Development and Use of Phosphorylation-Specific Capture and Detection Antibodies

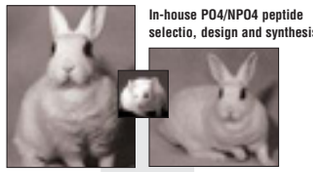
Michael D. Melnick, Bradley L. Smith and Christopher Bunker • Cell Signaling Technology, Inc., Danvers, MA

Capture antibody technologies rely on reagents that are specific and sensitive. CST is a leader in the development and production of antibodies that detect the site-specific phosphorylation of a protein. CST is applying its phosphorylation-specific antibody development expertise and life sciences experience to the development of capture antibody technologies including sandwich ELISAs, Bio-Plex bead-based assays and phospho-peptide immuno-affinity capture/LC-MS/MS methodologies for phospho-proteomics.

This program begins with novel rabbit and mouse fusion technologies combined with early screening of bleeds for detection of native proteins. It is clear that rabbits are the preferred source for phospho-antibodie. The desirability of monoclonals is also clear. Following the fusion, hybridomas are screened by multiple methods to identify potential capture and detection antibodies. In parallel, the hybridomas are tested by Western blot, immunoprecipitation, IHC and Flow cytometry. The resulting capture or detection clones are optimized for use in ELISAs, bead-base Bio-Plex assays or other applications. The development cycle depends greatly on having the appropriate cell system to demonstrate sensitivity. Negative controls are also included to determine specificity and cross-reactivity. This program is reliably producing phospho-specific ELISA kits and Bio-Plex assays. A more specialized program is using a similar approach to produce phospho-motif antibodies capable of immuno-precipitating phospho-peptides for MS analysis. These programs demonstrate that multiple technologies and expertise are required in order to successfully develop phospho-specific capture antibody reagents.

CAPTURE/DETECTION ANTIBODY PAIR DEVELOPMENT

Target & Phospho-Specific Antibody Development



In-house PO4/NPO4 peptide selectio, design and synthesis

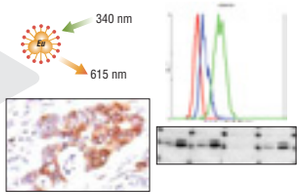
Polyclonal Antibodies Monolonal Antibodies

Monolonal Antibodies

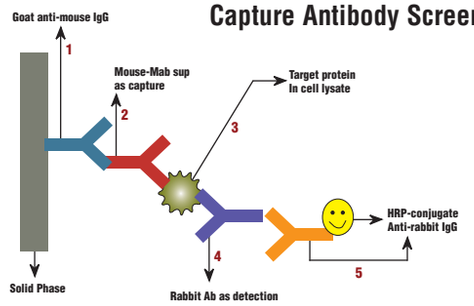
Hybridoma Screening

Protein A-Purified
Affinity Purified:
(-) non-PO4 affinity
(-) PO4 affinity

APPLICATION VALIDATED

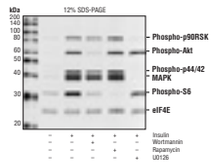


Capture Antibody Screen



Cell Lysate & Western Optimization

PathScan® Multiplex Western Cocktail I:
Phospho-p90RSK, Phospho-Akt, Phospho-p44/42 MAPK and Phospho-S6 Ribosomal Protein Detection Kit #7100



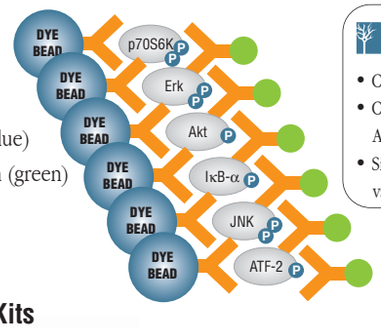
Western blot analysis of extracts from CHO cells, untreated or insulin-treated following pretreatment with wortmannin (PI3 kinase inhibitor), rapamycin (mTOR inhibitor) and/or U0126 (MEK inhibitor) as indicated, using #7100.

MULTIPLE SANDWICH ELISA FORMATS

Bio-Plex® Bead Suspension Array System: CST Development Partner

Two-color flow cytometry based sandwich immunoassay system

ID unique bead popultion (blue)
Quantity protein population (green)



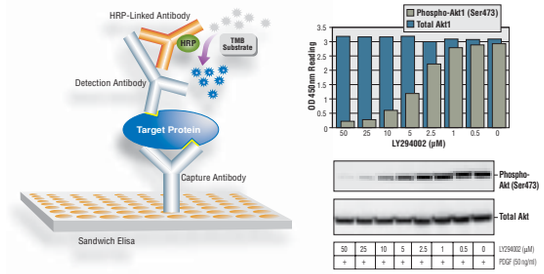
Cell Signaling TECHNOLOGY®

- Cell signaling biology
- Capture/detection Antibody Dev
- Single-plex pair validation

BIO-RAD

- Assay Multiplexing
- Software solutions
- Off the shelf production

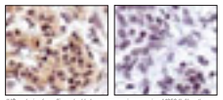
PathScan® ELISA Kits



VALIDATED FOR MULTIPLE APPLICATIONS

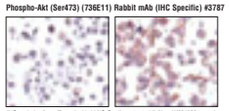
Antibody IHC Validation

Phospho-Akt (Thr308) (244F1) Rabbit mAb #4056

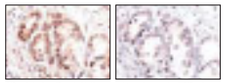


IHC analysis of paraffin-embedded pancreas carcinoma, using #4056 (left) or the same antibody preincubated with antigen specific peptide (right).

Phospho-Akt (Ser473) (736E11) Rabbit mAb (IHC Specific) #3787

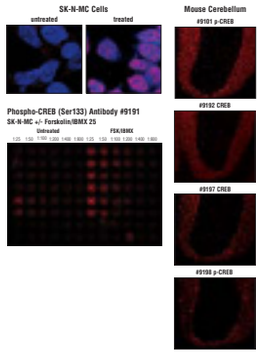


IHC analysis of paraffin-embedded LNCaP cells, untreated (left) or LY294002-treated (right), using #3787.

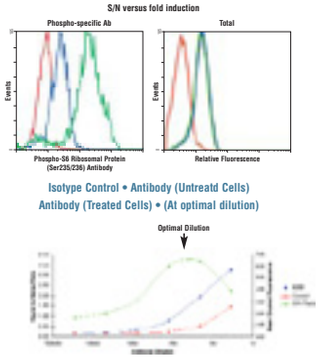


IHC analysis of paraffin-embedded human prostate carcinoma, using #3787 preincubated with an irrelevant peptide (left) or Phospho-Akt (Ser473) Blocking Peptide (IHC Specific) (#1140) (right).

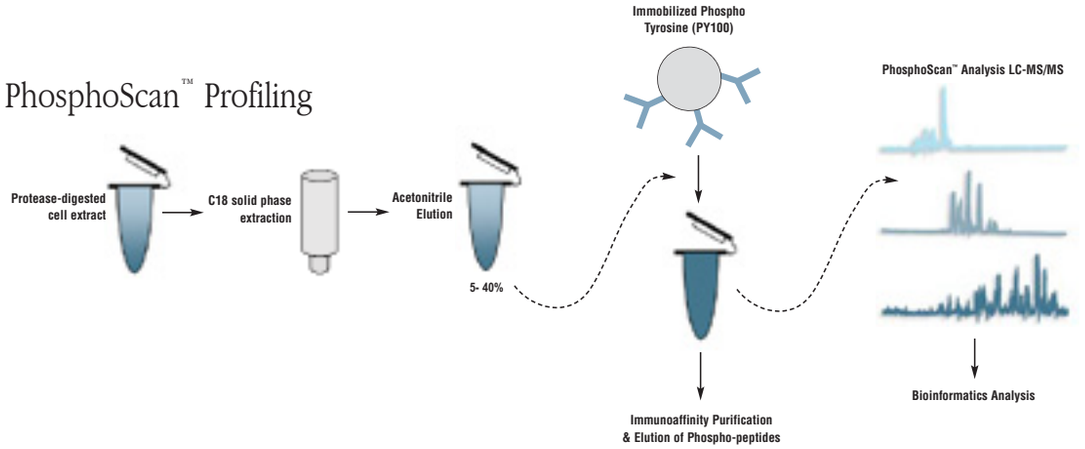
Antibody IF/ICC Validation



Antibody Flow Validation



PHOSPHO-PEPTIDE MOTIF CAPTURE AND LC-MS/MS ANALYSIS



Cell Signaling Technology Patent Pending
Rush, J. et al., Nature Biotech, Jan 05