Knockout and Knockdown Antibody Validation

Testing antibody performance against genetically modified samples

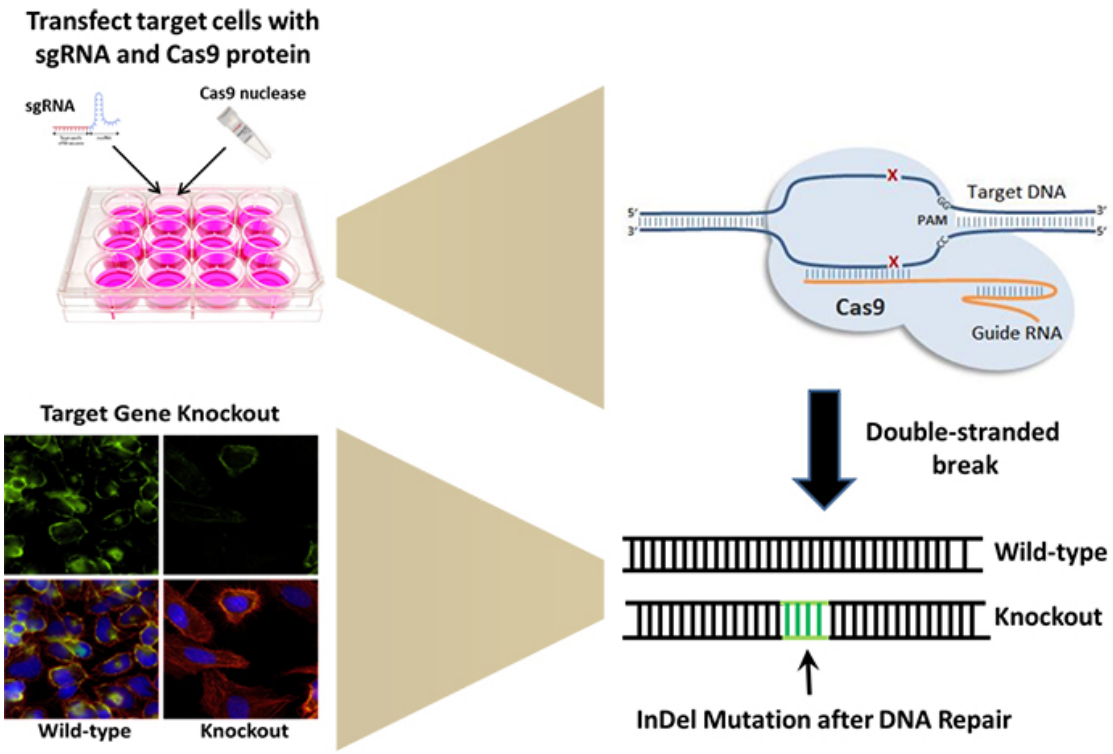
Validating an antibody's specificity is crucial to ensuring the absence of nonspecific binding and, therefore, the highest level of functionality. Testing antibody performance against genetically modified samples is one way to verify that an antibody recognizes a specific target. This can be done using a variety of methods, including mouse knockout models, dominant negative mutants, morpholinos, siRNA, and most recently, gene editing.

Thermo Fisher Scientific is committed to providing the best antibodies available. We are currently retesting the entire portfolio of Invitrogen™ antibodies to verify that they are specific to the indicated targets.

CRISPR-Cas9 in knockout cell models

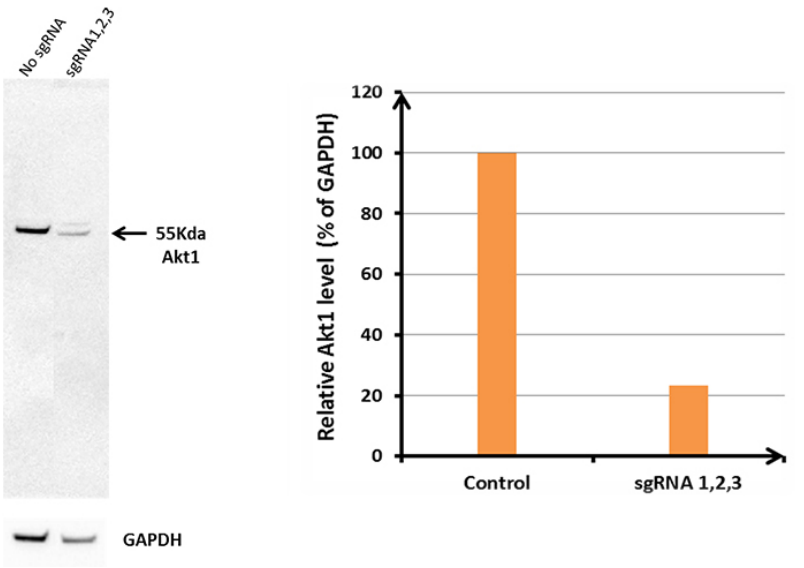
Utilizing the CRISPR-Cas9 system, scientists can create knockout cell models that can be subsequently used as robust controls for validating antibody specificity. The CRISPR-Cas9 system employs a noncoding single guide RNA (sgRNA) molecule to "guide" the CRISPR-associated Cas9 endonuclease to its intended target gene, where it cleaves the DNA. This DNA cleavage results in target gene knockout. In this way, CRISPR-Cas9 technology is used to ablate the target protein's expression in appropriate cell models, thus making it a suitable negative control for verifying antibody specificity.

CRISPR-Cas9 can also be used to simultaneously test antibody specificity for multiple signaling proteins in a pathway by knocking out expression of upstream mediators. This multiplexing capability allows for streamlined and high-throughput antibody validation. Considering this advantage, CRISPR-Cas9 technology becomes the preferred solution for validating antibodies by genetic modification.

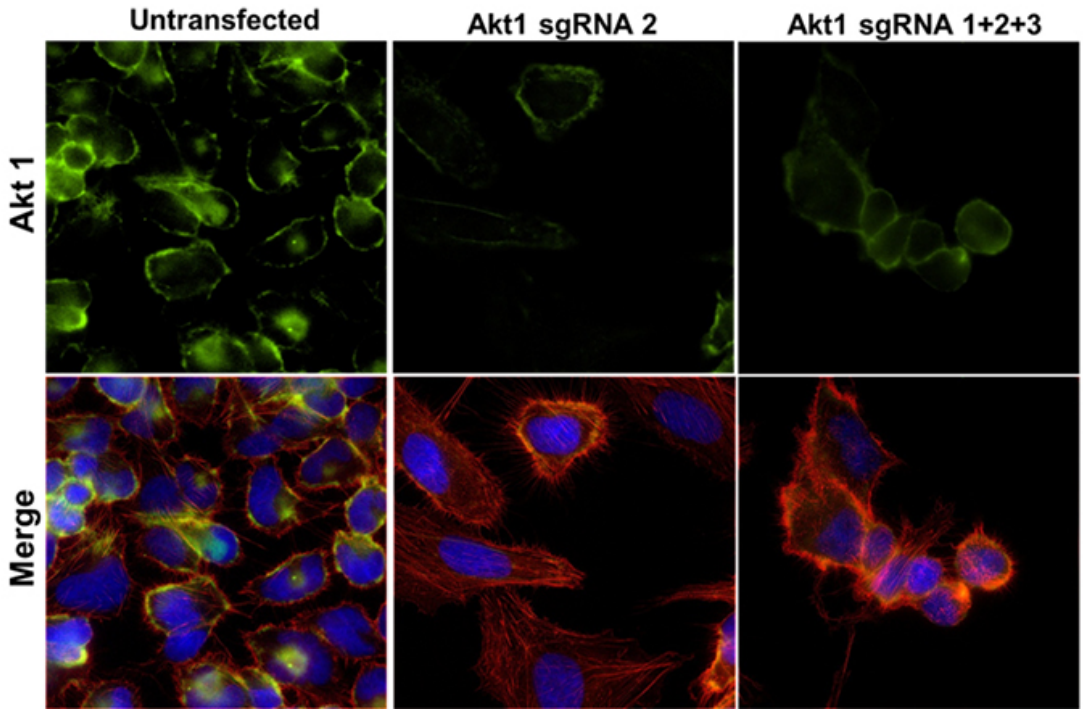


Schematic of CRISPR-Cas9 knockout–mediated validation of antibody specificity.

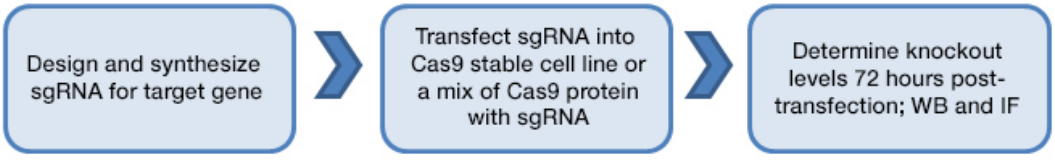
In the example below, U2OS cells stably expressing Cas9 endonuclease were transfected for 72 hours with three different sgRNAs targeting the *Akt1* gene individually and also in combination. Analysis by western blot (WB) and immunofluorescence (IF) using Invitrogen™ AKT1 Mouse Monoclonal Antibody (Cat. No. **AHO1112**) showed significant decreases in protein expression, confirming specificity of the anti-AKT1 antibody. Note that lysates for WB were generated from mixed cell populations and not individual clones; therefore, lower levels of AKT1 were still present.



AKT1 antibody showed specificity by CRISPR-mediated knockdown in western blot. Western blot analysis of AKT1 was performed by loading 15 µg of U2OS-Cas9 cell lysate. AKT1 was detected at ~55 kDa using AKT1 antibody (Cat. No. **AH01112**). Data were normalized to a GAPDH loading control. A mix of sgRNA 1, 2, and 3 showed reduction in AKT1 protein expression, confirming specificity.



sgRNA 2 and a mix of sgRNA 1, 2, 3 caused significant decreases in protein expression. U2OS cells stably expressing Cas9 nuclease were plated on coverslips and transfected after 24 hours with multiple sgRNAs directed against the *Akt1* gene (sgRNA1, sgRNA2, sgRNA3, mix of sgRNA1, 2, 3.). At 72 hours post-transfection, cells were fixed, permeabilized, and probed with AKT1 antibody (Cat. No. **AHO1112**). Nuclei and cytoskeleton were stained using Invitrogen™ *SlowFade*™ Gold Antifade Mountant with DAPI and rhodamine phalloidin, respectively. Images were captured at 60x magnification using a Nikon Ti-U microscope. Cells transfected with sgRNA 2 and with a mix of sgRNA 1, 2, and 3 showed significant reductions in AKT1 signal, thus confirming antibody specificity.



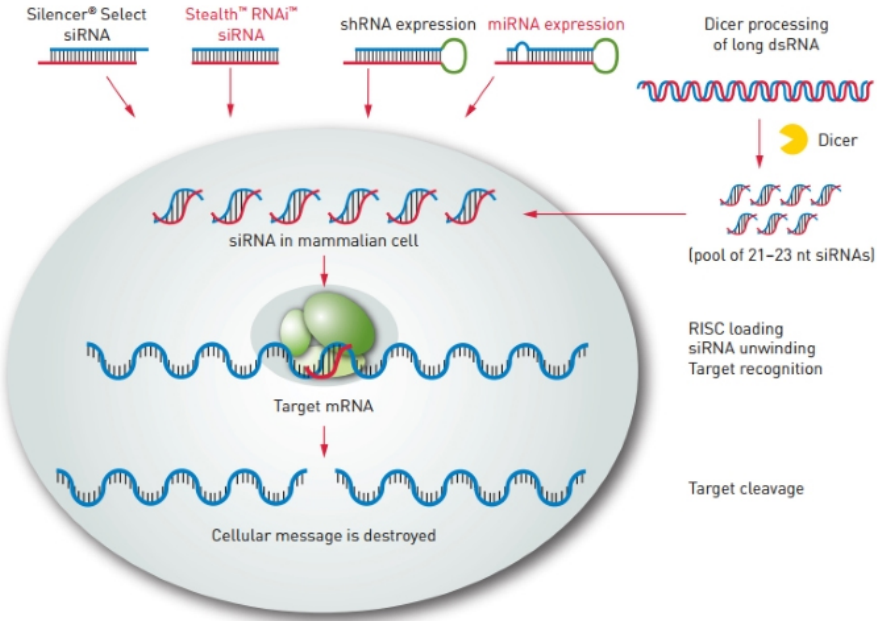
CRISPR-Cas9 experimental workflow for demonstrating antibody specificity.

RNAi in knockdown strategies

RNA interference (RNAi) technology takes advantage of a cell's natural machinery to effectively knock down expression of a gene of interest. It is a widely used method to validate antibody specificity.

In mammalian cells, short pieces of double-stranded RNA, otherwise known as short interfering RNA (siRNA), initiate the degradation or knockdown of a specific, targeted cellular mRNA. In this process, the antisense strand of the siRNA duplex becomes part of a multi-protein complex called the RNA-induced silencing complex (RISC). RISC then identifies the complementary mRNA and cleaves it at a specific site. Next, this cleaved message is targeted for degradation, ultimately resulting in the loss of protein expression.

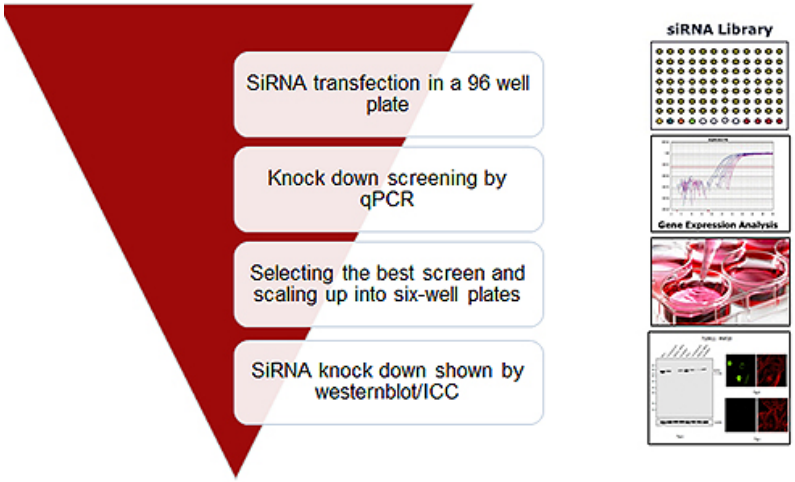
There are different methods for performing RNAi experiments. RNAi can be achieved by transfecting target cells with a pool of synthetic small RNAs or a pool of siRNA obtained via *in vitro* cleavage (*in vitro* dicing of target RNA). RNAi can also be achieved by transfecting cells with short hairpin RNA (shRNA) vectors. shRNA is processed within the cell, and the *in vivo*–generated siRNA can then target its specific mRNA molecule for degradation. Nontargeting controls can be used to test for specificity of the knockdown.



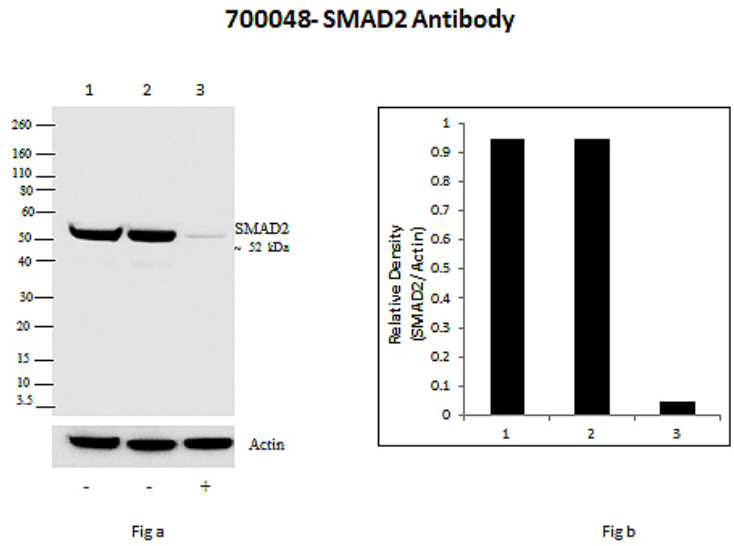
Principle of siRNA-mediated knockdown of target mRNA. The figure shows the use of *in vitro*–synthesized siRNA in a typical RNAi experiment.

In the example below, Invitrogen™ ***Silencer*™ Select siRNAs** are transfected into cells in a 96-well format, followed by RT-qPCR. The most efficient knockdown (based on cell type and siRNA transfection conditions) is identified through gene expression analysis. The chosen screen is then scaled up for validation of antibody specificity through western blotting and immunocytochemistry (ICC).

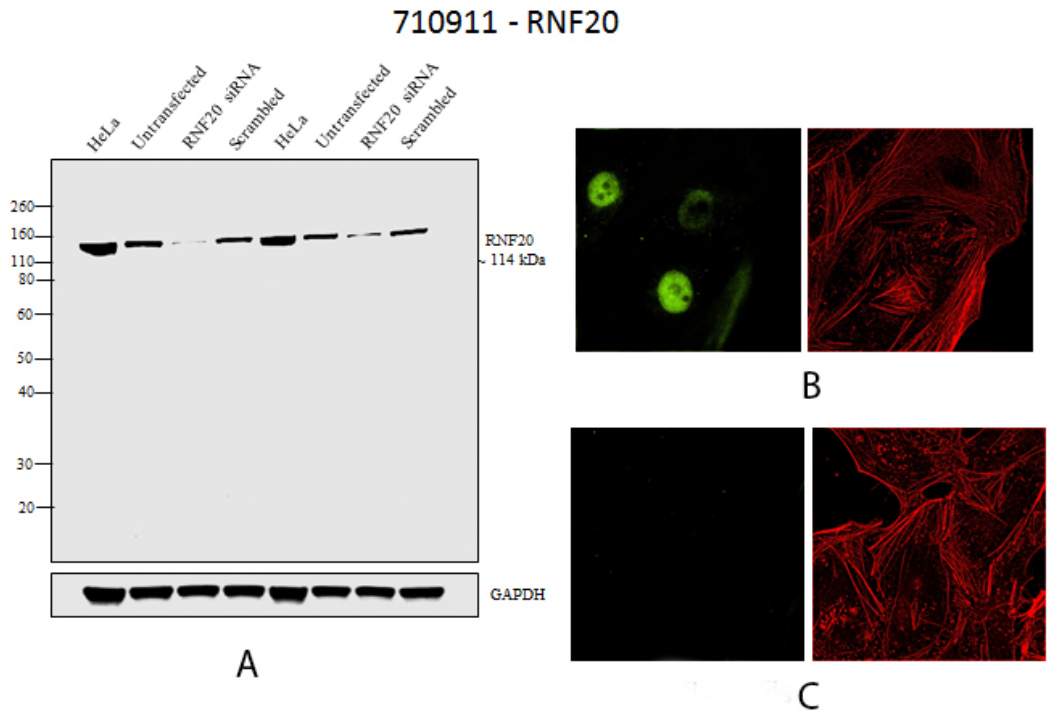
SiRNA transfection plan



Schematic of the knockdown strategy used to validate antibody specificity.



Western blot for antibody validation. (A) Western blot showing knockdown of SMAD2 (lane 3) in HeLa whole cell lysates after transfection with SMAD2-targeting siRNA, using Invitrogen™ SMAD2 ABfinity™ Rabbit Monoclonal Antibody (Cat. No.**700048**). The knockdown is shown along with untreated and scrambled RNA as controls in lanes 1 and 2, respectively. Detection of actin was used as a loading control. **(B)** Relative quantitation of the knockdown of SMAD2 bands on the western blot when compared to the untreated and scrambled siRNA as the positive control. The intensity of each band is normalized using the relative intensities of the actin bands.



Immunocytochemistry for antibody validation. (A) Western blot showing knockdown of RNF20 in HeLa whole cell lysates after transfection with RNF20-targeting siRNA, using Invitrogen™ RNF20 ABfinity™ Rabbit Oligoclonal Antibody (Cat. No. **710911**). The knockdown is shown along with untreated and scrambled RNA as specificity controls and GAPDH as loading control. **(B, C)** Immunocytochemistry images with Invitrogen™ Lipofectamine™ control and siRNA-transfected cells showing knockdown of RNF20. Images were taken at 60x magnification.

Verifying target specificity of Invitrogen antibodies using genetically modified samples

Invitrogen antibodies that have been verified against genetically modified samples to bind their target are indicated with a “verified specificity” symbol in search results and on relevant product pages. The data showing the verification will be provided on each product page.