EGFR pathway: using CRISPR knockout to validate antibody performance

Introduction

Invitrogen™ antibodies are validated* using a two-pronged approach—determining the specificity of the antibody for the target protein, and functional testing of the antibody in various applications. To ensure that the antibody binds to the intended target, its specificity is verified by one or more of the most appropriate specificity validation methods to test the antibodies based on the application, cell type, and cell function the antibody is utilized in. This note illustrates specificity verification using epidermal growth factor receptor (EGFR) as the model protein.

The EGFR pathway is one of the most thoroughly studied oncogenic pathways. It plays a crucial role in several cellular functions such as regulation of growth, survival, proliferation, and differentiation. EGFR is a tyrosine kinase receptor that upon ligand-induced activation recruits the GRB2–SOS complex to its cytoplasmic domain either directly or through the adaptor protein SHC. SOS then activates RAS, which in turn activates RAF, a major downstream effector. RAF leads to the phosphorylation of extracellular regulated kinases 1 and 2 (ERK1 and ERK2), mediated through MEK1 and MEK2 (Figure 1). These activated kinases are imported into the nucleus where they phosphorylate specific transcription factors involved in cell proliferation.

The dysregulation of EGFR and its downstream effectors has been implicated in various cancers. Due to gene amplification, A-431 human epidermoid carcinoma cells have elevated EGFR levels and therefore are an ideal cell model to study the EGFR pathway.

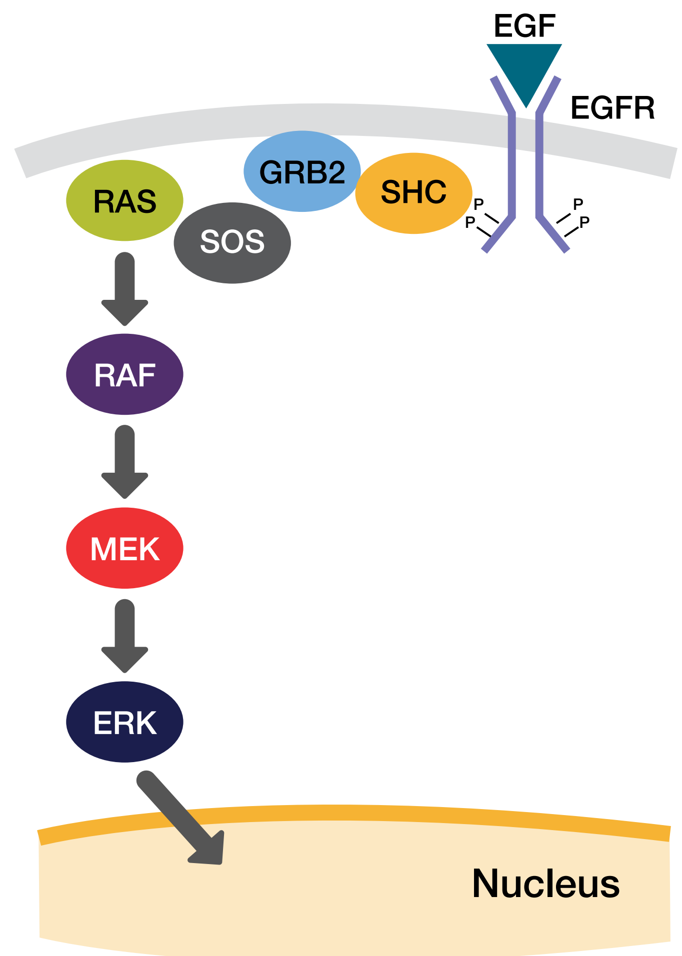

Figure 1. EGFR pathway showing downstream effectors.

Assessing antibody specificity using CRISPR-Cas9 technology

To assess the specificity of the antibody, the EGFR protein signal is measured in control cells and in cells in which the target gene has been knocked out using CRISPR-Cas9 technology. The lack of binding by the anti-EGFR antibody after elimination of EGFR protein expression demonstrates that the antibody is specific. The effectiveness of CRISPR-Cas9 knockout of EGFR is shown in Figure 2.

The CRISPR-Cas9 system is a powerful tool to study pathway dynamics and signaling cascades. The specificity of antibodies against phosphorylated downstream targets can be determined by knocking out key upstream nodes in a pathway. Treatment of A-431 cells with EGF results in activation of EGFR, which further leads to the phosphorylation of all downstream targets. In contrast, when EGFR-knockout cells are treated with EGF, the phosphorylation cascade is blocked. Figure 3 highlights the effect of knocking out EGFR on downstream phosphorylation events.

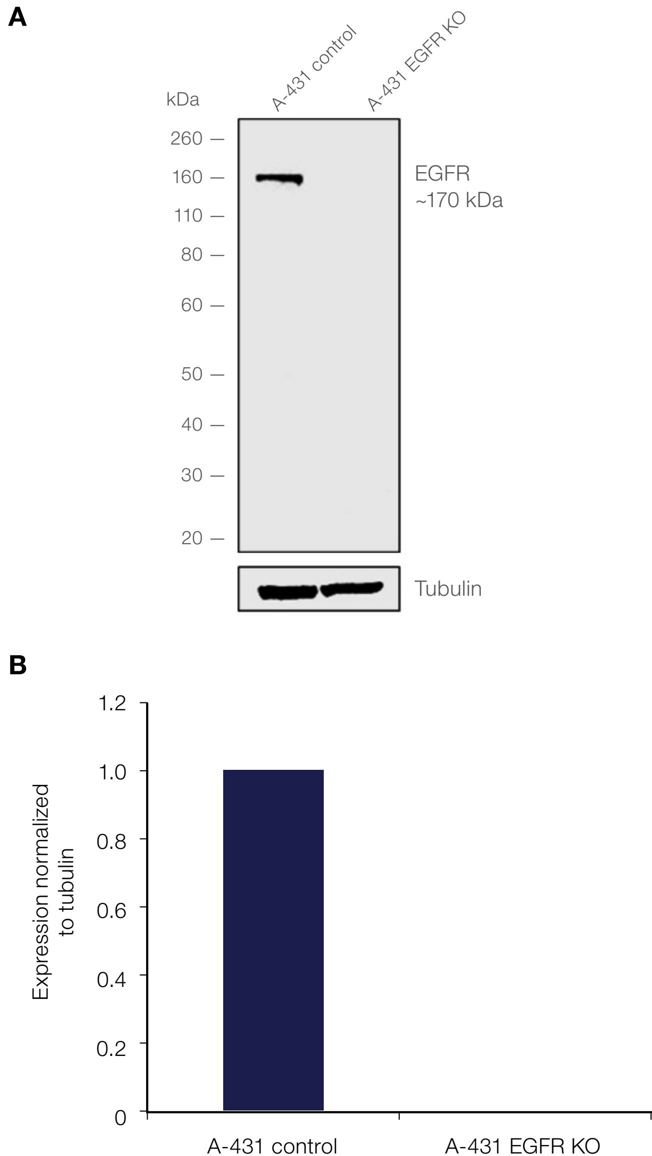

Figure 2. Analysis of EGFR knockout. (A) Western blot analysis of EGFR was performed using whole-cell extracts of A-431 control cells and A-431 EGFR knockout (KO) cells. EGFR was detected at ~170 kDa using Invitrogen™ EGFR Monoclonal Antibody (Cat. No. MA5-13269, 1 µg/mL). **(B)** Densitometric analysis of the western blot shows the absence of signal in the CRISPR-mediated knockout, which confirms that the antibody is specific to EGFR.

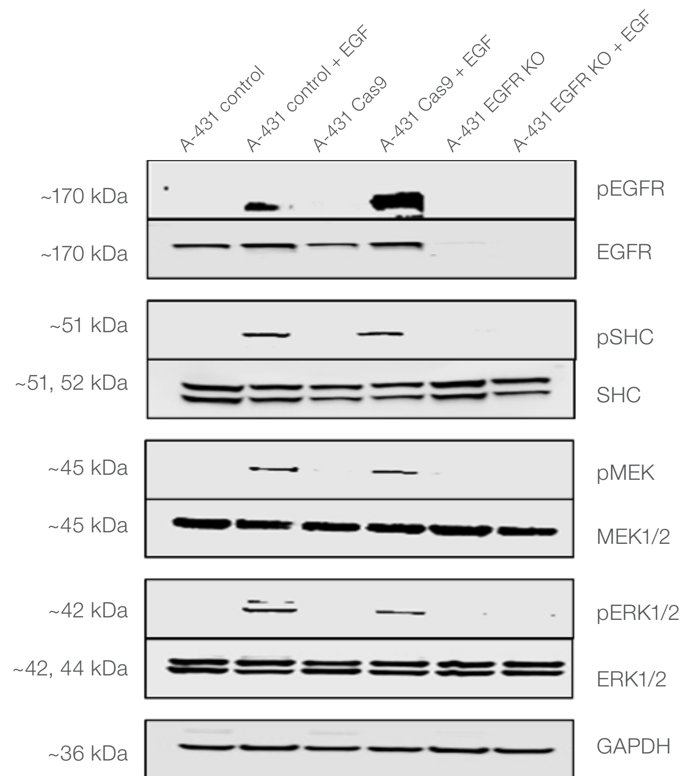

Figure 3. Effect of EGFR knockout on downstream proteins. Western blot analysis of phosphorylation of EGFR and its downstream targets SHC, MEK, and ERK was performed with whole-cell extracts of (left to right): A-431 control cells, A-431 control cells treated with EGF, A-431 Cas9 cells, A-431 Cas9 cells treated with EGF, A-431 EGFR KO cells, and A-431 EGFR KO cells treated with EGF. Phospho-EGFR was detected at ~170 kDa using Invitrogen™ Phospho-EGFR (Tyr1068) Polyclonal Antibody (Cat. No. PA5-17848), phospho-SHC was detected at ~51 kDa using Invitrogen™ Phospho-SHC (Tyr239, Tyr240) Polyclonal Antibody (Cat. No. 44-830), phospho-MEK was detected at ~45 kDa using Invitrogen™ Phospho-MEK1/MEK2 (Ser217, Ser221) Monoclonal Antibody (Cat. No. MA5-15016), and phospho-ERK1/2 was detected at ~42 kDa using Invitrogen™ Phospho-ERK1 (Thr202, Tyr205) Polyclonal Antibody (Cat. No. PA5-13036). Upon induction with EGF (200 ng/mL for 10 min), phosphorylation of the downstream targets SHC, MEK, and ERK was observed in control cells (second lane from left) and not in EGFR knockout cells (last lane on right).

Other methods for assessing antibody specificity

In addition to gene knockout mediated by the CRISPR-Cas9 system, antibody specificity can also be demonstrated by several other methods, depending on their relevance to the target. Here we discuss some of these specificity methods.

Cell treatment

Cells can be treated with either inducers or inhibitors to demonstrate antibody specificity. Phosphorylation and subsequent activation of EGFR is induced by the ligand EGF; this process can be abrogated by the specific EGFR inhibitors gefitinib and afatinib, which bind to its ATP-binding site. Altered expression of phospho-EGFR, upon EGF treatment alone or along with EGFR antagonists, observed in a given application demonstrates antibody specificity (Figures 4 and 5).

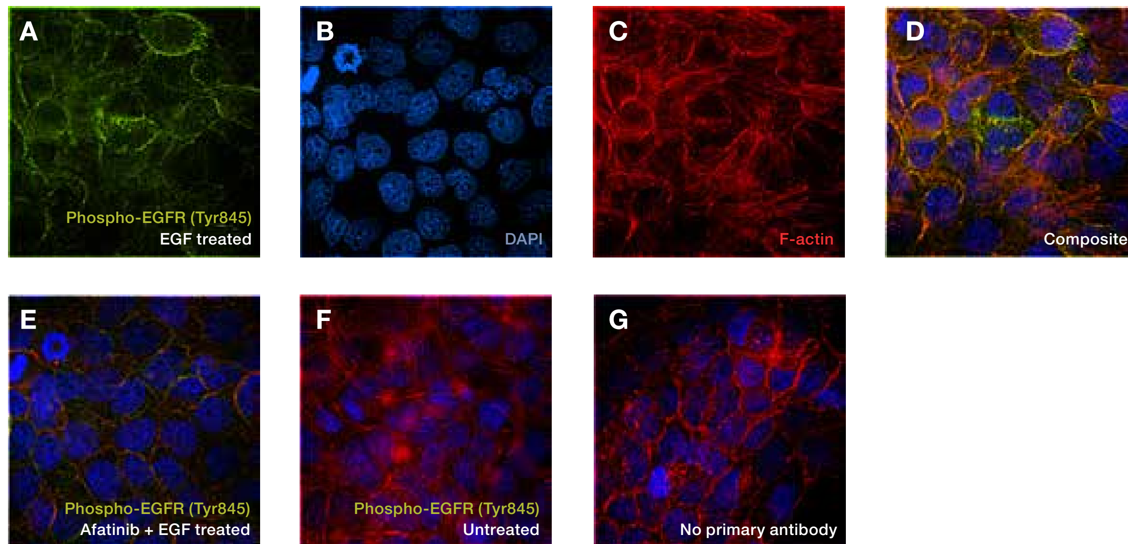

Figure 4. Immunofluorescence analysis of phospho-EGFR in the presence of inhibitor. (A) Phospho-EGFR (green) staining after EGF treatment (200 ng/mL for 10 min) was performed using Invitrogen™ Phospho-EGFR (Tyr845) Polyclonal Antibody (Cat. No PA5-17850) followed by Goat Anti–Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor™ 488 conjugate (Cat. No. A27034). **(B)** Nuclei (blue) were stained with Invitrogen™ SlowFade™ Gold Antifade Mountant with DAPI (Cat. No. S36938). **(C)** F-actin (red) was stained with Invitrogen™ Rhodamine Phalloidin (Cat. No. R415, 1:300 dilution). **(D)** The merged image shows membrane localization of phospho-EGFR. **(E)** Cells treated with the antagonist afatinib (1 µM for 6 hr) followed by EGF (200 ng/mL for 10 min) show reduced phospho-EGFR staining. **(F)** Control cells not treated with EGF do not show a signal. **(G)** Control cells not treated with primary antibody show minimal background.

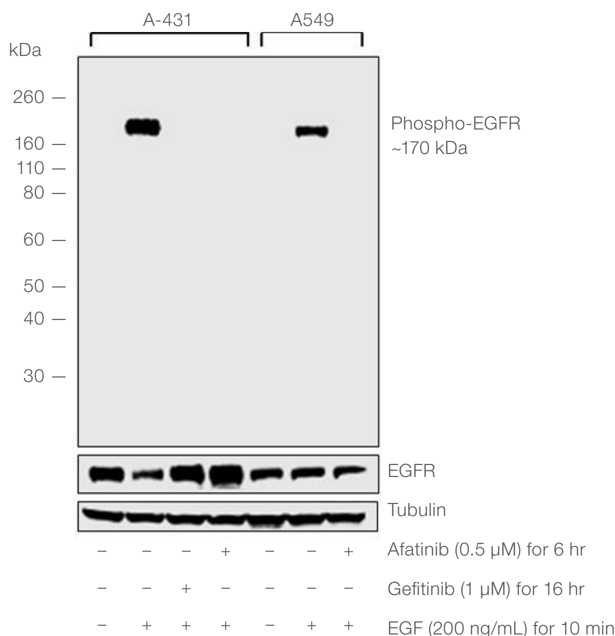

Figure 5. Western blot analysis of phospho-EGFR in the presence of inhibitors. The assay was performed on membrane-enriched extracts (30 µg lysate) of (left to right): A-431 cells, A-431 cells treated with EGF (200 ng/mL for 10 min), A-431 cells treated with gefitinib followed by EGF (1 µM gefitinib for 16 hr, then 200 ng/mL EGF for 10 min), A-431 cells treated with afatinib followed by EGF (0.5 µM afatinib for 6 hr, then 200 ng/mL EGF for 10 min), A549 cells, A549 cells treated with EGF (200 ng/mL for 10 min), and A549 cells treated with afatinib followed by EGF (0.5 µM afatinib for 6 hr, then 200 ng/mL EGF for 10 min). The blot was probed with Phospho-EGFR (Tyr1068) Polyclonal Antibody (Cat. No. PA5-17848, 1:1,000 dilution). A 170 kDa band corresponding to phospho-EGFR was detected, and an increase upon EGF treatment was observed in both cell lines. Pretreatment with the EGFR antagonists gefitinib and afatinib resulted in inhibition of EGFR phosphorylation in both cell lines.

invitrogen

Relative expression

Antibody specificity can be demonstrated by detection of differential basal expression of the target across cell models due to their inherent genetic constitution. EGFR expression is reported to be higher in A-431 cells than in other cancer cell lines, such as A549 lung adenocarcinoma cells and HeLa cervical cancer cells. This characteristic differential EGFR expression pattern observed in a western blot is further evidence that the antibody is indeed specific to EGFR. This approach is shown in Figure 6.

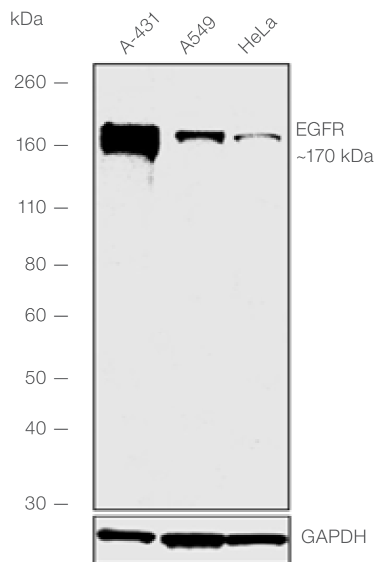

Figure 6. Detection of differential expression. Western blot analysis was performed on membrane-enriched extracts (30 µg lysate) of A-431, A549, and HeLa cells. The blot was probed with EGFR Monoclonal Antibody (Cat. No. MA5-13269, 2 µg/mL). A higher level of EGFR was observed in A-431 cells than in A549 and HeLa cells.

Conclusion

In summary, we have described the use of CRISPR-Cas9 technology as an effective method for verifying target protein specificity when performing antibody validation in the EGFR pathway. Additionally, we have highlighted other methods for demonstrating antibody specificity, including the use of cell treatments to alter protein expression and the use of cell lines with defined relative protein expression. The use of these methods to verify antibody specificity helps ensure proper functionality and instills confidence that our antibodies will provide superior performance in your research.

* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic use.

Find out more at **thermofisher.com/antibodyvalidation**

ThermoFisher SCIENTIFIC

For Research Use Only. Not for use in diagnostic procedures. © 2018 Thermo Fisher Scientific Inc. All rights reserved.
All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. **COL22631 0618**