Generation of Post-Translational Modification Specific Antibodies Targeting Phosphorylated Epidermal Growth Factor Receptor

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Abstract

Phosphorylation is a crucial form of post-translational modification (PTM) involved in the regulation of protein function and cell signaling. Phosphorylation of a protein causes conformational changes that lead to a change in activation status, or protein-protein binding events that rely on the recognition of phospho-motifs. Kinases and phosphatases are enzymes that catalyze the transfer or removal of a phosphate group respectively, and mutations in these enzymes have been shown to cause various diseases including cancer. The ability to differentiate between the phosphorylation states of a specific protein is instrumental in understanding the role of this PTM in complex signaling pathways and developing drugs to treat diseases.

Using phosphorylated and nonphosphorylated peptides as antigens, and in vitro guided selection strategies, recombinant antibodies were generated using Human Combinatorial Antibody Libraries (HuCAL®) technology, resulting in specialized reagents that can differentiate between the epidermal growth factor receptor (EGF R) in its dephosphorylated and phosphorylated (pTyr1045) forms. These antibodies were validated in western blotting (WB) by comparing phosphorylation levels in treated and untreated cell lysates, and by dephosphorylating proteins on western blot membranes. The two best antibodies, detecting total EGF R and phosphorylated EGF R only, were tested side-by-side to enable assessment of the impact of the different treatments.

Introduction to HuCAL Technology

- HuCAL technology combines a synthetic naïve library of human antibody genes that cover more than 95% of the structural human immune repertoire, with phage display to select the genes from the library that encode for antibodies binding specifically with high affinity to just about any given antigen
- 7 VH and 6 VL consensus framework sequences represent the diversity of human antibody germline genes, leading to 42 framework combinations in the master library. The framework genes are designed as modules and codon optimized for expression in both prokaryotic and eukaryotic expression systems. All six complementarity determining regions (CDRs) are diversified using trinucleotide mutagenesis technology, do not contain cysteines, and are designed for a minimum occurrence of glycosylation sites
- HuCAL PLATINUM® is a library of >10^{10} human antibodies in Fab format. The modularity enables easy optimization for affinity and/or specificity
- Implementation of SpyTag technology enables site-directed conjugation and fast switching between stable monovalent, bivalent, and Ig-like antibody formats

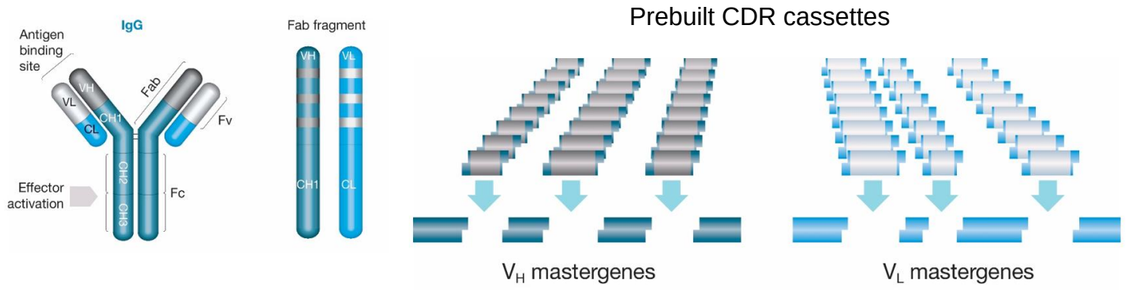


Fig. 1. Schematic image showing an antibody and a Fab fragment, and the HuCAL technology design concept.

Objective and Project Strategy

Generation of a pair of antibodies to detect total EGF R and EGF R phosphorylated on tyrosine residue 1045 (pTyr1045). The anti-phospho-specific antibody should not cross-react with nonphosphorylated EGF R.

Antibody selection with additional integrated affinity maturation steps ('pool maturation' RapMAT®):
first and second rounds of phage display on the peptide alternating between forms coupled to BSA or transferrin (Trf); cloning of LCDR3 cassette into pool of Fab genes; third and fourth rounds of panning using higher stringency on biotinylated peptides coupled to streptavidin beads.

- Phospho-specific antibody: panning on EGF R pTyr1045, CEDSFLQR(Yp)SSDPTGA-TRF (round 1), CEDSFLQR(Yp)SSDPTGA-BSA (round 2), and EDSFLQR(Yp)SSDPTGA-Ttds-Bio (rounds 3 and 4); blocking with EGF R-pep-TRF, CEDSFLQRYSSDPTGA
- Total EGF R antibody: panning on EGF R-pep-TRF (rounds 1 and 3) and EGF R-pep-BSA (rounds 2 and 4)
- Subcloning of pools into bivalent Fab format for primary ELISA screening
- Secondary screening by WB
- Affinity determination in bivalent format
- Testing of antibodies in WB, comparing phosphorylation levels in both treated versus untreated cell lysates, and by dephosphorylating proteins on WB membranes

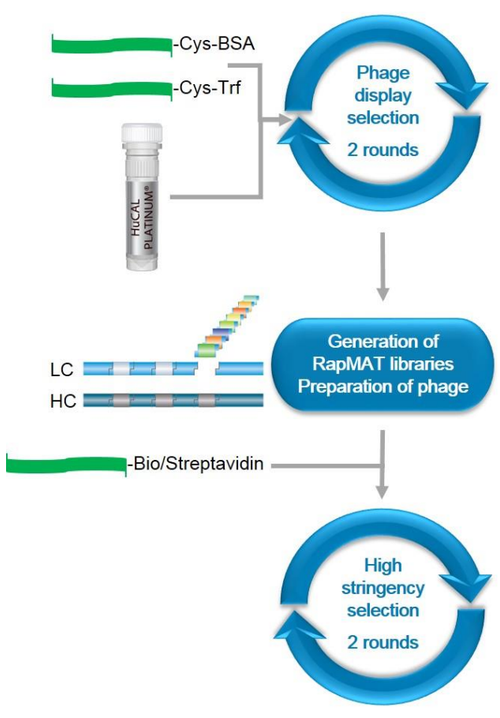


Fig. 2. Rapid affinity maturation (RapMAT) process flow.

Specificity ELISAs for Anti-EGF R and Anti-EGF R (pTyr1045) Antibodies

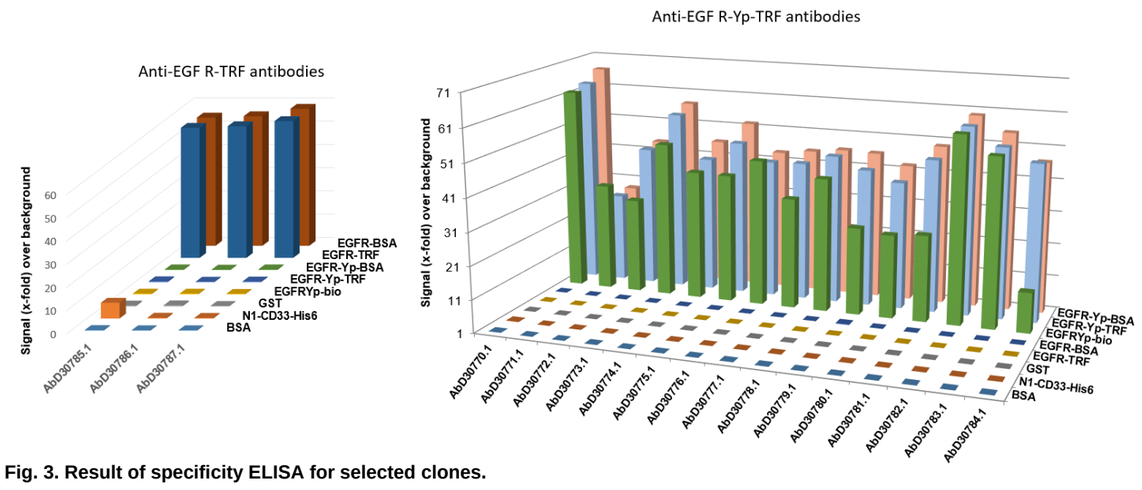


Fig. 3. Result of specificity ELISA for selected clones.

Affinity Determination of Selected Antibodies in Bivalent Fab Format

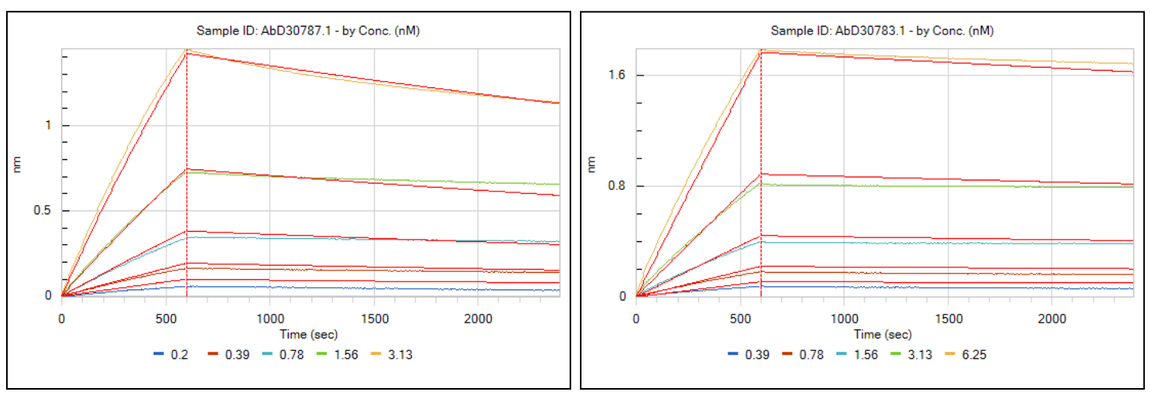


Fig. 4. Affinity determination of best antibodies from panning, clone AbD30787.1 and AbD30783.1.

Sample ID	Specificity	k_a	k_d	K_D
(Antibody clone)		[1/Msec]	[1/sec]	[nM]
AbD30787.1	Total EGF R	1.11 x 10^5	1.29 x 10^{-4}	1
AbD30783.1	EGF R pY1045-specific	3.05 x 10^3	4.63 x 10^{-5}	15

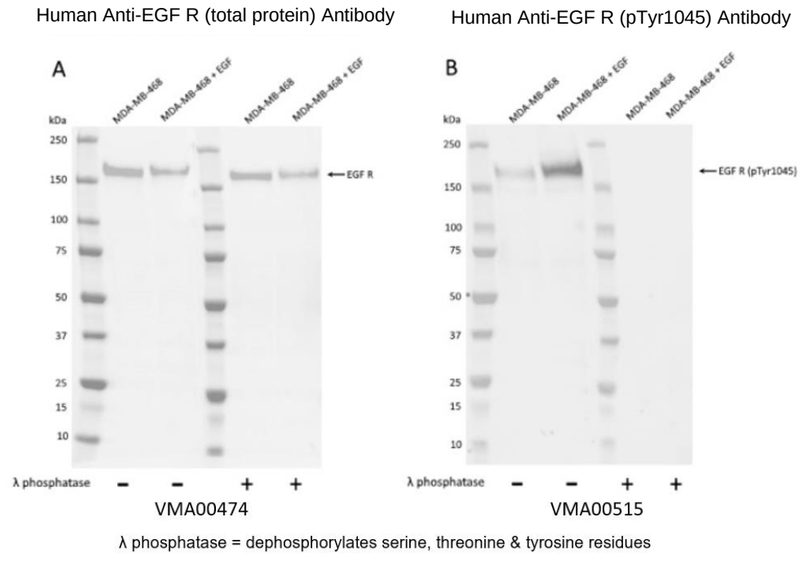
Affinity determinations were performed with the antibodies in a bivalent format, therefore the measurements are not for a true 1:1 interaction and are subject to avidity effects. The reported K_D value is an observed one for this specific measurement. The intrinsic affinity is likely to be lower.

Performance of Antibodies in Western Blotting

The following criteria were assessed to determine success in WB:

1. The phospho-specific antibodies should detect the phosphorylated EGF R protein; there should be an increase in the signal intensity after stimulation with EGF due to phosphorylation of Tyr1045, and a reduction after lambda phosphatase treatment.
2. The phospho-specific antibody must not detect the nonphosphorylated EGF R; there must be a significant reduction (≥90%) in signal intensity in both simulated and unstimulated lysates following phosphatase treatment.
3. The total EGF R antibodies should detect EGF R in both phosphorylated and nonphosphorylated forms, independent of phosphatase treatment.

Two antibodies, clone AbD30783 (Cat. #VMA00515) with pTyr1045 specificity and clone AbD30787 (#VMA00474) with total EGF R specificity, generated the best results in the assessment.



λ phosphatase = dephosphorylates serine, threonine & tyrosine residues

Fig. 5. Western blot analysis of untreated and EGF treated whole cell lysates (MDA-MB-468 cells) probed with A, Human Anti-EGF R Antibody (#VMA00474) and B, Human Anti-EGF R Antibody (pTyr1045) (#VMA00515). Membranes were treated with (+) and without (-) lambda protein phosphatase. Human Anti-EGF R Antibody (#VMA00474) detects EGF R independent of phosphatase treatment. With Human Anti-EGF R Antibody (pTyr1045) (#VMA00515), there is a significant reduction in phosphorylated band intensity post phosphatase treatment, and increase in signal intensity after EGF treatment.

Fig. 6. Quantification of western blot data using total protein normalization (TPN). Data show signal intensity increase after stimulation (cell lysate MDA-MB-468 versus MDA-MB-468 + EGF without (-) phosphatase treatment) and signal intensity reduction post phosphatase treatment (+).