Development and Validation of a Novel EGF Receptor-Neutralizing (D1D4J) Monoclonal Antibody

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INTRODUCTION

- The epidermal growth factor receptor (EGFR) is a cell-surface receptor for members of the EGF family. EGFR dimerizes upon ligand binding, which leads to autophosphorylation, downstream signaling and ultimately receptor internalization.
- In cancer, EGFR mutations and amplification have been linked to cell proliferation and survival.
- There are two FDA approved neutralizing monoclonal antibodies: cetuximab (BMS; mouse/human chimeric) and panitumumab (Amgen; fully humanized). However, these antibodies are often unavailable to researchers studying this important pathway.
- Various immunization strategies have been attempted at Cell Signaling Technology, Inc (CST) previously to generate an extracellular EGFR Rabbit mAb with limited success. A specifically designed whole cell immunization protocol described lead to suitable monoclonal candidates.
- Here we describe the development and validation of a new rabbit monoclonal EGFR-neutralizing antibody (clone D1D4J).

CONCLUSIONS

- D1D4J is a highly specific EGFR monoclonal antibody that recognizes the extracellular domain of human and mouse EGFR, and is the only commercially available extracellular Rabbit mAb on the market.
- D1D4J demonstrated strong and specific performance in Western blot (WB), immunofluorescence (IF) (live and fixed), flow cytometry (Flow), and immunoprecipitation (IP) assays.
- D1D4J also shows strong neutralizing ability as determined by multiple assays (WB and IF).
- D1D4J is a useful research tool to support further preclinical development of EGFR targeted therapies.
- EGF Receptor (D1D4J) XP® Rabbit mAb (Neutralizing) #54359 was validated using cell-based models and IF imaging. D1D4J binds EGFR and neutralizes EGF-induced activation and internalization. Compared to other Research Use Only (RUO) neutralizing antibodies on the market, D1D4J shows superior performance and is a useful research tool to study EGFR signaling.

Methods

Figure 1A: Whole Cell Immunization

Rabbits were immunized with mammalian cells expressing full length human EGFR protein

Figure 1B: Serum Analysis and Rabbit Selection

Rabbit serum tested for EGFR reactivity by flow cytometry

Cell Systems Used for Analysis	Over-expression or Endogenous	EGFR Expression Level
293 (Mock)	transfected mock	-
293 O/E hEGF Receptor	transfected	++++
A549	endogenous	+
A-431	endogenous	++
Jurkat	endogenous	-
CAMA-1	endogenous	-

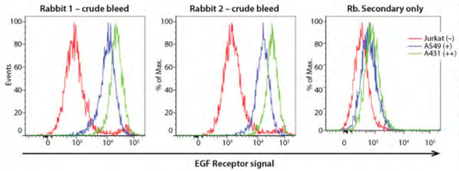
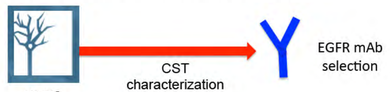


Figure 1C: Monoclonal Antibody Creation



Assessing Specificity

D1D4J was tested according to validation procedures at CST. D1D4J was validated and optimized for WB, IF, FC, and IP.

Figure 2A: D1D4J detects EGFR in human and mouse by WB and does not cross react with other ErbB/HER family members

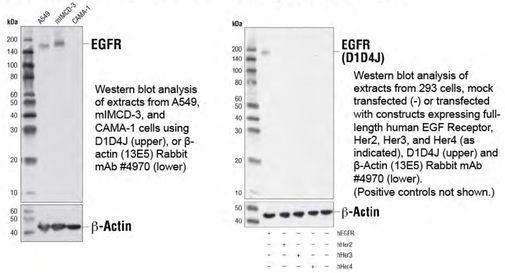
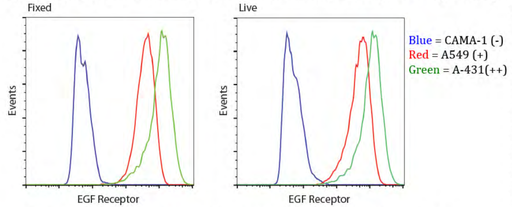
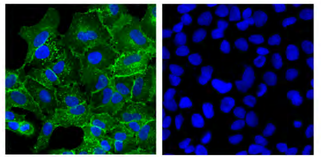


Figure 2B: D1D4J detects EGFR in fixed and live cells by Flow Cytometry



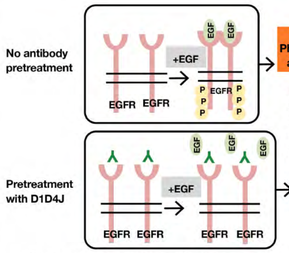
Flow cytometric analysis of fixed, unpermeabilized(left) or live (right) CAMA-1 cells (blue), A549 cells (red), and A-431 cells (green) using D1D4J. Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) #14412 was used as a secondary antibody.

Figure 2C: D1D4J detects EGFR by Immunofluorescent analysis



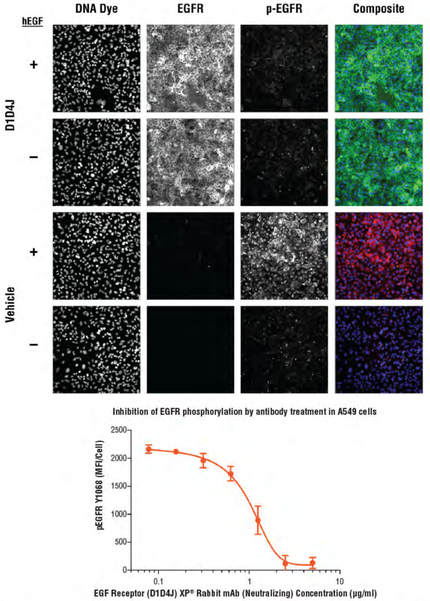
Confocal immunofluorescent analysis of A549 (left) and CAMA-1 (right) using D1D4J (green). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).

Assessing Neutralizing Activity



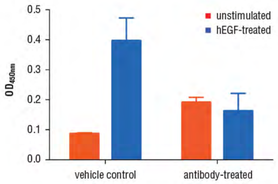
Neutralizing Treatment Method:
Serum-starved A549 cells were pre-incubated (1-2hr, 5ug/mL) with D1D4J, or vehicle, and subsequently stimulated with hEGF #8916 (15min, 100ng/ml; +).

Figure 3A: D1D4J neutralizing activity confirmed by IF analysis



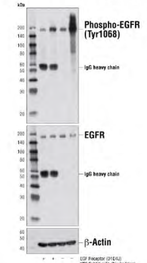
IF images (upper) of treated A459 cells pre-incubated with D1D4J (5ug/mL, 1 hr) or vehicle (PBS); and subsequently stimulated with hEGF #8916 0.....10 (15 min, 100 ng/ml) as shown. Cells were then fixed and stained for activated EGFR (p-EGFR Tyr1068 in red); D1D4J was also labeled post-fixation (in green). Blue pseudocolor = Hoechst 33342 (fluorescent DNA dye). Phospho-EGFR signal was quantified for the full titration range of D1D4J treatment using an ArrayScan™ VTI HCS Reader (bottom).

Figure 3B: D1D4J neutralizing activity confirmed by PathScan® ELISA analysis



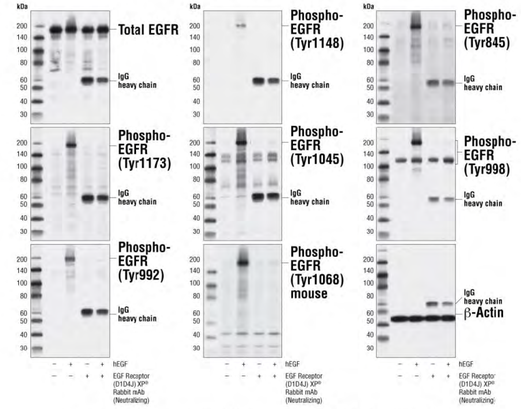
ELISA analysis of cell extracts from treated or control A549 cells. The extracts were subsequently left unstimulated (red) or stimulated with hEGF #8916 (15 min, 100 ng/ml; blue), using PathScan® Phospho-EGF Receptor (panTyr) Sandwich ELISA Kit #7911.

Figure 3C: D1D4J pre-treatment reduces EGFR phosphorylation



WB analysis of extracts from treated (+) or control (-) A549 cells using p-EGFR(Tyr1068) (D7A5) #3777 (upper), D1D4J (middle), or β-actin (13E5) Rabbit mAb #4970 (lower).

Figure 3D: D1D4J neutralizes EGFR phosphorylation at numerous tyrosine residues



WB analysis of extracts from treated (+) or control (-) A549 cells using: Total EGFR (D38B1) #4267 (left , upper), p-EGFR (Tyr1173) #4407 (left, middle), p-EGFR (Tyr992) #2235 (left, lower), p-EGFR (Tyr1148) #4404 (center, upper), p-EGFR (Tyr1045) #2237 (center, middle), p-EGFR (Tyr1068) (1H12) Mouse mAb #2236 (center, lower), p-EGFR (Tyr845) (D63B4) #6963 (right, upper), p-EGFR (Tyr998) (C24A5) #2641 (right, middle), β-Actin (13E5) #4970 (right, lower).

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