# A COMMITMENT TO ANTIBODY PERFORMANCE

Reproducibility is a guiding principle of research. If someone else can't replicate the work, it isn't science. Achieving reproducibility requires explicit protocols and standards, alongside reliable reagents and tools. And key among these reagents are antibodies. This series of three articles will explore how Thermo Fisher Scientific is ensuring that its antibodies are consistently of the highest quality.


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For ThermoFisher SCIENTIFIC by natureresearch CUSTOM MEDIA

# ANTIBODY BOOTCAMP — RISING TO THE FITNESS CHALLENGE

Scientists and suppliers are EMBRACING STRATEGIES to improve the performance of research antibodies and tackle the reproducibility crisis.

Antibodies are invaluable tools in the life sciences. Their high specificity and selectivity for unique protein targets make them indispensable research reagents. Scientists worldwide spend nearly US$2.5 billion a year on antibodies to detect and quantify the expression of proteins in cells and tissues[1].

However, lately, the quality of these reagents has come under intense scrutiny. Not all of them seem to be as selective and specific as was assumed, leading to incorrect, inconsistent and irreproducible results[2]. Alarm bells sounded in 2012 when independent laboratories were unable to replicate the results of 47 out of 53 landmark cancer research papers[3].

"The field has been hampered by antibodies that recognize the wrong (or multiple) protein isoforms and antibodies that don't work well in particular applications," says Andrew Waters, a postdoctoral researcher at the University of North Carolina, Chapel Hill. Waters's own dissertation work was significantly delayed because of an antibody that recognized a nonspecific protein of the same molecular weight as his target protein.

Antibody underperformance can significantly drain research time and money. Months, sometimes years, can be spent trying to replicate experiments or proceed with work that is based on incorrect conclusions. To address this growing problem, researchers need to be aware of the issues surrounding these reagents — and antibody manufacturers need to set higher quality standards.

**Common issues and how to avoid them**

Although antibodies are designed to recognize a target protein, they may not be able to do so in all applications — namely, those that alter the target protein's structure. Thus, antibodies should be verified in the application of interest.

**"OUR AIM IS TO BUILD TRUST WITH THE SCIENTIFIC COMMUNITY AND HELP ADVANCE THEIR RESEARCH."**

Antibody performance can also be hampered by binding to off-target proteins when the target is expressed at low levels or has many isoforms. These potential obstacles can be assessed by using appropriate positive and negative controls prior to carrying out the experiment.

Different batches of antibody can produce dramatically different results. Because antibodies are often referred to simply by brand name, it is important to check the manufacturer's lot number and characterization data. This information is often omitted in published articles, making it very hard to track down the actual antibody that was used — and reproduce the findings.

Lack of training in the use of research antibodies compounds these risks. "Many young scientists fail to appreciate the need to confirm that their antibody works in their set-up," says Giovanna Roncador, head of the Monoclonal Antibody Unit at Centro Nacional de Investigaciones Oncológicas in Madrid.

With colleagues from the European Monoclonal Antibodies Network (EuroMabNet), Roncador has produced a comprehensive set of guidelines to avoid common pitfalls in research antibody use[4]. Their recommendations include: defining the target antigen and the experimental techniques that will be used to identify it; conducting a thorough search of the literature to find information on existing antibodies; assessing the available validation data and determining what further validation measures are required; and providing all the necessary protocol and antibody details so others can reproduce the findings.

Other organizations are helping with training: societies such as ISAC (International Society for Advancement of Cytometry) and ICCS (International Clinical Cytometry Society) are producing webinars and educational materials to help junior scientists select and handle research antibodies.

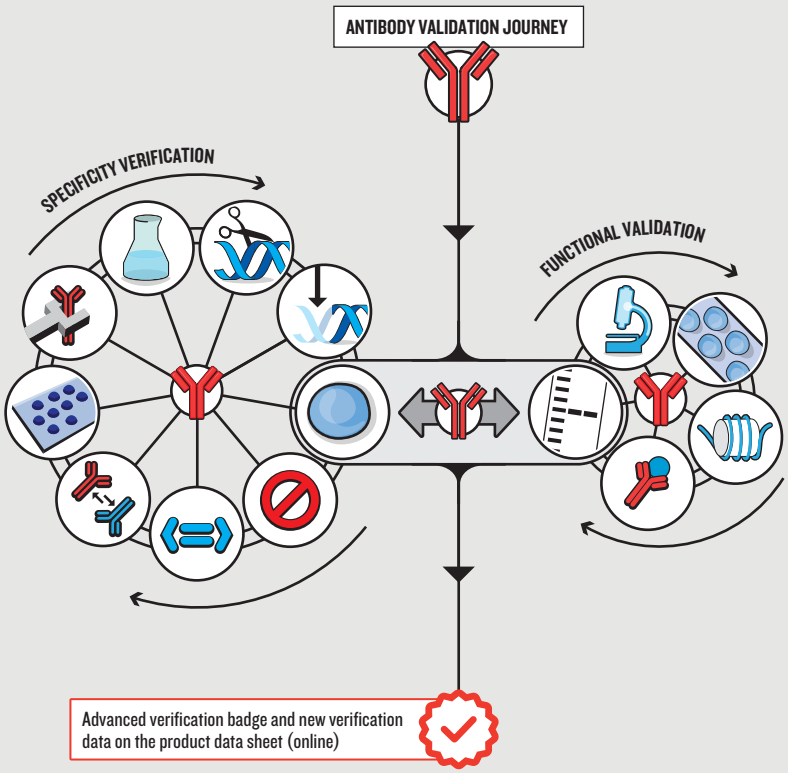
However, determining an antibody's sensitivity, specificity and reproducibility in a given application — across experiments and over time — is a complex and costly process that researchers can't do on their own. Experts from industry and academia have come together to develop standard guidelines for antibody validation.

**Establishing validation standards**

The International Working Group for Antibody Validation (IWGAV) is a consortium of leading protein scientists formed in 2015, and supported by the global life sciences company Thermo Fisher Scientific. The IWGAV has proposed five approaches for antibody validation: using genetics; using an orthogonal (non-antibody) strategy; using independent antibodies binding to the same target; correlating antibody labelling with the expression of tagged proteins; and immunoprecipitation followed by mass spectrometry[5]. At least one of these strategies should be used when validating an antibody for a specific application. Thermo Fisher has used these recommendations as the basis for its own internal systematic approach for

**TWO-PART APPROACH FOR ANTIBODY VERIFICATION**

Rigorous antibody validation is achieved by testing that the antibody binds to the right target in the application of interest. This involves using at least one of nine specificity tests in the applications shown below.




**SPECIFICITY VERIFICATION**

- Knockout—expression testing using CRISPR-Cas9 cell models
- Knockdown—using RNAi to reduce the expression of genes of interest
- Cell treatment—modulating cell signalling pathways to detect an enrichment, depletion or translocation of the antibody target
- Neutralization—functional blocking of protein activity by antibody binding
- Relative expression—using naturally occurring variable expression to confirm specificity
- IAV—independent antibody validation using two differentially raised antibodies against the same target
- Peptide array—using arrays to test reactivity against known protein modifications
- Orthogonal method—correlation using both antibody-dependent and -independent methods
- IP-MS—immunoprecipitation (IP) followed by mass spectrometry (MS) to identify antibody targets

**FUNCTIONAL VALIDATION**

- Western blotting
- Immunofluorescent imaging
- Flow cytometry
- Chromatin immunoprecipitation (ChIP)
- Immunohistochemistry

verifying the specificity and functionality of antibodies created for its Invitrogen brand (see 'Two-part approach for antibody verification').

Deepa Shankar, director for research and development at Thermo Fisher, explains: "We want to help researchers make an informed choice by producing the most compelling data showing that an antibody works." Her team is devoted to validating the company's large antibody portfolio — testing them using Thermo Fisher's two-part approach. "We spend a lot of time ensuring that we test our antibodies in the right environment, in multiple models and in different applications," she says. "Our aim is to build trust with the scientific community and help advance their research."

Detailed testing protocols and results, as well as published antibody data, are collated on the company's website. "Customer feedback is really positive," says Shankar. "We are seeing a growing number of publications using our antibodies demonstrating that they are working."

In recognition of these efforts, Thermo Fisher won the 2018 CiteAb Award for the best antibody validation initiative. "Rigorous validation procedures are not in place in many laboratories. Lack of awareness, resources and funds means researchers are relying on vendors to provide good antibodies," explains Paul Wallace, director of the Department of Flow & Image Cytometry, Roswell Park Comprehensive Cancer Center in Buffalo, New York, and a panel member on Thermo Fisher's Antibody Validation Forum. "I am very impressed by how Thermo Fisher is taking responsibility for the quality of its antibody products — and is open to dialogue with users."

## Bright outlook

The first step in solving any problem is to recognize that it exists. Since the issue of antibody validation was exposed, it has been openly discussed — and many initiatives set up to find the best solutions. "We are making headway, but a lot more still needs to be done to figure out what are the best strategies to address the problem," says Wallace. Agreeing to the need for antibody validation standards is a significant first step. Given the importance of reproducibility for the advancement of science, it is in the interest of all researchers and suppliers to step up to the challenge of implementing these standards. ■

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# SPECIFIC ANTIBODIES NEED SPECIFIC VALIDATION

To be useful for research, an antibody must be RIGOROUSLY EVALUATED in a test that accounts for the biology of the target antigen.

One of the most utilized tools in biomedical research is the monoclonal antibody. These proteins have the potential to seek out and bind to any desired target, and can be used for cell imaging, cell sorting, immunoassays and many other applications.

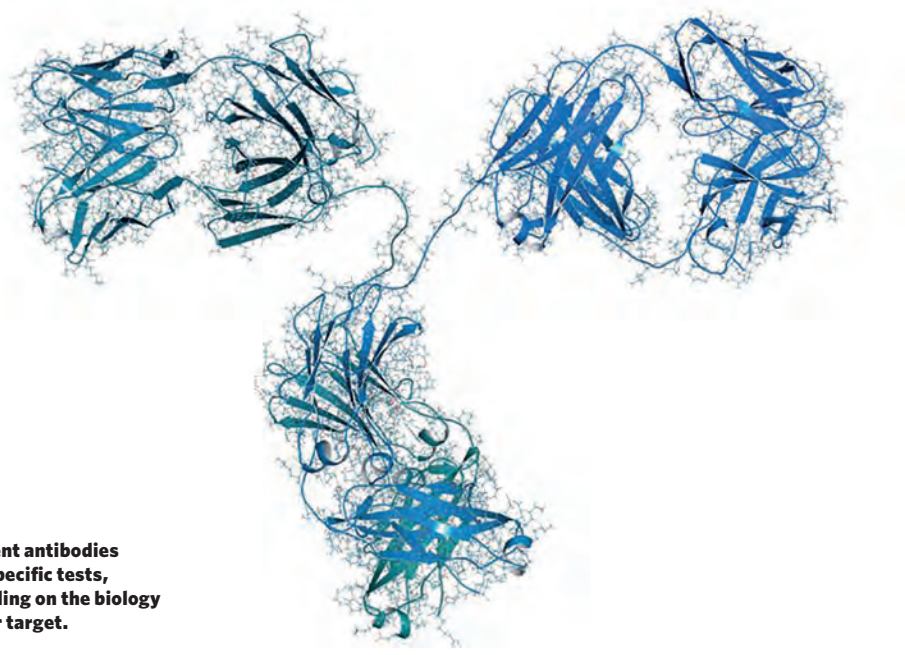
But these lab workhorses don't always run true. Depending on the nature of its target, an antibody might be inconsistent in certain tests — binding to the wrong target to give false positive results, for instance. Given the prevalence of research antibody use — this is potentially a billion-dollar problem.

A major goal is to develop antibody validation strategies so that researchers can have confidence that an antibody is suitable for their particular needs — and that their results will be reproducible.

Thermo Fisher Scientific has developed a two-part antibody validation platform to test, not only the specificity of its Invitrogen™ antibodies (that they bind to the right target), but also their suitability for different applications. However, the same test is not appropriate for all antibodies: Thermo Fisher uses an appropriate test for each protein target, depending on its biological function. Certain antibodies will be best tested using CRISPR-Cas9 to knock out the gene that encodes the target protein, and checking that the antibody no longer binds to anything. Other antibodies might be tested using immunoprecipitation followed by mass spectrometry to check that they are bound to the right targets.

Thermo Fisher is developing and refining antibody validation tests based on biological function of the target antigen. Here are two case studies of specific proteins and their specificity tests.

**"CERTAIN ANTIBODIES WILL BE BEST TESTED USING CRISPR-CAS9."**



**Different antibodies need specific tests, depending on the biology of their target.**

**Knockouts for cancer**

The epidermal growth factor receptor (EGFR) is a well-studied protein: dysregulation in the EGFR pathway is implicated in various cancers. In order to test whether antibodies are specific to EGFR or to any of its downstream targets, researchers can knock out critical proteins in the EGFR pathway and see how the antibody-binding signal changes.

In recent years, the CRISPR-Cas9 system has become known as the most reliable and powerful way to knock out a gene. This makes it ideal for testing antibody specificity within a signalling cascade. Thermo Fisher researchers took a standard human carcinoma line (A-431) and used a western blot to get a baseline for the binding signal. They then used CRISPR-Cas9 to eliminate the target gene and create EGFR knockouts. A western blot of protein extracted from these knockout cells showed that there was no longer any signal for a target protein (Figure 1).

Further tests confirmed the result. The signalling cascade downstream of EGFR includes proteins such as RAS, RAF, MEK and ERK. Activation of EGFR by epidermal growth factor (EGF) leads to phosphorylation of these

downstream proteins, which can be detected using other antibodies that recognize these phosphorylated states. However, adding EGF to the EGFR-knockout cells should not result in any downstream phosphorylation. Adding the same antibodies that recognize phosphorylated targets produced no signals. Thus, Thermo Fisher researchers are confident that the anti-EGFR antibody is target-specific.

**An array of modifications**

In the cell nucleus, DNA is tightly packaged — wrapped around histone proteins to form chromatin. Studying histones is difficult, as they can be affected by a number of chemical changes, known as post-translational modifications (PTMs). For example, residues on a histone can gain one or more methyl, acetyl or phosphoryl groups, which each have an effect on cellular function.

Certain techniques, such as chromatin immunoprecipitation (ChIP), western blotting, immunofluorescence and immunohistochemistry, use antibodies against specific histone PTMs to understand the state of the histone and its binding. However, several histone modifications have similar DNA-binding patterns; an antibody that has not been rigorously tested against all histone PTMs might bind to the wrong type and deliver a false-positive result.

Thermo Fisher tested its histone PTM-specific antibodies using an array of peptides bearing a variety of PTMs. If an antibody is truly specific to one PTM, it will bind only to those spots that carry that PTM. Thermo Fisher researchers measured the signals using a specificity factor: the average intensity of all spots containing a particular PTM divided by the average intensity of all spots without it (Figure 2). The antibodies showed a 4- to 190-fold higher specificity factor for their target PTM state than non-target states, giving confidence that they are highly selective.

Thermo Fisher has seven other specificity tests beyond genetic knockout and peptide arrays. These include using RNAi to knock down gene expression, a differentially raised antibody to independently verify targeting, and naturally occurring variable expression to confirm specificity. Only through such careful and rigorous testing can researchers be confident that their lab workhorses are up to the job — and that their work will stand up to the closest scrutiny. ■

*Find application notes on these Invitrogen antibodies and more about Thermo Fisher Scientific's two-part testing approach at thermofisher.com/antibodyvalidation*

**FIGURE 1:**
**ANTIBODY SPECIFICITY TESTING WITH A GENE KNOCKOUT**

Starting with the A-431 cell line, CRISPR-Cas9 was used to knock out the epidermal growth factor receptor (EGFR). A western blot shows that antibodies to EGFR (Cat. No. MA5-13269, 1 µg/mL) bind to the control cells but not to the EGFR KO cells. Tubulin protein was used as a loading control.

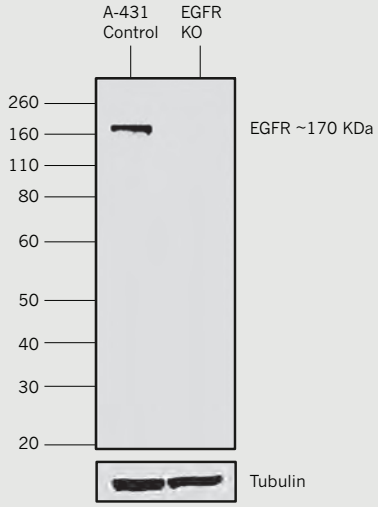
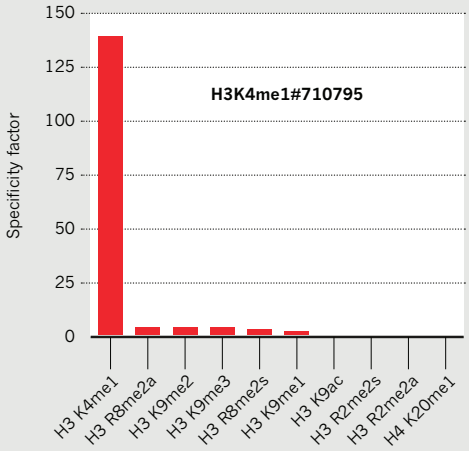



**FIGURE 2:**
**TESTING OF SPECIFICITY OF ANTIBODIES TO HISTONE PTMS**

An antibody is needed to distinguish the type of methylation on a specific lysine residue. A candidate antibody was tested against a peptide array with spots carrying lysine residues that are mono-, di- or tri-methylated. The specificity factor showed that the antibody (Cat. No. 710795) recognized only one type of methylation, meaning it is highly selective.




**"STUDYING HISTONES IS DIFFICULT, AS THEY CAN BE AFFECTED BY A NUMBER OF CHEMICAL CHANGES."**

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# NEURODEGENERATIVE DISEASE RESEARCH NEEDS SMART ANTIBODIES

Ensuring that researchers get specific and reliable antibodies requires **EXTENSIVE QUALITY CONTROL** testing behind the scenes.

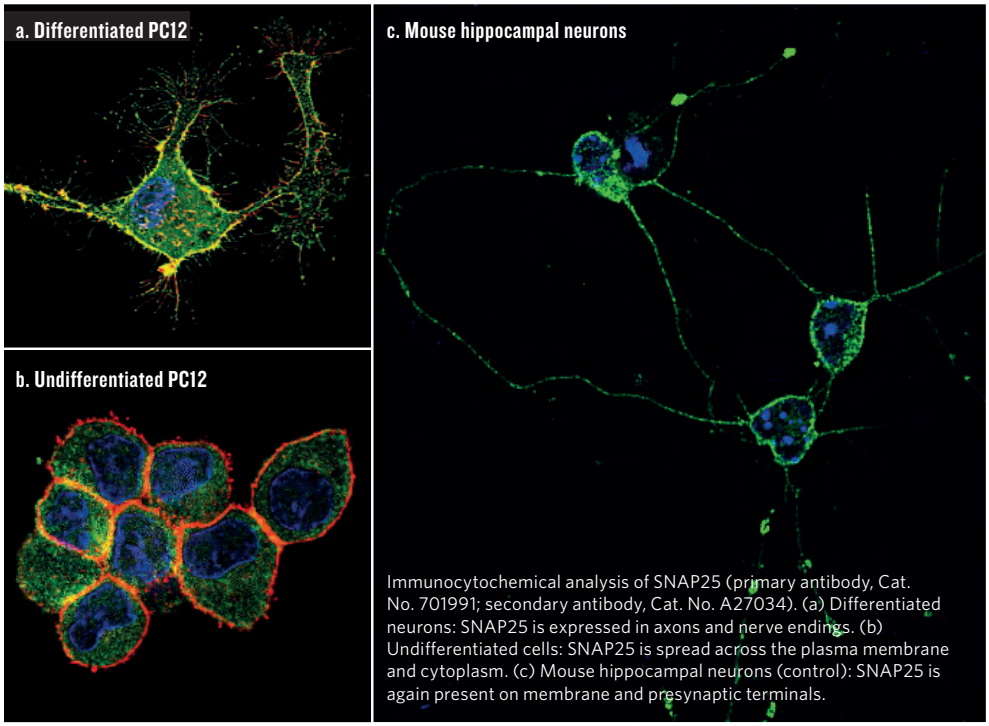
Neurodegenerative diseases are a growing burden worldwide. Common conditions such as Parkinson's and Alzheimer's diseases, epileptic encephalopathy and amyotrophic lateral sclerosis (ALS) threaten the lives of millions of people, and current therapies are only minimally effective. Tremendous amounts of research time and money are being invested in this area, driven by an urgent need to understand these diseases at the molecular level.

Research in this field has already uncovered some biological insights. Most neurodegenerative diseases share common pathogenic mechanisms. Defects in axonal transport have been implicated in Parkinson's and Alzheimer's diseases, and there is evidence that vesicular transport across synapses — the junctions between neurons — is also impaired in many neurodegenerative conditions.

Antibodies are important reagents to further neuroscience research. Not only can they help researchers study proteins involved in the pathogenesis of these diseases, but they can also be used in biomarker tests for early detection. Thermo Fisher Scientific has developed antibodies against three critical targets: SNAP25, VAMP1 and OPTN. Both SNAP25 (synaptosomal-associated protein, molecular mass of 25 kDa) and VAMP1 (vesicle-associated membrane protein-1) are important members of the SNARE family of proteins, which are involved in vesicular transport of neurotransmitters across synapses[1]. OPTN (optineurin) is a Golgi complex-associated protein that is involved in many intracellular processes including autophagy flux, which is disrupted in several neurodegenerative disorders[2]. OPTN mutation has also been reported as a causative factor in glaucoma and ALS3.

Finding antibodies against these targets is only the first step. For them to be useful to the neuroscience community, the antibodies need to be well characterized, highly specific, and certain to work in relevant applications such as western blotting and cell and tissue immunofluorescence. As such, Thermo Fisher extensively tests its Invitrogen™ antibodies using a two-part validation approach: using target specificity models and relevant functional applications. Using tools such as CRISPR-Cas9 or RNAi-mediated gene knockdown, and relative expression of specific proteins in different cell types, Thermo Fisher can provide neuroscientists with high-quality antibodies to use in their experiments to obtain reliable, reproducible results.

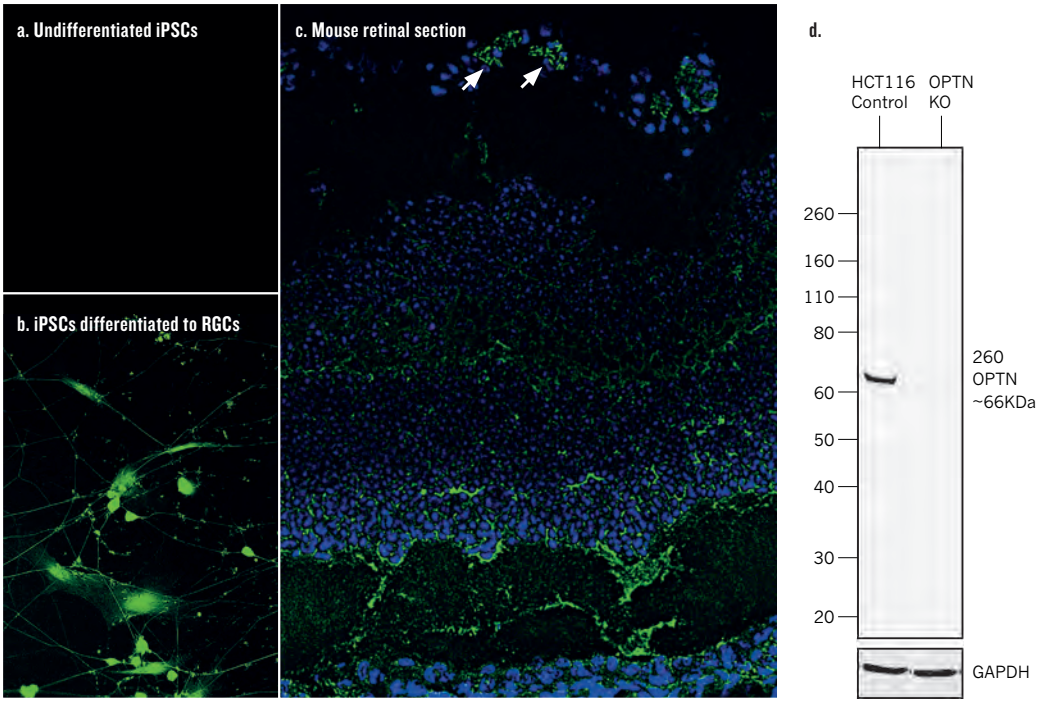
**FIGURE 1: SNAP25 ANTIBODY SPECIFICITY TESTS**




Immunocytochemical analysis of SNAP25 (primary antibody, Cat. No. 701991; secondary antibody, Cat. No. A27034). (a) Differentiated neurons: SNAP25 is expressed in axons and nerve endings. (b) Undifferentiated cells: SNAP25 is spread across the plasma membrane and cytoplasm. (c) Mouse hippocampal neurons (control): SNAP25 is again present on membrane and presynaptic terminals.

**FIGURE 2: OPTN ANTIBODY SPECIFICITY TESTS**

Immunofluorescence (IF) of optineurin (OPTN) (primary antibody, Cat. No. 702766; secondary antibodies, Cat. No. A27034 for IF and A27036 for western blotting) in (a) undifferentiated induced pluripotent stem cells (iPSCs) and (b) retinal ganglion cells differentiated from iPSCs. Green represents OPTN. (c) Tissue IF of OPTN on adult mouse eye cryosection, with expression localized in the ganglion cell layer of the retina (white arrows). Green represents OPTN and blue represents nuclei. (d) Antibody specificity demonstrated by CRISPR-Cas9 mediated knockout of OPTN (OPTN KO).




**"MOST NEURODEGENERATIVE DISEASES SHARE COMMON PATHOGENIC MECHANISMS."**

### Three tests for three antibodies

SNAP25 is known to be enriched in specific areas of neurons. As a control, Thermo Fisher scientists added Invitrogen SNAP25 monoclonal antibodies to a primary culture of mouse hippocampal neurons, and confirmed that they localized to the hippocampal membrane and presynaptic terminals as expected. In order to test their specificity, Thermo Fisher scientists used the PC12 cell line. These cells are of embryonic origin and easily divide until treated with nerve growth factor (NGF), whereupon they terminally differentiate into neuron-like cells. In undifferentiated PC12 cells, the SNAP25 antibody was distributed throughout the cytoplasm. When NGF was added and the cells differentiated, the antibodies were redistributed to the membrane of developing axons and nerve endings (Figure 1).

The same model system was used to test the VAMP1 oligoclonal antibody, where tissue from rat and mouse brains serves as the control. In a western blot experiment, the antibody detected a single band at 16 kDa, corresponding to the molecular mass of VAMP1. To validate the specificity, the antibody was again added to PC12 cells. In undifferentiated cells, no VAMP1 was detected, but in NGF-treated, differentiated cells, western blotting showed a band at 16 kDa. Tissue immunofluorescence of mouse brain sections confirmed that VAMP1 was localized in the hippocampal regions (data not shown).

HCT116 cell lysates were used to test the specificity of the OPTN monoclonal antibody. Western blot analysis showed a single band at the expected size of 66 kDa. The signal was absent in lysates of cells in which OPTN had been knocked out using CRISPR-Cas9, confirming the specificity of this antibody. OPTN was distinctively localized to the retinal ganglion cells (RGCs) in adult mouse eye tissue. Consistent with this, tissue immunofluorescence of mouse eye sections using the OPTN antibody showed a strong signal in the ganglion cell layer. Similarly, immunofluorescence in RGCs differentiated from induced pluripotent stem cells (iPSCs) picked up a strong signal that was absent in undifferentiated iPSCs (Figure 2).

By using the appropriate test for each antibody, based on the biology of its target protein and the intended application, Thermo Fisher is able to confirm specificity. Having reliable antibodies will give researchers confidence in their studies of the mechanisms of neurodegenerative disease, as well as in applying them to downstream diagnostic tests or treatments. ■

*For a detailed explanation of these validation strategies, please go to thermofisher.com/antibodyvalidation.*

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