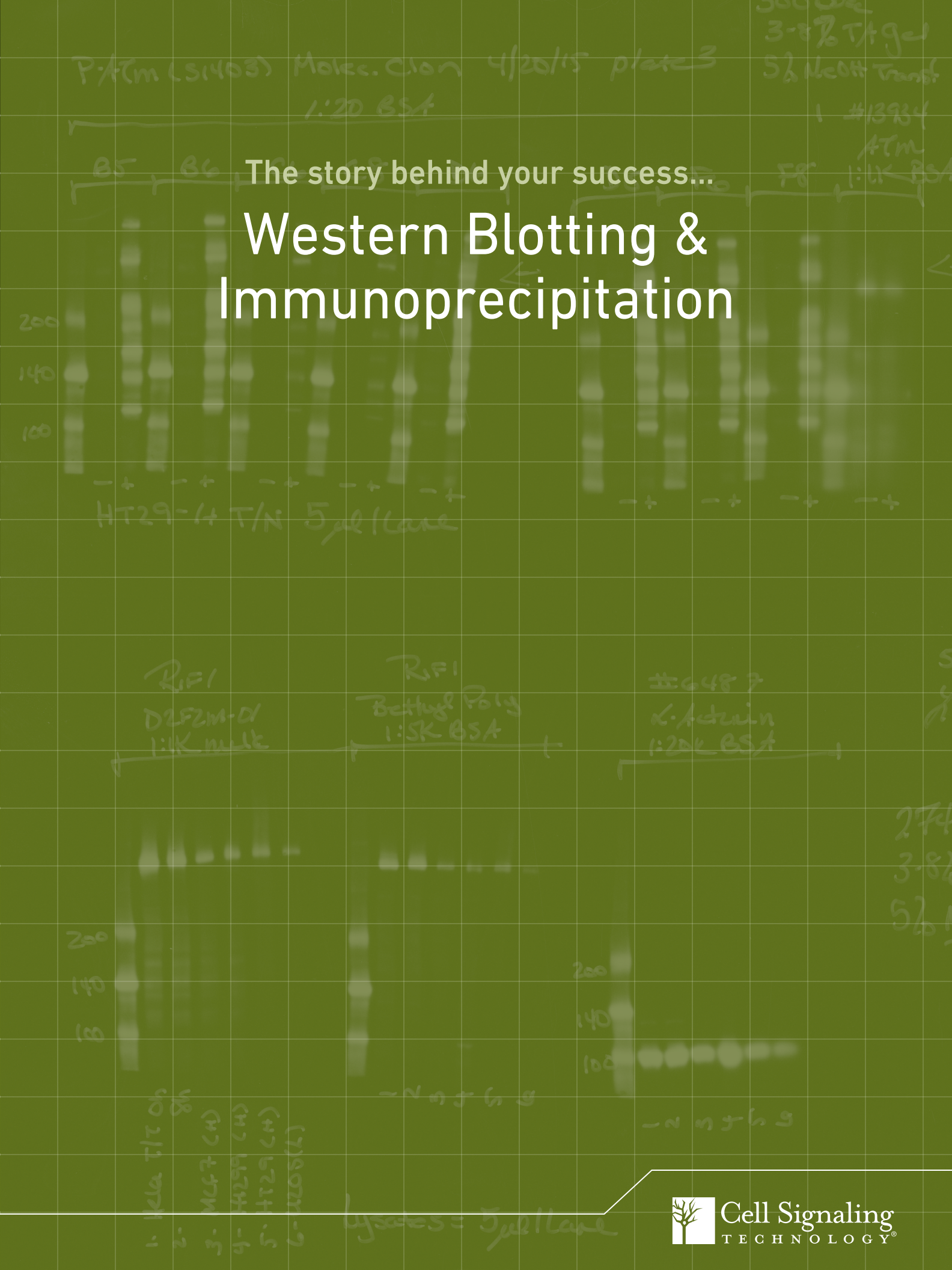
The story behind your success...

# Western Blotting & Immunoprecipitation



Cell Signaling TECHNOLOGY®

# Why choose Cell Signaling Technology?

## Western Blotting & Immunoprecipitation

Western blotting (WB) is widely used to analyze specific protein expression in cell or tissue extracts, and immunoprecipitation (IP) is employed to enrich a specific protein or protein complex from a heterogeneous cell or tissue extract. At Cell Signaling Technology (CST) we understand that western blotting and IP experiments are time consuming and that their success has a critical impact on your research progress. For that reason, we thoughtfully develop antibodies and provide optimized protocols along with reference information and technical support to make your western blotting and IP experiences successful.

CST provides extensive western blotting reference information from our rigorous in-house testing to support your experiments.

**Specified Dilutions and Buffers:** You save time by skipping antibody titrations, because optimal dilutions and dilution buffers are identified for every CST™ antibody. These specifications are generated and supported by extensive in-house testing and are provided on the datasheet that accompanies every product.

**Controls:** Target-specific positive and negative control cell extracts are recommended, as they can ensure confidence in the results from your experimental cell extracts and help with troubleshooting your experiment. CST provides target-specific control extracts for many of our antibodies. See the table of Control Cell Extracts on the CST website for a full listing.

**Protocols:** Minimize your time and reagent waste by using CST product-specific protocols. The protocols, which we optimize for each antibody, are provided to you so that you can start your experiment using proven methods.

## Contents



**Troy, PhD, Product Scientist** (left) has been with CST since 2010, **Christina, Product Scientist** (center) has been with CST since 2007, **Kenneth, Production Associate** (right) has been with CST since 2011.

## CST Technical Support

At CST, providing exceptional customer service and technical support are top priorities. Our scientists work at the bench daily to produce and validate our antibodies, so they have hands-on experience and in-depth knowledge of each antibody's performance. In the process, these same scientists generate valuable reference information that they use to answer your questions and help troubleshoot your experiment by phone or email.

**www.cellsignal.com/support** (USA & Europe) | **www.cst-c.com.cn/support** (China) | **www.cstj.co.jp/support** (Japan)

# It takes hundreds of westerns

**to develop a single monoclonal antibody.**

Monoclonal antibody development at CST starts at the target identification step. Here, the target protein's research relevance and the scientific community's interest are evaluated, and target specific applications (western blotting, flow cytometry, etc.) are prioritized. Once the source material is available, honing in on the best possible antibody to meet your research needs begins. CST antibody development is performed entirely in-house, from target selection to specificity and applications testing, using standardized protocols that can be reliably repeated by different users and from lot-to-lot.

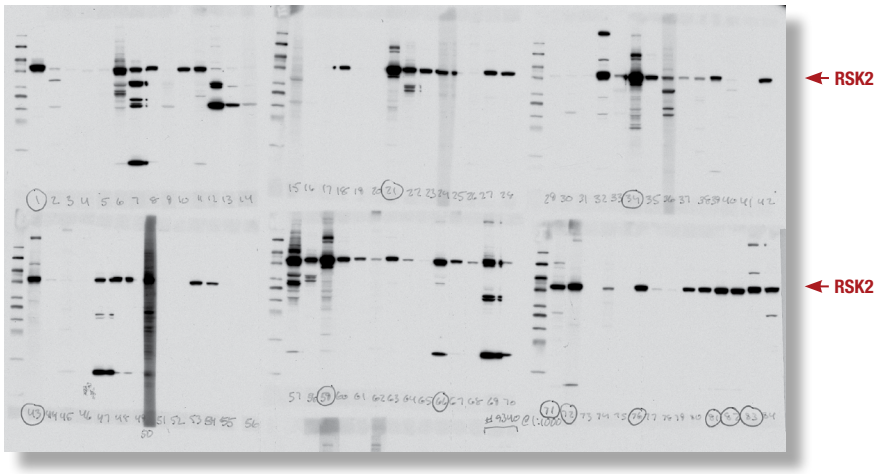
## CST Development of RSK2 mAb

### 1. ELISA screening

First, ELISA screening is performed on as many as 960 clonal cell populations.

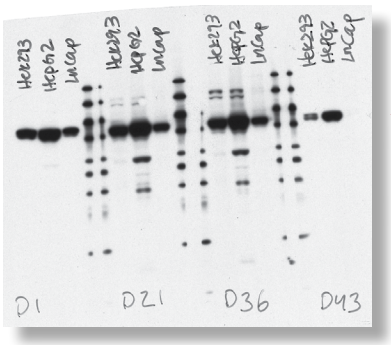
### 2. Western blot screening

The clones that are positive by ELISA are screened by western blot (WB) using an appropriate cell lysate (in this case, HeLa cell lysates).




### 3. Testing of cell lines

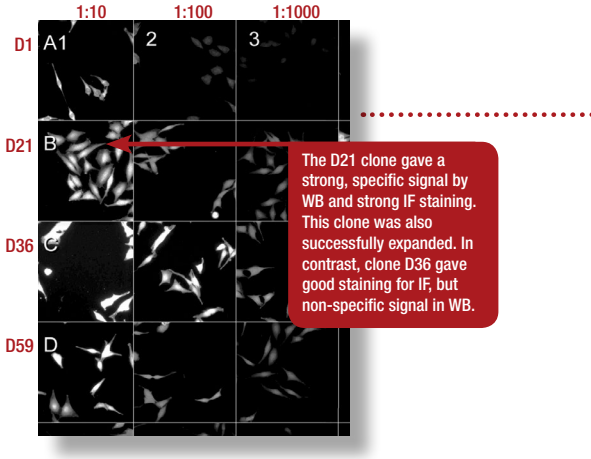
Clones that are positive by WB are identified and retested on extracts from multiple cell lines.



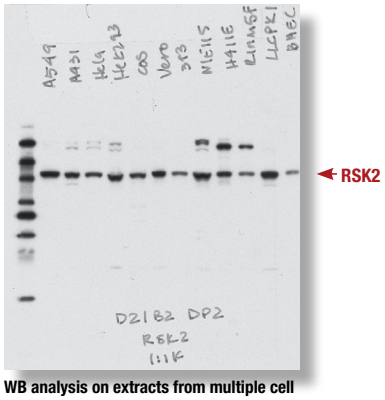

### 4. Screening by other key applications

Clones that demonstrate specific WB signals are further screened for desired applications such as immunohistochemistry (IHC), immunofluorescence (IF) or flow cytometry (F).

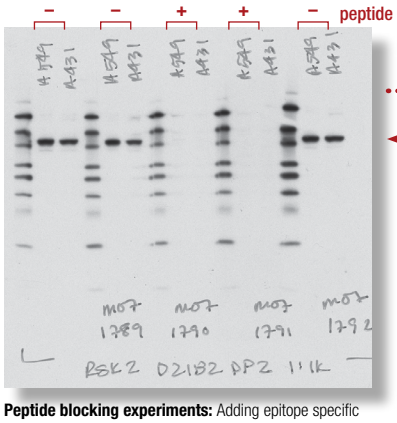
**IF screening in a 96-well format:** HeLa cells were serum-starved and then treated with 50 ng/ml EGF for 20 min. IF performance of supernatants from clones was assessed and images were generated using a high content imager. Clones that perform well in high priority applications are expanded to develop sustainable antibody source material.



## 5. Further screening of selected clone



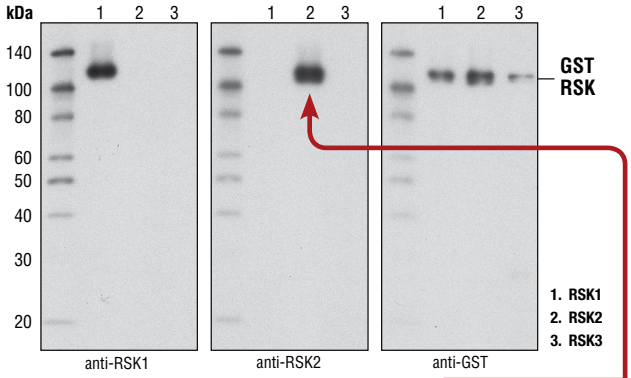

**WB analysis on extracts from multiple cell lines**: WB on lysates from multiple cell lines demonstrates species cross-reactivity.




**Peptide blocking experiments:** Adding epitope specific peptide (+) to the primary antibody solution blocks antibody binding to RSK2. This ensures that the WB signal is specific for RSK2 protein detection.

## 6. Confirming antibody specificity

Checking for cross-reactivity with other RSK family proteins.

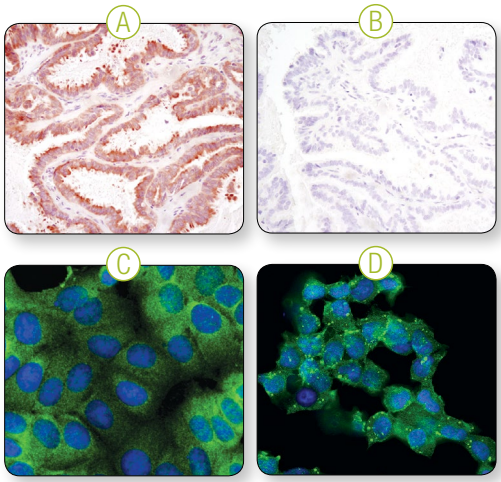



**RSK2 (D21B2) XP® Rabbit mAb #5528:** WB analysis of recombinant GST-tagged RSK1, RSK2, and RSK3 (2 µg each) using RSK1 (D6D5) Rabbit mAb #8408 (left), #5528 (center), or GST (91G1) Rabbit mAb #2625 (right). These WBs confirm that #5528 (center) is specific for RSK2 and #8408 (left) is specific for RSK1. The GST WB validates sample quality.

**Confirmation that RSK2 (D21B2) XP® Rabbit mAb #5528 is specific for RSK2 and does not cross react with RSK1 or RSK3.**

## 7. Final application validation

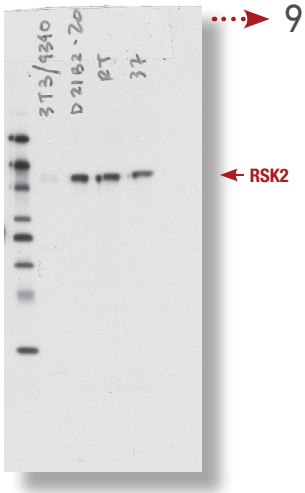
Final antibody material is tested for applications such as IHC and IF, and optimal dilutions are determined.




**RSK2 (D21B2) XP® Rabbit mAb #5528:** IHC analysis of paraffin-embedded human ovarian serous adenocarcinoma using #5528 in the presence of control peptide **(A)** or antigen-specific peptide **(B)**. Confocal IF analysis of MCF7 cells, untreated **(C)** or treated with sodium arsenite (500 µM, 1 hr) **(D)** using #5528 (green). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye). Localization of RSK2 to "stress granules" in arsenite-treated cells is very similar to that seen by Eisinger-Mathason et .al. (2008) *Molecular Cell*, 722–736.

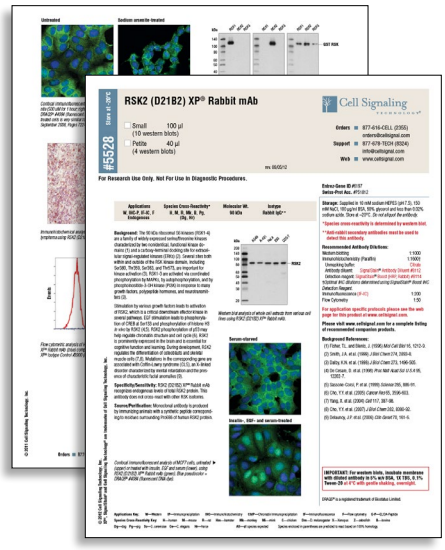
## 8. Shipping stability test

Cell extracts were analysed by WB using RSK2 antibody that had been stored at -20ºC, room temperature (RT), or 37ºC for 1 week. No deterioration of signal was seen when the antibody was stored at RT or 37ºC, so this antibody can be shipped at RT thereby reducing the environmental impact of shipping.




## 9. Ready for release

RSK2 (D21B2) XP® Rabbit mAb #5528 is released for sale with a technical datasheet containing specific dilution recommendations and species cross-reactivity.



# It's not you, it's your antibody.

**The accuracy of western blot results is dependent on the quality of the primary antibody used.**

## CST Validation experiments
### include testing in biologically relevant systems:

1. **Detection of Endogenous Protein Levels**
   Testing samples from cell lines and/or tissues that are known to either express or not express the protein of interest demonstrates that the antibodies are capable of detecting endogenous protein levels.

2. **Phospho-specificity**
   Antibody testing on lysates that are untreated or treated with phosphatase to remove phosphate groups confirms that the antibody is phospho-specific.

3. **Biologically Relevant Treatments**
   Testing samples from cell lines that are treated with growth factors, cytokines, or chemical activators or inhibitors to induce or inhibit target expression and/or modification confirms that the antibody recognizes its specific target protein or protein modification.

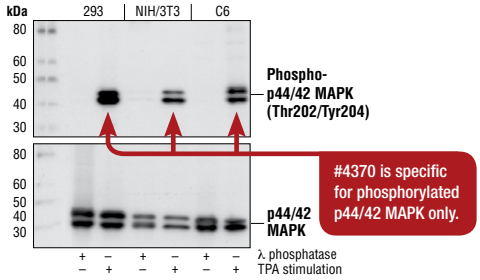
4. **siRNA Knockdown**
   Testing samples from cell lines transiently transfected with siRNA to knock down target protein expression demonstrates antibody target specificity.

5. **Lot-to-Lot Consistency**
   All new antibody lots are compared to previous lots of the same antibody in parallel experiments to ensure that antibodies perform consistently in continuing research.

6. **Immunoprecipitation validation**
   Mouse or rabbit immunoglobulin (IgG) conjugated to beads in the absence of primary antibody are used ensuring that non-specific binding to beads or the IgG would be detected.

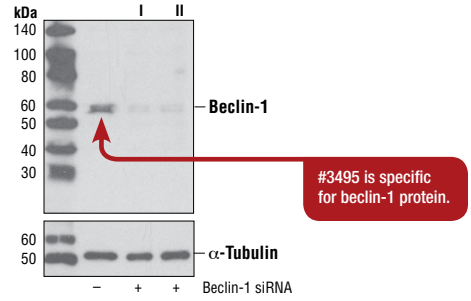
   A control sample of 10% lysate demonstrates lysate integrity and provides a point of reference to evaluate the ability of the antibody to enrich the target protein.

## Western Blot of phospho-specificity




**Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370:** WB analysis of extracts from 293, NIH/3T3, and C6 cells, treated with λ phosphatase (to inhibit phosphorylation) or TPA #4174 (to stimulate phosphorylation), using #4370 (upper), or p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695 (lower).

## siRNA knockdown to test specificity




**Beclin-1 (D40C5) XP® Rabbit mAb #3495:** WB analysis of extracts from HeLa cells, transfected with 100 nM SignalSilence® Control siRNA (Unconjugated) #6568 (-), SignalSilence® Beclin-1 siRNA I #6222 (+) or SignalSilence® Beclin-1 siRNA II #6246 (+), using #3495 (upper) or α-Tubulin (11H10) Rabbit mAb #2125 (lower). The Beclin-1 (D40C5) XP® Rabbit mAb confirms silencing of Beclin-1 expression, while the α-Tubulin (11H10) Rabbit mAb is used to control for protein loading.

# The Power of IP and co-IP

Immunoprecipitation (IP) and co-immunoprecipitation (co-IP) are widely used techniques for the enrichment of specific proteins and subsequent identification of protein-protein interactions and novel members of protein complexes. IP is also often used to enrich for a protein of interest for further experiments such as mass spectrometry or structure-function studies.

The CST IP protocol details the preparation of cell or tissue lysates under non-denaturing conditions, in the presence of protease and phosphatase inhibitors, to preserve the native state of both the target protein and associated proteins. The primary concern in an IP experiment is preventing binding of non-specific proteins to either the antibody or IP beads. Non-specific binding can be experimentally addressed by adding a lysate pre-clearing step prior to IP and by adding multiple washes following IP. The enrichment step can be performed using antibodies and beads in multiple formats and each type offers benefits as well as limitations. The immunoprecipitated protein is detached from the antibody-bead complex and denatured before analysis by western blot.

Successful IP and co-IP experiments require specific and sensitive antibodies to minimize enrichment of non-specific proteins. CST™ antibodies recommended for IP are validated in-house using CST companion reagents, including IP beads and buffers, to ensure reliable IP and co-IP data. Directly conjugated and biotinylated antibodies are also available, which offer convenience and decrease experimental running times. In the absence of IP-validated antibodies for a target, a protein can be engineered to carry a specific tag (eg. GST or Myc-Tag) that is recognized by a Tag-specific antibody or bead conjugated antibody validated for IP.

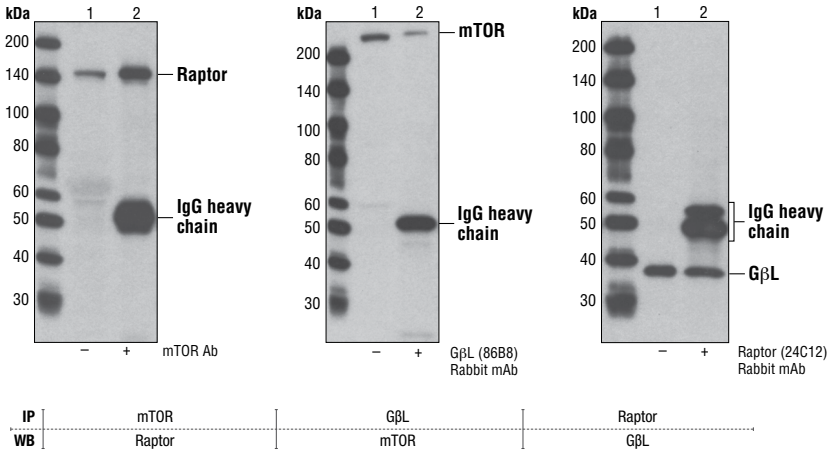
CST antibodies recommended for IP are validated in-house using CST companion reagents, including IP beads and buffers, to ensure reliable IP and co-IP data.

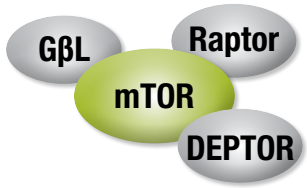
### Options for Primary Antibody:

- Unconjugated primary antibody followed by protein A or protein G coupled agarose, Sepharose®, or magnetic beads.
- Biotinylated primary antibody followed by streptavidin coupled beads.
- Primary antibody directly conjugated to Sepharose® or magnetic beads.

### IP Bead Formats:

- Agarose and Sepharose® beads have higher protein binding capacity, but require repeated centrifugation steps, which increases experimental time and can lead to loss of bead-bound protein.
- Magnetic beads are convenient because they decrease experimental time by using a magnetic rack to pellet beads. In addition, they are amenable to high throughput experiments.




**mTORC1 Complex**

co-IP of 3 proteins from the mTORC1 complex demonstrates the effectiveness of CST antibodies to mTOR, GβL, and Raptor. All data were generated using HeLa cell extracts and the antibodies indicated. Lane 1 is 10% input, lane 2 is the IP.

## Cell Signaling Technology Antibody Performance Guarantee

As a company of scientists, we understand how important it is to work with antibodies that are specific and consistent from lot-to-lot. To ensure product performance, we validate all of our antibodies in-house, in multiple research applications. If a product does not perform in your experiment as described on our website or datasheet, please contact your local CST office or nearest distributor within 12 months of product receipt. The same expert scientists who produced your antibody will guide you through a few simple troubleshooting steps and, if your issue is not resolved, we will replace the antibody at no cost to you, or provide you with a credit.

For complete terms and conditions, please visit **www.cellsignal.com/abguarantee**

# What does XP® mean for you?

## Exceptional performance monoclonal antibodies generated using XMT® technology.

**XMT technology provides access to a broad range of antibodies unattainable with conventional monoclonal technologies.**

XP monoclonal antibodies show superior performance in key applications, with utility across multiple applications. XP antibodies are rigorously validated for multiple applications so that the antibody can be used to assay protein expression or modification by the method most relevant to your research.

CST has successfully used XMT technology to develop high quality antibodies for challenging and clinically applicable targets, and continues to pursue targets of high value to the research community.

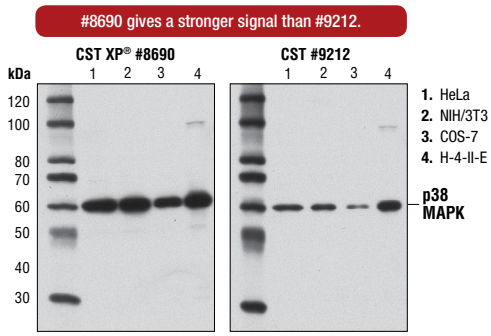
## p38 MAP Kinase antibody comparison

p38 MAP kinase plays an important role in the cellular response to cytokines and stress. It is activated by a variety of stresses including osmotic shock, inflammatory cytokines, lipopolysaccharide, UV light, and growth factors. Activated p38 phosphorylates other MAP kinases; transcription factors such as ATF-2, Max, and MEF2; and other substrates such as tau and keratin 8. Biological consequences of p38 activation include the regulation of processes such as inflammation, cell cycle, cell differentiation, development, senescence, and tumor suppression.

p38 MAPK (D13E1) XP® Rabbit mAb #8690 gives a stronger signal than p38 MAPK Antibody #9212. The XP antibody demonstrates a higher sensitivity, meaning that you can detect lower levels of protein expression by western blot and save valuable sample by running less material on your gels.

| APPLICATIONS | XP® #8690 | #9212 |
|---|---|---|
| Western blot | 1:1000 | 1:1000 |
| Immunohistochemistry | 1:400 | 1:200 |
| Immunofluorescence | 1:200 | 1:100 |
| Flow cytometry | 1:800 | 1:200 |

Recommended dilutions for CST™ antibodies #8690 and #9212 in validated applications.



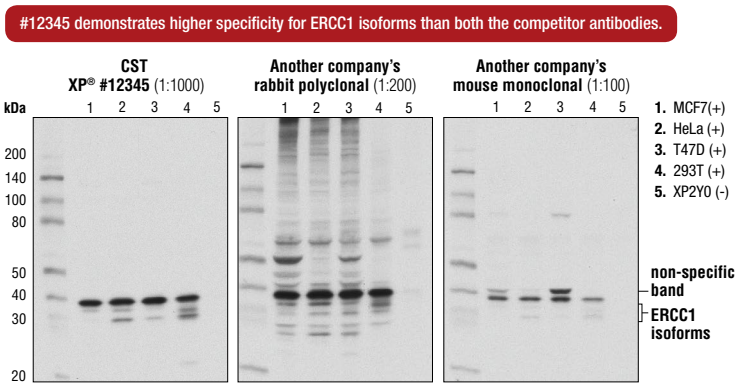

**p38 MAPK (D13E1) XP® Rabbit mAb #8690:** WB analysis of extracts from various cell lines using #8690 (left) or p38 MAPK Antibody #9212 (right). Both antibodies were diluted 1:1000, and the films were exposed for an equal length of time.

## Performance testing

At CST, we regularly test the performance of our antibodies against those from other providers. That is how we know our antibodies are among the best available.

Shown to the right is a comparison of CST ERCC1 (D6G6) XP® Rabbit mAb #12345 and two different competitor antibodies. Western blot analysis was performed using our optimized western blotting protocol and the other suppliers' recommended protocols, where available, ensuring that the antibody comparison testing was performed using optimal protocol conditions for each antibody.

CST ERCC1 (D6G6) XP® Rabbit mAb #12345 demonstrates higher specificity for ERCC1 isoforms than both the competitor antibodies and superior sensitivity to the target protein than the competitor mouse monoclonal antibody.




**ERCC1 (D6G6) XP® Rabbit mAb #12345:** WB analysis of extracts from various cell lines using ERCC1 antibodies including #12345 and other companies' rabbit polyclonal and mouse monoclonal antibodies.

# CST Scientists

Cell Signaling Technology is a company founded in 1999 by scientists who saw a need in the research community for high quality, reliable antibodies. CST product scientists maintain current and future antibody lots and are at the bench every day to better help customers solve their research challenges.

**Nicole, Product Scientist**
has been at CST since 2006.

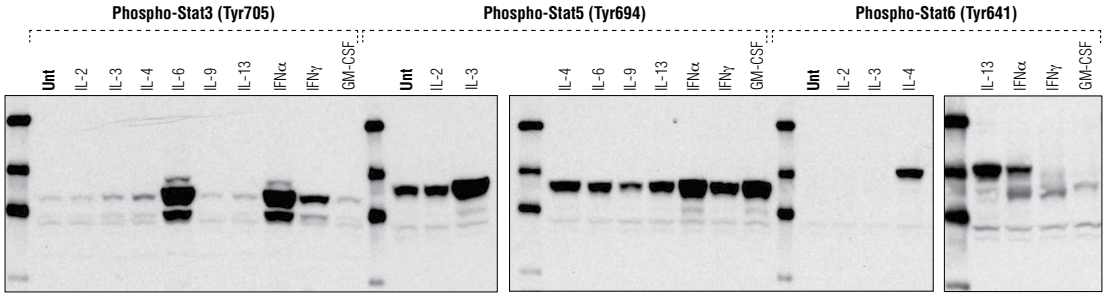
Our scientists take personal pride in their antibodies and are dedicated to developing the most comprehensive portfolio of high quality products.

"In order to provide the best support possible, I wanted to have experience working with the sample models that our customers are using. I don't feel that it is enough to work with one or two cell lines and one treatment. Rather, I need experience with multiple cell lines and multiple treatments. It helps me to help the customer better. For the Stats in particular, it was important to see how the different proteins respond to a wide variety of cytokines, especially since responses are highly dependent on cell type. The data is used as reference material for technical support, but also the numerous positive controls we generated have helped other departments at CST develop models for testing Stat antibodies in other applications."

*- Nicole, Production Scientist*

# Stat protein phosphorylation profiling

TF-1 cells were untreated (Unt) or treated with cytokines and growth factors known to induce Stat activity. WB analysis was performed with 3 different CST phospho-Stat antibodies. This data provides reference material for technical support as well as positive experimental controls.




**Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145:** Phosphorylation is induced by IL-6, IFNα, and IFNγ. | **Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb #9359:** High basal level of phosphorylation but further phosphorylation can be stimulated by IL-3, IFNα, and GM-CSF | **Phospho-Stat6 (Tyr641) Antibody #9361:** Phosphorylation is stimulated by IL-4, IL-13, and IFNα.

## Cell Signaling Technology Antibody Citations for Western Blotting

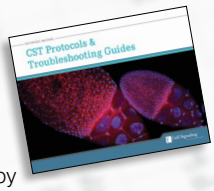
Scientists in the research community perform the ultimate validation of our products. CST antibodies are continually cited in top-tier journals covering a wide range of research areas. References are listed on individual product webpages.

# Western blotting tools for success

Information to support your western blotting experiment is available in our white paper "A Guide to Successful Western Blotting", available by request at:


**www.cellsignal.com/wbsuccess**.

We also provide you with the protocols that our scientists used to validate each antibody so you don't have to spend time on optimization. Request a copy of our Protocols & Troubleshooting Guide at: **www.cellsignal.com/protocolguide**




Watch our Western Blotting Protocol and Troubleshooting Guide videos to learn how small changes to the protocol can affect the final outcome of your blot. To see videos, go to: **www.cellsignal.com/wbvideos**

# What are Motif Antibodies?

**Motif specific antibodies are developed and validated to recognize proteins containing a particular post-translational modification in the context of a kinase substrate or binding motif.**

The activity of expressed proteins is often regulated by post-translational modification (PTM) of key amino acid residues. The function of an individual PTM may be to activate or inhibit signaling, block an active site, or target a protein for destruction. Motif specific antibodies are developed and validated to recognize a target protein containing a particular PTM in the context of a specific kinase substrate or binding motif. These motif antibodies are widely used to study substrate and biomarker deviation from basal phosphorylation, methylation, acetylation, or ubiquitination in response to stimuli. Motif antibodies are also used to assay for global signaling changes associated with disease progression.

## Commonly asked motif antibody questions

**Harry, Product Scientist**
has been at CST since 2006.

**"I see a band at a specific molecular weight on a western blot. How can I identify the protein?"**

This is a challenging question, as the band may be composed of one or more proteins. However, it is possible to use the PhosphoSitePlus® resource from CST to identify putative proteins within specific molecular weights. Once potential proteins are identified, target specific antibodies can be used to identify the protein of interest. Alternatively, you can utilize our PTMScan® products and technology for more extensive evaluation of your sample.

**"I have a protein with a specific sequence. Do you have a motif for this sequence?"**

Cell Signaling Technology (CST) product scientists have detailed knowledge of antibody cross-reactivity based on mass spectrometry data so we will be able to assist you in the selection of the optimal motif antibody.

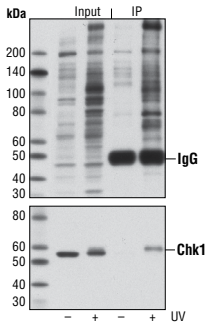
## Motif enrichment

A motif antibody may be used in conjunction with a total antibody to study a protein target that does not have a commercially available, site-specific phospho-antibody.

- The PhosphoSitePlus database from CST can be used to identify the sequence of a protein of interest so that an optimal motif antibody can be identified.
- Motif antibodies are used to enrich for target PTM proteins by IP prior to western blotting with a target specific antibody.

### Example of a motif specific antibody.

**Phospho-ATM/ATR Substrate (S*Q) (D23H2/D69H5) Rabbit mAb #9607:** IP of HeLa cell extracts, untreated (-) or UV-treated (+, 2 hr) (lanes 3 and 4), using #9607. 10% input is shown in lanes 1 and 2. WB analysis was performed using the same antibody (upper) and Chk1 (2G1D5) Mouse mAb #2360 (lower). These WBs show that multiple proteins are enriched by the S*Q motif antibody (upper) and one of these proteins is Chk1 (lower).




## PhosphoSitePlus®

PhosphoSitePlus, the online resource from CST, integrates encyclopedic information on experimentally determined protein modification sites, upstream and downstream regulation of these modifications, and powerful analytical tools for investigating the structural and biological significance of protein modifications. Many cutting-edge features make it the premier resource in protein modification research available today: expansive and continuously curated content; molecular renderings to visualize the location of modification sites; on-the-fly generation of kinase substrate sequence logos; browsing of high-throughput content by disease, cell line, and tissue; new search interfaces that retrieve modification sites and proteins by subcellular localization, sequence and motifs, domains, responsiveness to treatments, disease, and tissue or cell type.

**www.phosphosite.org**

# Our mission...

To deliver the world's highest quality research, diagnostic, and therapeutic products that accelerate biological understanding and enable personalized medicine.

Cell Signaling Technology, Corporate Headquarters, Danvers, Massachusetts

Cell Signaling TECHNOLOGY®

Cell Signaling Technology (CST) is a private, family-owned company, founded by scientists and dedicated to providing high quality research tools to the biomedical research community. Our employees operate worldwide from our U.S. headquarters in Massachusetts and our offices in the Netherlands, China, and Japan.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