys27) mono-methyl	**A1**	H3 (Thr6) phospho/(Lys9) tri-methyl
E	H3 (Lys9) non-methyl	**V**	H4 (Lys20) mono-methyl	**JJ**	H3 (Arg2) asymmetric-di-methyl/ (Lys4) di-methyl	**SS**	H3 (Arg26) asymmetric-di-methyl/(Lys27) di-methyl	**B1**	H3 (Lys4) di-methyl/(Thr6) phospho
F	H3 (Lys9) mono-methyl	**W**	H4 (Lys20) di-methyl	**KK**	H3 (Arg2) asymmetric-di-methyl/ (Lys4) tri-methyl	**TT**	H3 (Arg26) asymmetric-di-methyl/(Lys27) tri-methyl	**C1**	H3 (Lys4) mono-methyl/(Thr6) phospho
G	H3 (Lys9) di-methyl	**X**	H4 (Lys20) tri-methyl	**LL**	H3 (Arg8) symmetric-di-methyl/ (Lys9) mono-methyl	**UU**	H3 (Lys27) mono-methyl/(Ser28) phospho	**D1**	H3 (Lys4) tri-methyl/(Thr6) phospho
H	H3 (Lys9) tri-methyl	**Y**	H2A (Lys5) non-methyl	**MM**	H3 (Arg8) symmetric-di-methyl/ (Lys9) di-methyl	**VV**	H3 (Lys27) di-methyl/(Ser28) phospho	**E1**	H3 (Thr6) phospho/(Lys9) di-methyl
I	H3 (Lys27) non-methyl	**Z**	H2A (Lys5) mono-methyl	**NN**	H3 (Arg8) symmetric-di-methyl/ (Lys9) tri-methyl	**WW**	H3 (Lys27) tri-methyl/(Ser28) phospho	**F1**	H3 (Thr6) phospho/(Lys9) mono-methyl
J	H3 (Lys27) mono-methyl	**AA**	H2A (Lys5) di-methyl						
K	H3 (Lys27) di-methyl	**BB**	H2A (Lys5) tri-methyl						
L	H3 (Lys27) tri-methyl	**CC**	H3 (Thr3) phospho/ (Lys4) mono-methyl						
M	H3 (Lys36) non-methyl								
N	H3 (Lys36) mono-methyl	**DD**	H3 (Thr3) phospho/ (Lys4) di-methyl						
O	H3 (Lys36) di-methyl	**EE**	H3 (Thr3) phospho/ (Lys4) tri-methyl						
P	H3 (Lys36) tri-methyl								
Q	H3 (Lys79) non-methyl								

ELISA

ELISAs are also widely used to evaluate antibody specificity, as shown in **Figure 2**. Here, a competitive ELISA was used to validate an N6-Methyladenosine (m6A) (D9D9W) rabbit mAb. This was achieved by incubating the antibody in microplate wells precoated with an m6A oligonucleotide in the presence of increasing concentrations of differentially modified adenosine. The results show that antibody binding to m6A is blocked only by the free m6A peptide, which is indicative of specificity for the target.

Figure 2



Specificity of N6-Methyladenosine (m6A) (D9D9W) was determined by competitive ELISA. The graph depicts the binding of the antibody to a pre-coated m6A oligonucleotide in the presence of increasing concentrations of differentially modified adenosine. As shown in the graph, antibody binding is only blocked by free m6A nucleoside.

Assessing antibodies against nonprotein targets

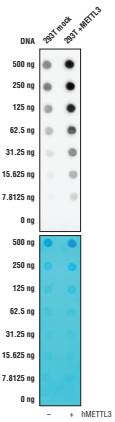
During the validation of antibodies against small molecules or nonprotein targets, it is critical to determine that the antibody specifically and sensitively binds the moiety of interest without detecting any biosimilars. This is because off-target binding can interfere significantly with any experimental results, rendering any data useless. In these instances, it is sensible to compare the reactivity of the antibody against both the intended antigen and against other compounds of similar size and shape, for example by running an ELISA.

Dot blot

A simpler approach to an ELISA is to use a dot blot technique where the antibody being validated is screened against samples that have been blotted on to a membrane. This method is frequently used as a complementary strategy to evaluate antibody specificity for a PTM, as illustrated in **Figure 3**. However, it is important to note that, while dot blots are a quick and easy way to test antibody specificity, the experimental design must always be carefully thought out and well controlled for any data to be relevant.

In this validation experiment, the same N6-Methyladenosine (m6A) (D9D9W) rabbit mAb mentioned previously was tested against RNA from 293T cells in the presence or absence of expression of METTL3, a protein that induces production of N6-Methyladenosine. Antibody binding to the target is clearly influenced by the concentration of METTL3, providing confidence in its specificity for the target.

Figure 3



Total RNA purified from 293T cell extracts, either mock transfected (-) or transfected with a DNA construct expressing full-length human METTL3 (hMETTL3; +), were blotted onto a nylon membrane, UV cross-linked, and probed with N6-Methyladenosine (m6A) (D9D9W). The top panel shows the antibody detecting more methylated adenosine in cells overexpressing METTL3, while the bottom panel shows the membrane stained with methylene blue.

Peptide competition

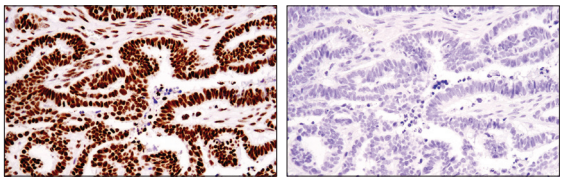
During a peptide competition assay, the antibody being validated is incubated with the immunizing peptide prior to use in a downstream application. In theory, antibody binding to the peptide will prevent detection of the antigenic target. However, peptide competition should never be considered validation in isolation, because a peptide antigen will block antibody binding to all proteins to which the antibody binds, even those that bind nonspecifically. It is imperative that other validation strategies as described in this handbook be used in combination with peptide competition assays.

Despite this, peptide competition does have utility in validating antibodies against PTMs, since it allows direct comparison of a modified peptide to a nonmodified peptide. **Figure 4** shows evaluation of a Tri-Methyl-Histone H3 (Ly36) (D5A7) rabbit mAb by IHC using this approach. Following antibody incubation with a nonmodified peptide (left) or a tri-methyl-blocking peptide (right), ovarian carcinoma sections were subject to IHC analysis. The results provide supporting evidence that the staining of the antibody is specific for the methyl-modification.

Throughout this handbook, it has been heavily stressed that no one validation strategy is better than another and that none of the approaches discussed here should ever be used in isolation. Each antibody-based application provides a unique set of conditions, presenting very different challenges relative to antibody specificity, sensitivity, and functionality. As just one example, an antibody that displays exquisite specificity by western blot may be nonspecific in an IHC assay or ineffective in a functional assay. This emphasizes the importance of validating every single antibody using strategies and protocols consistent with the desired application in the intended model system.

At CST, we employ the hallmarks described here to assure confidence in our antibody reagents, indicating those applications in which researchers can expect our antibodies to perform appropriately. Ultimately, it is up to the end users of our products to ensure that any antibody works in the intended application, protocol, and model system. Taking the time to optimize each experimental system individually is critical to producing precise results that accelerate research.

Figure 4

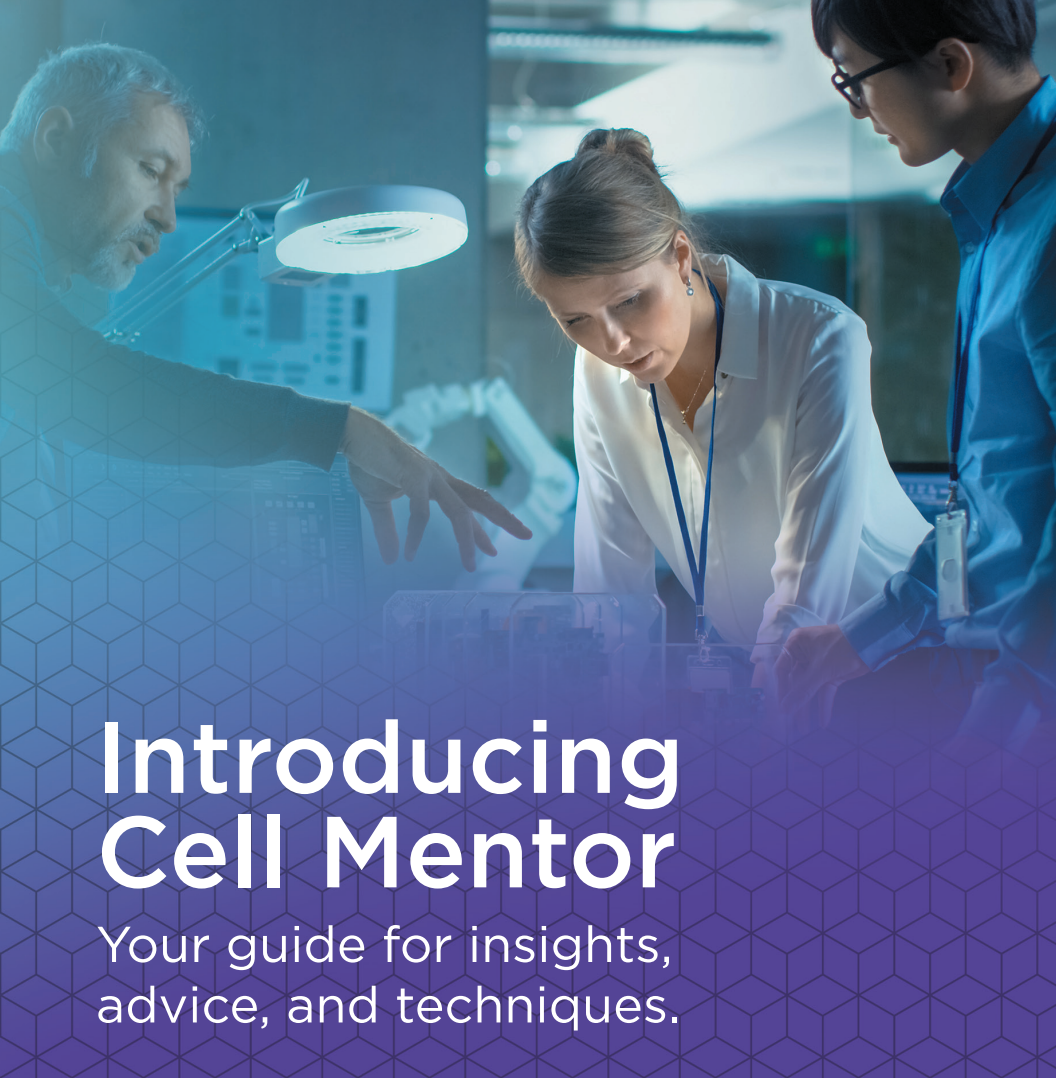


IHC analysis of paraffin-embedded human serous papillary carcinoma of the ovary using Tri-Methyl-Histone H3 (Lys36) (D5A7) in the presence of non-methyl peptide (left) or K36 tri-methyl peptide (right).

CST validation of histone modification antibodies:

CST modification-specific histone antibodies are validated using a peptide array assay. In a single experiment, our arrays assess reactivity against known modifications across all histone proteins as well as evaluating the effects of neighboring modifications on the adility of the antibody to detect a single modification site. This provides confirmation that our antibodies are performing as expeected.

- Peptides with mono-, di-, tri, methyl-, acety-, or unmodified lysine are spotted onto nitrocellulos either alone or in combination with a known neighboring histone modification.
- The histone modification antibody is applied to the array at three concentrations, allowing assessment of antibody reactivity while ensuring that the antibody concentration is not saturating the assay.



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