The life science research community faces ongoing concerns about data reproducibility, especially the validity of antibody-based reagents. Currently there are no standardized or widely accepted guidelines for antibody validation by researchers or manufacturers. In the former case, wide variability in antibody validation practices within the research community have been highlighted in a recent survey by the Global Biological Standards Institute. In the second case, a recent article in Nature discusses the variable standards and performance of antibodies and antibody suppliers in the market and the resulting negative consequences for researchers. R&D Systems, a recognized leader in the development and validation of antibodies, is committed to helping our customers attain the best possible results with our products.

R&D Systems, a Bio-Techne brand, has developed antibodies for the scientific community for 30 years and we leverage our knowledge and expertise to manufacture the highest quality products. This whitepaper highlights examples of how we approach antibody development and validation using highly selective criteria for the release of monoclonal and recombinant monoclonal antibodies.

Antibody Development Process Overview

- **Antigen Design**
  Each project is carefully reviewed to best match the antigen and host animal to the desired final specificity and application. The antigen design can be either a peptide (usually 10-25 amino acids long) or a recombinant protein. Peptides are a preferred choice for immunohistochemistry (IHC) and western blot (WB) applications in most cases, while a full-length recombinant protein has the advantage of raising antibodies to folding-specific epitopes. This is especially crucial for applications that require reagents for native targets, such as immunoassays and flow cytometry.

- **Feasibility Testing of Antibodies**
  The selection process for monoclonals includes two stages. An initial screen to select the best clones is followed by further application specificity testing and optimization. This initial screen includes high homology targets as well as any tags or conjugation partners used during immunizations, to exclude false positives and cross-reactive clones. Direct ELISA is used to narrow down clones with the desired specificity before performing application testing. The ELISA test is repeated several times at various development phases and used as part of the quality control process. Once a panel of monoclonal antibodies is selected, all clones are subjected to multiple application validation assays (such as WB, IHC, ICC, and Flow).
Specificity and Sensitivity Testing

Each selected panel is further tested on models designed to carefully test the desired specificity and sensitivity of the antibodies. For western blot, this can be done by choosing known positive and negative models (Figure 1). In the case of phosphorylated targets, kinase activators and inhibitors can be utilized (Figure 2). In some cases, low expression levels of a specific protein combined with closely related proteins within the same family make it very challenging to test the antibody panels. This may require the utilization of transfectant models to further investigate the reactivity of a chosen clone (Figure 3).

Western blot data that show specificity with the expected molecular weight and expression profile are a very useful tool to support other applications such as IHC and flow cytometry. However, since some applications have antibodies that recognize folded epitopes, many flow cytometry or assay reagents are not compatible with western blot. Therefore, western blot results cannot be used as supportive evidence for these applications. This is where the careful choice of positive and negative cell models is crucial to the validation of the reagent. For IHC, that may require testing across various tissue or cancer types, to exclude off-target reactivity. Knockout models in mice have also been used in some cases, but are not readily available across a wide variety of targets and therefore play a very minor role during validation. Recent advances in CRISPR and other gene editing technologies will provide further toolsets to address the gap more efficiently going forward, especially in validating western blot negative reagents for flow cytometry and immunocytochemistry (ICC).

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**Figure 1: Detection of human, mouse and rat ERK1/ERK2 by western blot in various cell lines**

Western blot shows lysates of HeLa, PC-12, and TS1 cell lines. PVDF membrane was probed with anti-ERK1/ERK2 monoclonal antibody (Catalog # MAB1576) followed by HRP-conjugated goat anti-mouse IgG secondary antibody (Catalog # HAF007). For additional reference, recombinant ERK1 and ERK2 proteins (2 ng/lane) were included. A specific band for ERK1/ERK2 was detected at approximately 44 and 42 kDa (as indicated).

**Figure 2: Specificity testing of phospho-specific antibodies using a kinase activator and an inhibitor**

Detection of human phospho-ERK1 (T202/Y204) or phospho-RSK (S380) by western blot. Western blot shows lysates of HeLa cells that were incubated with control solutions or with 200 nM PMA (Tocris, catalog # 1201) for 20 minutes, either with or without 10 µM U0126 (Tocris, catalog # 1144) pretreatment for one hour. PVDF membranes were probed with 0.5-1 µg/mL of the anti-ERK1/2 (T202/Y204) monoclonal antibody (Catalog # MAB18251) or anti-RSK1 (S380) monoclonal antibody (Catalog # MAB79671) followed by HRP-conjugated goat anti-mouse IgG secondary antibody (Catalog # HAF018). Specific bands were detected for phospho-ERK1 (T202/Y204) and phospho-RSK1 (S380) at approximately 43 and 41 kDa, respectively (as indicated). RSK1 is downstream from the ERK1/2 signaling cascade and therefore is less affected by the inhibitor.
Human CEACAM-6 specificity testing: MAB3934

Release Decision and Quality Control

Upon completion of testing across all required applications, the data is reviewed by a team of scientists and the best performing clones are made available commercially. R&D Systems’ antibodies are tested far beyond simple positive validation via western blot or ICC, as is common for many commercially available antibodies. Prior to release, the production and bottled lots undergo a stringent quality control test, running the optimized conditions for the various applications under ISO9001 quality controlled document management.

The antibody will be released only if the results obtained during the development phase can be repeated. Since lot-to-lot consistency is crucial for long-term studies in the research community, this testing is repeated on all new lots and compared to the previous lot, resulting in high lot-to-lot consistency (Figure 4). To ensure that customers have success with our antibodies, experimental conditions and sample types that are used in-house to validate the antibodies are included on the website and product datasheet.

HEK293 human embryonic kidney cell lines transfected with human CEACAM-6/CD66c or related CEACAM transfectants fused to eGFP were stained with mouse anti-human CEACAM-6/CD66c monoclonal antibody (Catalog # MAB3934) followed by APC-conjugated anti-mouse IgG secondary antibody (Catalog # F0101B). Human CEACAM-6 antibody: MAB3934 (Clone 439424). Quadrant markers were set based on IgG2A control antibody staining (Catalog # MAB003).

Figure 3: Specificity testing for closely related family members by flow cytometry

Western blot shows lysates of A431 and NIH-3T3 cell lines. Lysates were loaded at 25 μg under reducing conditions. The membrane was blocked in 5% Milk (0.15M NaCl) for 1 hour. The anti-Caspase 1 monoclonal antibody (Catalog # MAB6215) was used at 0.1 μg/mL in 2% Milk (0.15M NaCl), 4 °C overnight. A donkey anti-mouse HRP (Catalog # HAF018 1:1000, 1 hour incubation) was used as the secondary. All washes were done for 30 minutes with 1x TBST between incubations.

Figure 4: Lot-to-Lot reproducibility of a mouse anti-Caspase 1 monoclonal antibody
Summary

R&D Systems uses a rigorous process to design, test and validate its monoclonal antibody products. Initial screening of our antibodies is often more extensive than full validation from other companies. Great care is taken to validate our antibodies in relevant applications before releasing them to customers. Continuing quality control and lot-to-lot validation ensures that our antibodies perform consistently and generate highly reproducible data over time.

References


