Dissect signaling pathways with multiplex western blots

Using fluorescent antibody conjugates in combination with the iBright Imaging System.

Signaling pathways control and coordinate all aspects of cell function. Dissecting these pathways leads to the identification of key receptors, enzymes, and signaling molecules that regulate both normal development and disease states. Great advances in examining signaling pathways have been made using CRISPR-Cas9 technology, which allows precise editing or excision of genes of interest, thus knocking out expression of targeted proteins. Using these gene knockouts, researchers can study the upstream and downstream interactions of the targeted protein.

Analyze gene knockouts with multiplex western blotting

The effect of gene knockouts on individual proteins in a signaling pathway can be determined using western blotting with pathway-specific antibodies. For example, antibody pairs that recognize a specific protein and corresponding phosphoprotein can be used to determine the phosphorylation state of a putative downstream protein in a signaling pathway. Probing the western blot with several fluorescently labeled antibodies that emit light at different wavelengths enables the simultaneous detection of multiple protein targets on a single blot. In contrast, traditional chemiluminescent western blotting requires multiple rounds of probing, stripping, and reprobing to collect the same pool of data.

By minimizing the need to run multiple protein gels or reprobe blots, multiplex western blotting saves a significant amount of time, effort, and cost. Furthermore, advances in western blot imaging equipment and software enable streamlined protein quantitation. To demonstrate its utility, we used multiplex western blotting to illustrate the efficient knockout of the gene encoding the epidermal growth factor receptor (EGFR), and the effects of this knockout on the downstream mitogen-activated protein kinase 1 (MEK1) (Figure 1). EGFR knockout cells created using CRISPR-Cas9 gene editing technology were treated with epidermal growth factor (EGF). Cell lysates were then prepared, separated by protein gel electrophoresis, transferred to a membrane, and probed using spectrally distinct fluorescent antibodies specific for EGFR and MEK1 and the corresponding phosphoproteins (Table 1). The blot was imaged using the Invitrogen™ iBright™ FL1000 Imaging System; the signals were normalized using an antibody specific for the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Figure 1 shows that EGF-induced phosphorylation of EGFR and MEK1 was observed in control cell lines (lanes 1–4) and not in the EGFR knockout cell lines (lanes 5 and 6).

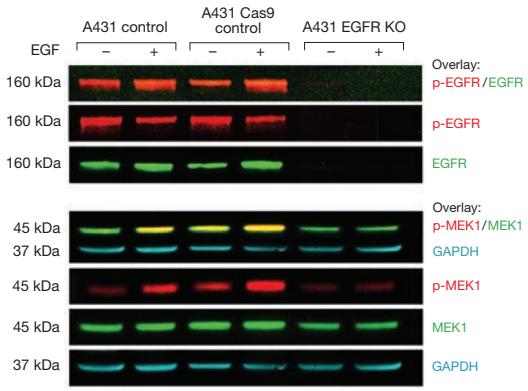

Figure 1. Western blot analysis of EGFR and its downstream target MEK1 using EGFR knockout cells. Electrophoresis was performed using an Invitrogen™ NuPAGE™ Bis-Tris 10% gel (Cat. No. NP030A) loaded with 30 µg of whole cell extracts from (1) A431 control cells, (2) EGF-treated A431 cells (200 ng/mL for 10 min), (3) A431 Cas9 control cells, (4) EGF-treated A431 Cas9 cells, (5) A431 EGFR knockout (KO) cells, (6) EGF-treated A431 EGFR KO cells, and Invitrogen™ Novex™ Sharp Pre-Stained Protein Standard (Cat. No. LC5800). Proteins were transferred to a nitrocellulose membrane using the Invitrogen™ iBlot™ 2 Gel Transfer Device (Cat. No. IB21001), blocked with 5% skim milk for 1 hr, and incubated with Invitrogen™ primary antibodies and Invitrogen™ Alexa Fluor™ Plus secondary antibodies (Table 1). Blots were imaged on the Invitrogen™ iBright™ FL1000 Imaging System (Cat. No. A32752).

Table 1. Primary and secondary antibodies, western blotting conditions, and imaging channels used to produce the data in Figure 1.

Target	Primary Ab* host (Cat. No.)	Secondary Ab* (Cat. No.)	Imaging channel
Phospho-EGFR (Tyr1068)	Rabbit (PA5-17848)	Alexa Fluor Plus 800 donkey anti–rabbit IgG (A32808)	800 nm
EGFR	Mouse (MA5-13343)†	Alexa Fluor Plus 680 donkey anti–mouse IgG (A32788)	680 nm
Phospho-MEK1 (Thr386)	Rabbit (702581)	Alexa Fluor Plus 800 donkey anti–rabbit IgG (A32808)	800 nm
MEK1	Mouse (MA5-15093)	Alexa Fluor Plus 680 donkey anti–mouse IgG (A32788)	680 nm
GAPDH	Goat (PA1-9046)	Alexa Fluor Plus 488 donkey anti–goat IgG (A32814)	488 nm

*Primary antibody (Ab) dilutions were determined empirically and ranged from 1:500 to 1:2,000; incubations were performed overnight at 4°C. Secondary antibody dilutions were 1:1,000, and incubations were performed for 1 hr at room temperature, protected from light. †MA5-13343 has been replaced with EGFR Antibody Cocktail (Cat. No. MA5-13697). Search our extensive antibody portfolio at **thermofisher.com/antibodies**.

Learn more about multiplex western blotting

Significant advances in instrumentation as well as in immunodetection have facilitated the migration from ECL to fluorescence-based western detection. Learn more about multiplex western blotting at **thermofisher.com/5steps-multiplexwesterns**. ■