20 Years of Antibody Validation at CST

Cell Signaling
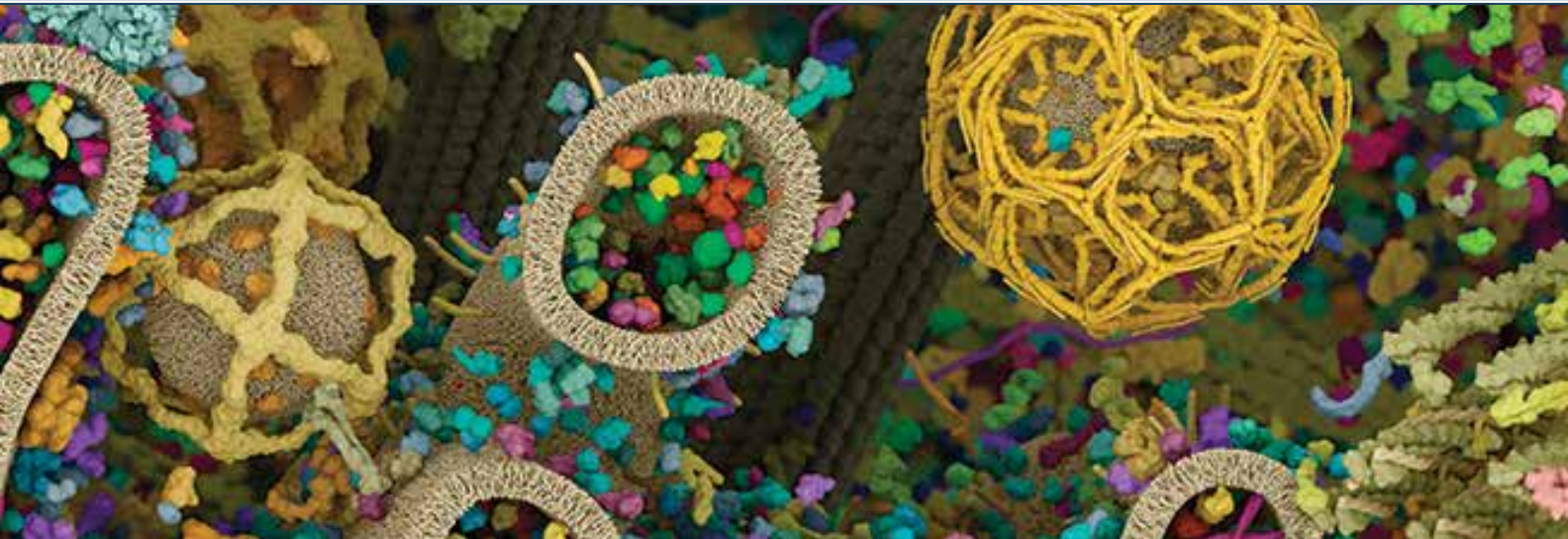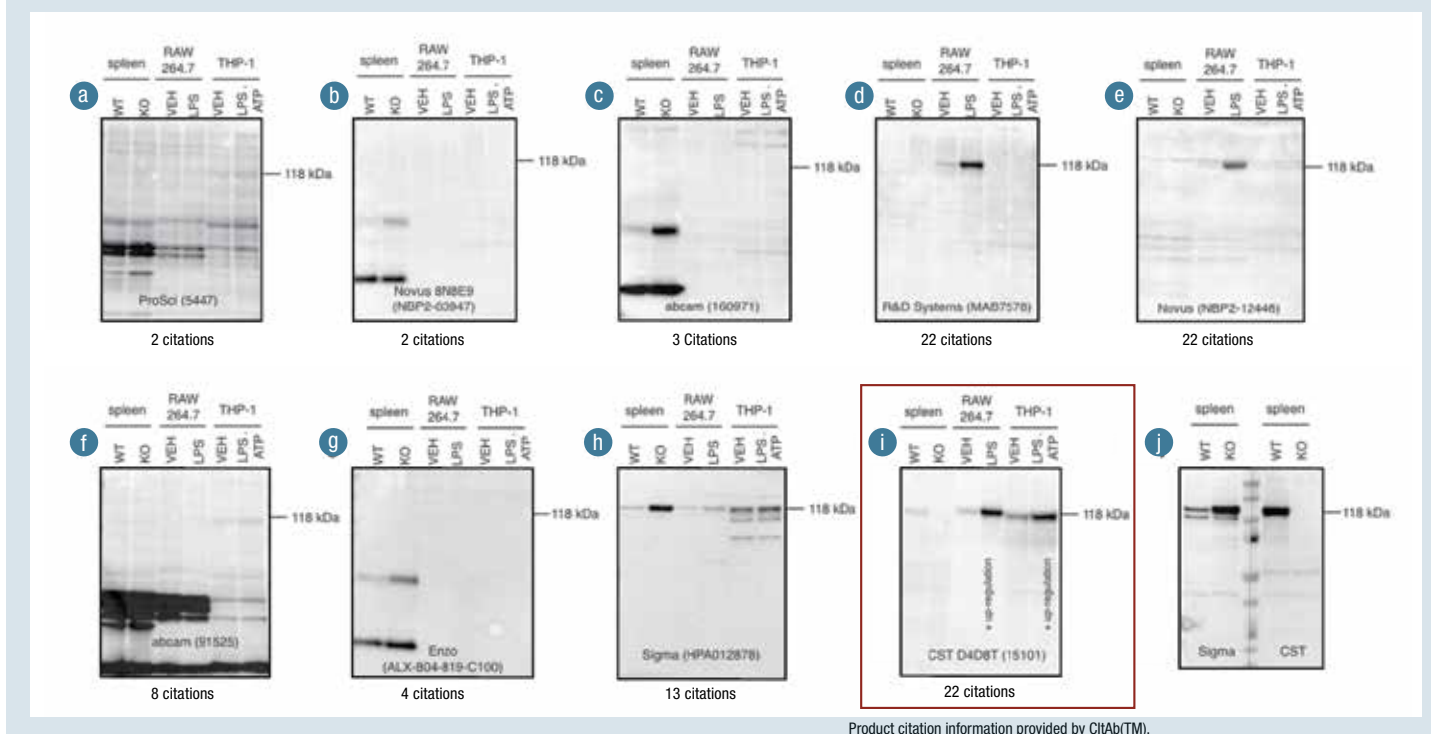TECHNOLOGY®

An Example of Using Unreliable, Poorly Validated Antibodies

A recent publication in *Scientific Reports* rigorously tested 9 commercially available antibodies and found that only one, from Cell Signaling Technology(CST), met all validation criteria. All of the tested antibodies were used in independently published research, and many presented contradictory conclusions about the mechanisms of macular degeneration.

Independent validation of the specificity of commercially available anti-NLRP3 antibodies by western blotting

Product citation information provided by CItAb(TM).

	Manufacturer	Reactivity	Host	[antibody]	Catalogue #	Lot #
anti-NLRP3	Cell Signaling Technologies	mouse, human	rabbit	WB 1:1000	D4D8T, 15101	3
	ProSci	mouse, human	rabbit	WB 1:1000	5447	6769-1204
	Novus Biologicals	mouse, human	mouse	WB 1:1000	8N8E9, NBP2-03947	-
	Novus Biologicals	mouse, human	rabbit	WB 1:400	NBP2-12446	080639650-14
	abcam	human	mouse	WB 1:1000	ab160971	GR228391-15
	R&D Systems	mouse	rat	WB 1:250	MAB7578	-
	abcam	mouse, human	rabbit	WB 1:500	ab91525	GR62279-6
	Enzo Life Sciences	human	mouse	WB 1:100	ALX-804-818-C100	11051424
	Sigma	human	rat	WB 1:1000	HPA012878	D106015

Images are licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Kosmidou C, Efstathiou NE, Hoang MN, Notomi S, et al. Issues with the Specificity of Immunological Reagents for NLRP3: Implications for age-related macular degeneration. Page 4-7. Sci Rep. 2018;8(1):461.

Validating the specificity of commercially available anti-NLRP3 antibodies by western blotting.

Nine commercially available anti-NLRP3 antibodies were tested in terms of their specificity against murine and human positive controls, including mouse spleen tissue, murine RAW 264.7 and human THP-1 macrophage cell lines. Antibody specificity was validated by testing protein expression in spleen tissue from Nlrp3 knockout mice as a negative control. RAW 264.7 cells were primed with (LPS 10 ng/mL) for 6 hours and were compared to vehicle untreated cells. THP-1 macrophages were primed with LPS (10 µg/mL) plus ATP (5 mM) for 3 hours and compared to vehicle control THP-1. 50 µg of total protein were loaded on a gel and blotted with anti-NLRP3 antibodies with the expected molecular weight at ~118 kDa

CST Quality Principles

Antibody validation is not a one-size-fits-all process. Our teams of scientists perform custom analysis of each antibody using the most biologically relevant models and assays, and we offer optimized protocols and expert technical support to ensure that our antibodies will work for your research.

☑ Specificity

- **Binary Models:** Analysis of panels of cell lines with high, medium, low, or no protein target expression levels including all relevant controls.
- **PTM-specificity:** Post-translational modification confirmation using appropriate kinase-specific activators, inhibitors, and other treatments (phosphatase, acetylase, PNGase, etc.). Peptide arrays transient expression of site-specific mutants to confirm site-specificity and effects of nearby PTM's on antibody specificity.
- **Genetic Inactivation:** Specificity confirmed with siRNA knockdown, knockout models, and other tools
- **Biologically Relevant Treatments:** Testing cell lines treated with growth factors, cytokines, or chemical activators/inhibitors to knowingly modify target expression, localization, posttranslational modification, etc.
- **On-and-Off Target Binding:** Analysis of multiple normal and diseased tissues to assess performance in a broad spectrum of tissues.

☑ Sensitivity

- Detection of endogenous protein levels across a wide array of cell- and tissue-based samples to verify reactivity and sensitivity.
- Optimized application validation including testing multiple protocols (buffers, conditions, etc.) and application-specific formulation.
- Titration to determine the optimal working concentration in each assay
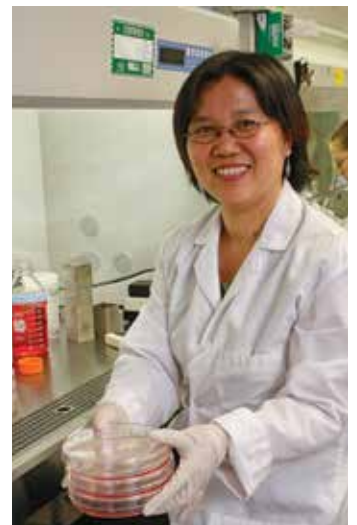
☑ Consistency

- Lot-to-lot validation (compare new lot to old lot) to ensure consistent performance.
- Recombinant monoclonal antibody technology allows highly controlled, reproducible production of each lot.
- Annual quality control testing for all products.
- Every new product is tested and verified by at least three different internal teams to ensure performance across multiple samples, users, and experiments.
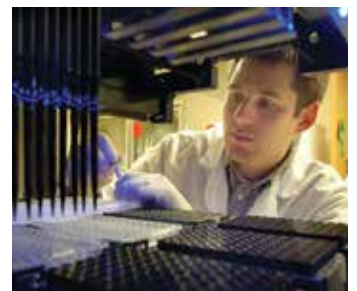
☑ Methodology

- Product-specific optimized protocols
- Recommendations for optimal dilutions and buffers

☑ Support

- Top-ranked global technical support
- The scientists who validated the antibody will help make sure it works in your lab
- Ongoing product testing and validation in new applications based on customer feedback and recommendations.

Only the Best Survive the CST Validation Process

1 Antigen Design

2 Antibody Development and Validation

Tests performed for each application

IHC	ChIP	Flow	IF	WB/IP	Application
IHC	ChIP	Flow	IF	WB/IP	Genetic models: knockout-derived tissues/cell lines, CRISPR
IHC	ChIP	Flow	IF	WB/IP	Endogenous binary models: positive vs negative expressing cells/tissues
IHC	ChIP	Flow	IF	WB/IP	Knock-down strategies, siRNA/shRNA
IHC	ChIP	Flow	IF	WB/IP	Testing across multiple tissues or cell lines with varied expression levels
IHC	ChIP	Flow	IF	WB/IP	Independent antibody verification using antibodies against the same target but a different antigen
IHC	ChIP	Flow	IF	WB/IP	Comparison to published data
IHC	ChIP	Flow	IF	WB/IP	Controls: Isotype/secondary, loading, quality, etc.
IHC	ChIP	Flow	IF	WB/IP	Multiple rounds of independent testing to verify consistency and reproducibility
IHC	ChIP	Flow	IF	WB/IP	Overexpression of target protein
IHC		Flow	IF	WB/IP	Tissue arrays and rodent tissues
	ChIP	Flow	IF	WB/IP	Primary cell lines/differentiated stem cells
IHC	ChIP		IF	WB/IP	Treatment induced up- or down-regulation of expression, PTM's and/or changes in localization
IHC		Flow	IF	WB/IP	Phosphatase treatment
IHC			IF	WB/IP	Animal models of disease
IHC			IF	WB/IP	Cell-cycle-dependent expression, localization
IHC			IF	WB/IP	Peptide blocking
IHC			IF		Xenografts (drug-treated and control)
IHC				WB/IP	Human normal and tumor tissues
	ChIP			WB/IP	Peptide ELISA, peptide arrays and dot blot
				WB/IP	Recombinant proteins
	ChIP				Positive vs. Negative loci
	ChIP				Independent complex subunit verification

Every CST antibody undergoes rigorous application-specific testing customized according to the target and needs of the individual antibody. Listed is a sampling of the methods we employ to ensure our antibodies perform as expected.

We select applications for testing based on the biological function and relevance of the target. During the validation process several antibodies against the same target may be tested - only the best performing, most reproducible antibodies make it in to our catalog.
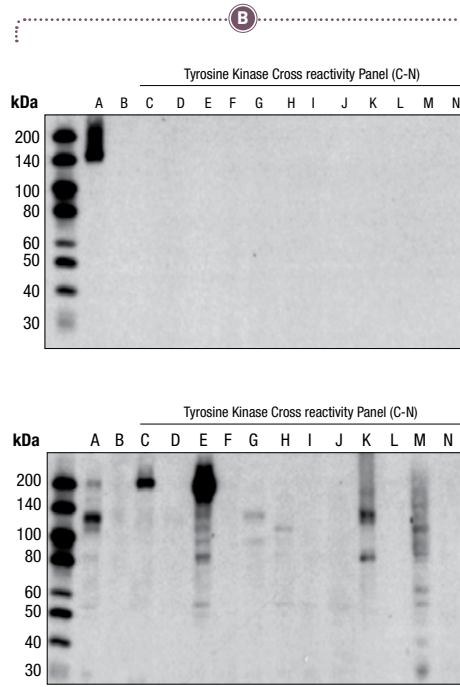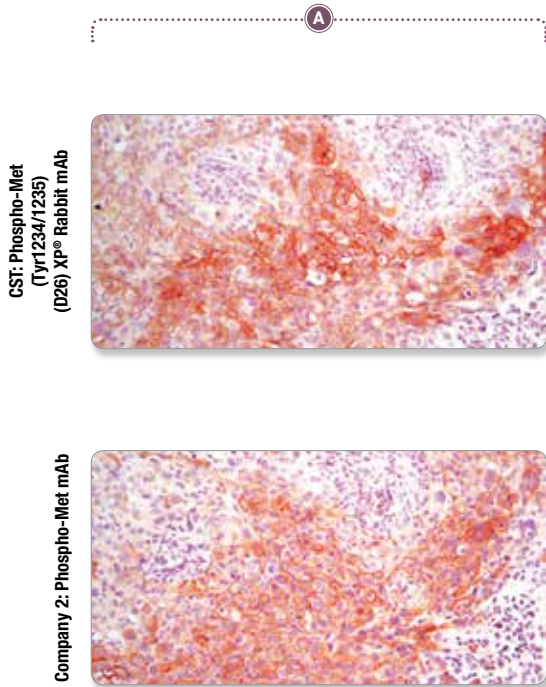
⑤

CST Validated Antibody

④

Stability Testing
and
Packaging Decisions

③

Independent QC
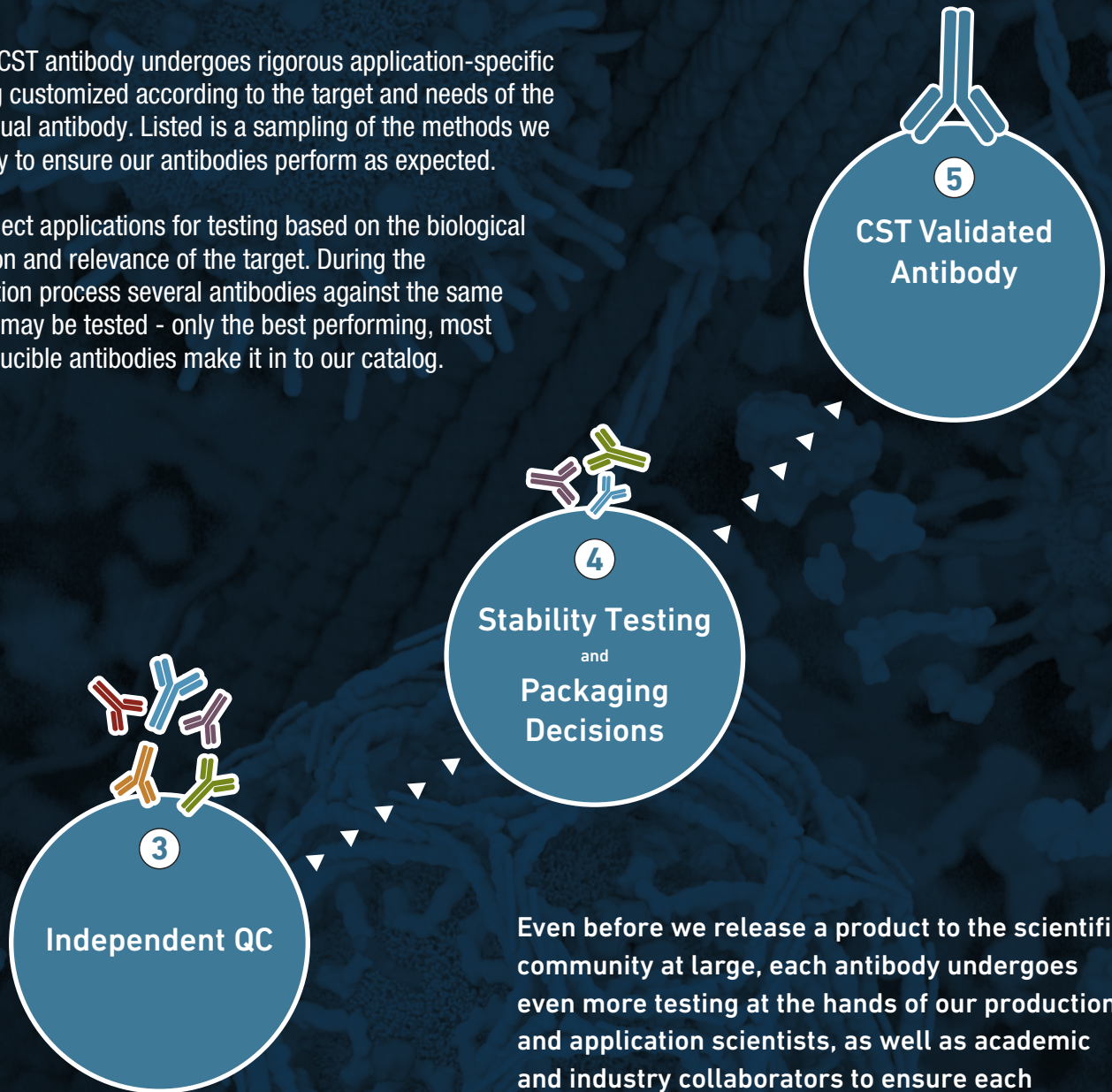
Even before we release a product to the scientific community at large, each antibody undergoes even more testing at the hands of our production and application scientists, as well as academic and industry collaborators to ensure each product is specific, functional and optimized for each approved application.

IHC

Is your antibody specific?

While an antibody may "look" like it is working appropriately, this is not always the case. In most instances, validation of the antibody in more than one application is required to prove specificity. Antibodies against phospho-MET (Tyr234/1235) were used to stain tissues (**A**) and while both look equivalent, western blot (**B**) shows that only the CST antibody (#3077) specifically detects phospho-MET while the other antibody broadly reacts with other RTKs, a common problem with antibodies against phospho-tyrosine residues.

A / **B**

CST: Phospho-Met (Tyr1234/1235) (D26) XP® Rabbit mAb

Company 2: Phospho-Met mAb

Tyrosine Kinase Cross reactivity Panel (C–N)

kDa: 200, 140, 100, 80, 60, 50, 40, 30

A B C D E F G H I J K L M N

Tyrosine Kinase Cross reactivity Panel
Cell Lines and Treatments

A. A431 + HGF
B. A431 - HGF
C. NIH/3T3 + PDGF
D. NIH/3T3 - PDGF
E. A431 + EGF
F. COS/FGFR1
G. CHO/IRS1,IR + Insulin
H. K562
I. SUPM2
J. L540
K. NIH/3T3/src
L. GST-Tie-2
M. SEM
N. H526+SCF

Phospho-Met (Tyr1234/1235) (D26) XP® Rabbit mAb #3077: Comparison of CST #3077 (top) and another company's product (Company 2 mAb, bottom) on HCC827 xenograft (**A**) gives the appearance of specific staining for both products. Western blot analysis of various cell lines and treatments using #3077 and the Company 2 antibody show a single band at 145 kDa in HGF-stimulated, but not unstimulated, A-431 cells (**B**). #3077 demonstrated no cross reactivity in control extracts treated with growth factors that activate other RTKs or that overexpress other RTKs or cytoplasmic tyrosine kinases (**top**). In contrast, the Company 2 phospho-Met antibody recognized several nonspecfic bands in these control experiments (**bottom**). Both membranes were developed on the same film with the same exposure time (10 sec).
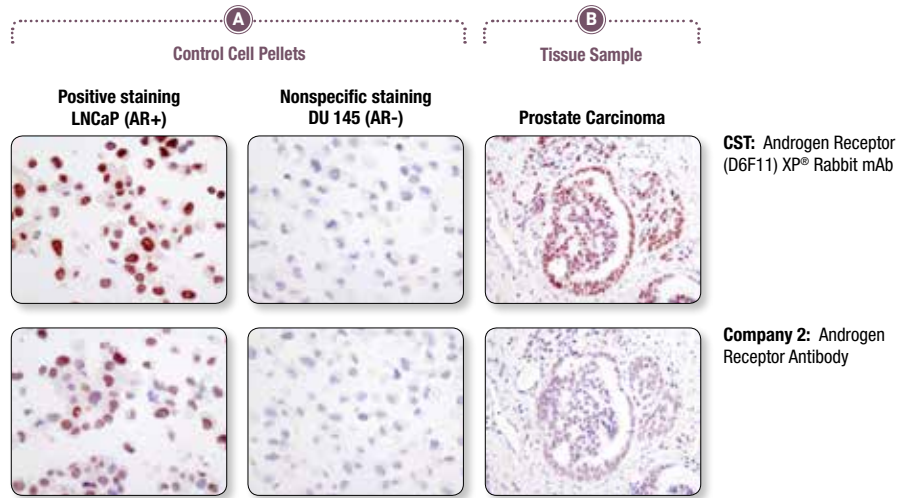
Is your antibody as sensitive as you need?

CST's validation process includes testing each antibody at a range of dilutions in positive and negative tissues and cell lines to determine the optimal working concentration for each assay. This is critical to ensure optimal sensitivity and signal to noise ratio.

Androgen Receptor (D6F11) XP® Rabbit mAb #5153: IHC analysis of #5153 was compared to another company's IHC-approved androgen receptor mouse monoclonal antibody. The optimal dilution of each antibody was individually evaluated to optimize androgen receptor (AR) staining in AR-expressing LNCaP cells and minimize nonspecific staining in AR null DU 145 cells **(A)**. The determined optimal dilution for each antibody was utilized in IHC analysis of paraffin-embedded human prostate carcinoma **(B)**.

A Control Cell Pellets   **B** Tissue Sample

Positive staining LNCaP (AR+)   Nonspecific staining DU 145 (AR-)   Prostate Carcinoma

CST: Androgen Receptor (D6F11) XP® Rabbit mAb

Company 2: Androgen Receptor Antibody

Is your antibody supported by optimized reagents and protocols?

Ensure results are reproducible in your lab and others by using CST protocols and companion reagents that are optimized to work with your antibodies and targets.
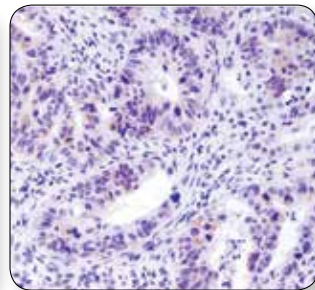
When **PLK1 (208G4) Rabbit mAb #4513** was first released several years ago, our IHC group could not recommend its use for IHC based on its lack of staining. At that time, the standard reagents for IHC analysis were those listed below.
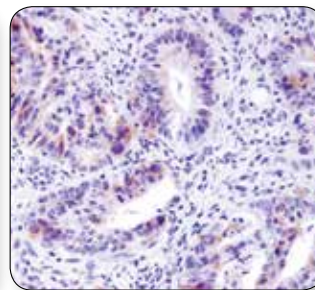
With the incorporation of **SignalStain® Antibody Diluent #8112**, we observed a slightly improved signal compared with our original conditions.

Changing the detection system to **SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114** further improved the signal. However, based on our rigorous standards, even a signal at this level did not warrant an IHC recommendation for this antibody.

Finally, when we changed the chromogen to **SignalStain® DAB Substrate Kit #8059**, we were able to produce a robust signal using this antibody, thus warranting an IHC recommendation. All changes in staining were achieved without altering the original antibody dilution.

Diluent: TBST/5% NGS
Detection: biotin-based
Chromogen: NovaRed™

Diluent: #8112
Detection: biotin-based
Chromogen: NovaRed™

Diluent: #8112
Detection: #8114
Chromogen: NovaRed™

Diluent: #8112
Detection: #8114
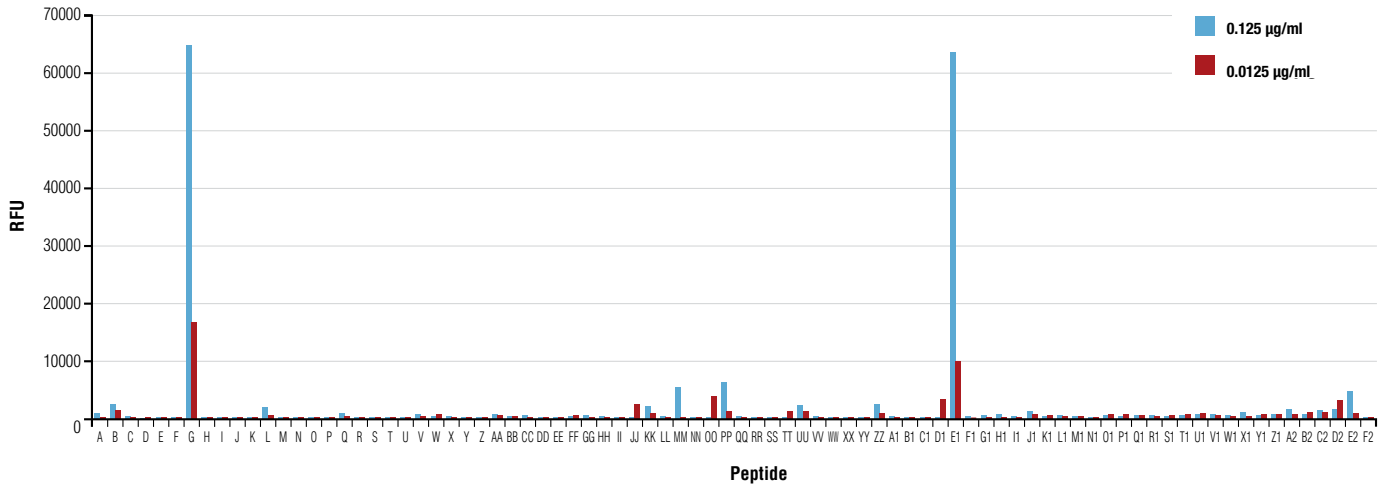**Chromogen:** #8059
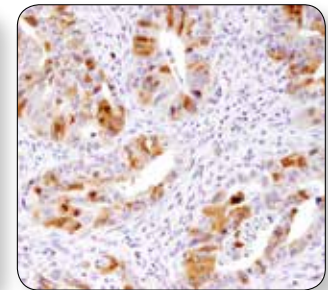
Optimized protocol

PLK1 (208G4) Rabbit mAb #4513: IHC analysis of paraffin-embedded human colon carcinoma using #4513 and various IHC reagents, as indicated

All improvements in staining were achieved solely by replacing reagents, without altering the original antibody dilution, demonstrating the impact of diluent, detection, and chromogen reagents on your IHC results.

ChIP

Is your antibody specific?

Antibodies targeted to histone modifications may bind non-specifically to similar, but off-target histone modifications. Conversely, their specific binding can be inhibited by steric hindrance due to modifications on neighboring residues. Validating histone modification antibodies with peptide arrays confirms specificity as well as the effects of neighboring modifications on the ability of the antibody to detect a single modification site.


A	H3 (Lys4) non-methyl	V	H4 (Lys20) mono-methyl	QQ	H3 (Lys9) tri-methyl/(Ser10) phospho	L1	H1.4 (Lys26) mono-methyl
B	H3 (Lys4) mono-methyl	W	H4 (Lys20) di-methyl	RR	H3 (Arg26) asymmetric-di-methyl/(Lys27) mono-methyl	M1	H1.4 (Lys26) di-methyl
C	H3 (Lys4) di-methyl	X	H4 (Lys20) tri-methyl	SS	H3 (Arg26) asymmetric-di-methyl/(Lys27) di-methyl	N1	H1.4 (Lys26) tri-methyl
D	H3 (Lys4) tri-methyl	Y	H2A (Lys5) non-methyl	TT	H3 (Arg26) asymmetric-di-methyl/(Lys27) tri-methyl	O1	H1.4 (Lys26) mono-methyl/(Ser27) phospho
E	H3 (Lys9) non-methyl	Z	H2A (Lys5) mono-methyl	UU	H3 (Lys27) mono-methyl/(Ser28) phospho	P1	H1.4 (Lys26) di-methyl/(Ser27) phospho
F	H3 (Lys9) mono-methyl	AA	H2A (Lys5) di-methyl	VV	H3 (Lys27) di-methyl/(Ser28) phospho	Q1	H1.4 (Lys26) tri-methyl/(Ser27) phospho
G	H3 (Lys9) di-methyl	BB	H2A (Lys5) tri-methyl	WW	H3 (Lys27) tri-methyl/(Ser28) phospho	R1	H2B (Lys5/Lys12/Lys15/Lys20)
H	H3 (Lys9) tri-methyl	CC	H3 (Thr3) phospho/ (Lys4) mono-methyl	XX	H3 (Lys9) mono-methyl/(Ser10/Thr11) phospho	S1	H2B (Lys5) mono-methyl
I	H3 (Lys27) non-methyl	DD	H3 (Thr3) phospho/ (Lys4) di-methyl	YY	H3 (Lys9) di-methyl/(Ser10/Thr11) phospho	T1	H2B (Lys5) di-methyl
J	H3 (Lys27) mono-methyl	EE	H3 (Thr3) phospho/ (Lys4) tri-methyl	ZZ	H3 (Lys9) tri-methyl/(Ser10/Thr11) phospho	U1	H2B (Lys5) tri-methyl
K	H3 (Lys27) di-methyl	FF	H3 (Arg2) symmetric-di-methyl/(Lys4)mono-methyl	A1	H3 (Lys4) mono-methyl/(Thr6) phospho	V1	H4 (Lys5/Lys8/Lys12/Lys16)
L	H3 (Lys27) tri-methyl	GG	H3 (Arg2) symmetric-di-methyl/(Lys4) di-methyl	B1	H3 (Lys4) di-methyl/(Thr6) phospho	W1	H4 (Lys5) mono-methyl
M	H3 (Lys36) non-methyl	HH	H3 (Arg2) symmetric-di-methyl/(Lys4) tri-methyl	C1	H3 (Lys4) tri-methyl/(Thr6) phospho	X1	H4 (Lys5) di-methyl
N	H3 (Lys36) mono-methyl	II	H3 (Arg2) asymmetric-di-methyl/(Lys4) mono-methyl	D1	H3 (Thr6) phospho/(Lys9) mono-methyl	Y1	H4 (Lys5) tri-methyl
O	H3 (Lys36) di-methyl	JJ	H3 (Arg2) asymmetric-di-methyl/(Lys4) di-methyl	E1	H3 (Thr6) phospho/(Lys9) di-methyl	Z1	H4 (Arg3) asymmetric-di-methyl/(Lys5) mono-methyl
P	H3 (Lys36) tri-methyl	KK	H3 (Arg2) asymmetric-di-methyl/(Lys4) tri-methyl	F1	H3 (Thr6) phospho/(Lys9) tri-methyl	A2	H4 (Arg3) asymmetric-di-methyl/(Lys5) di-methyl
Q	H3 (Lys79) non-methyl	LL	H3 (Arg8) symmetric-di-methyl/(Lys9) mono-methyl	G1	H3 (Lys56) non-methyl	B2	H4 (Arg3) asymmetric-di-methyl/(Lys5) tri-methyl
R	H3 (Lys79) mono-methyl	MM	H3 (Arg8) symmetric-di-methyl/(Lys9) di-methyl	H1	H3 (Lys56) mono-methyl	C2	H4 (Arg3) symmetric-di-methyl/(Lys5) mono-methyl
S	H3 (Lys79) di-methyl	NN	H3 (Arg8) symmetric-di-methyl/(Lys9) tri-methyl	I1	H3 (Lys56) di-methyl	D2	H4 (Arg3) symmetric-di-methyl/(Lys5) di-methyl
T	H3 (Lys79) tri-methyl	OO	H3 (Lys9) mono-methyl/(Ser10) phospho	J1	H3 (Lys56) tri-methyl	E2	H4 (Arg3) symmetric-di-methyl/(Lys5) tri-methyl
U	H4 (Lys20) non-methyl	PP	H3 (Lys9) di-methyl/(Ser10) phospho	K1	H1.4 (Lys26)	F2	H3 (Lys9) non-methyl

Is your antibody as sensitive as you need?

ChIP was performed with cross linked chromatin from 4 x 10^6 HeLa cells and either 1 µg of CTCF (D31H2) XP$^®$ Rabbit mAb #3418 or 1 µg of Competitor Polyclonal Antibody, using SimpleChIP$^®$ Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared from 5 ng enriched ChIP DNA using NEBNext$^®$ Ultra™ II DNA Library Prep Kit for Illumina$^®$ and sequenced on the Illumina NextSeq. The CST recombinant rabbit mAb provides higher signal and lower background than the Competitor Polyclonal Antibody in both whole genome analysis (**A**) and localized gene analysis (**B**). ChIP-validated antibodies are tested for sensitivity, which allows for analysis of low-abundance, low-stability interactions.
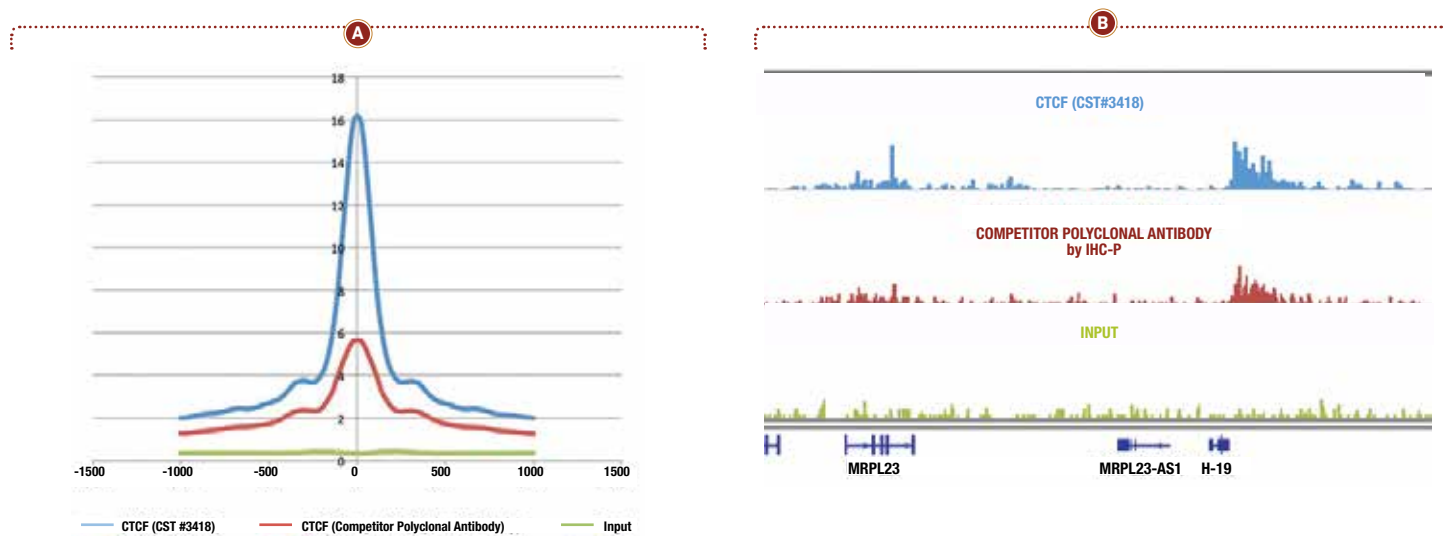
Is your antibody supported by optimized reagents and protocols?

SimpleChIP$^®$ Plus Chromatin IP Kits

In this example, Chromatin immunoprecipitations were performed with cross linked chromatin from 4 x 106 HCT 116 cells and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using SimpleChIP$^®$ Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared from 50, 5, or 0.5 ng enriched ChIP DNA or 50 ng Input DNA using SimpleChIP$^®$ ChIP-seq Multiplex Oligos for Illumina$^®$ (Dual Index Primers) #47538, pooled into one sample, and sequenced on an Illumina$^®$ Next-Seq platform. The figure shows binding across GAPDH, a known target gene of H3K4me3. Using reagents that protect chromatin integrity and ensure assay reliability.

All of our ChIP-validated antibodies are titrated in the ChIP assay to determine their optimal dilution factor. This information is provided with each antibody along with our optimized protocols, so you will have the information you need to plan successful experiments.
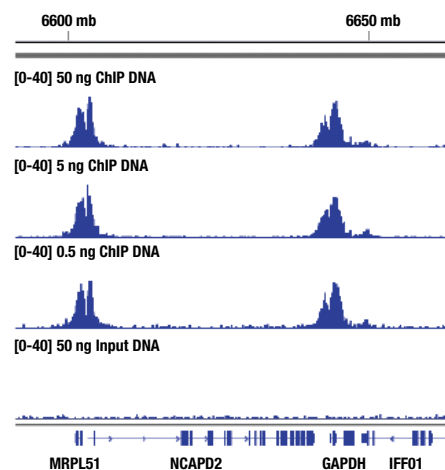
Flow

Is your antibody specific?

Btk (D3H5) Rabbit mAb #8547 selectively identifies B cells from human whole blood.
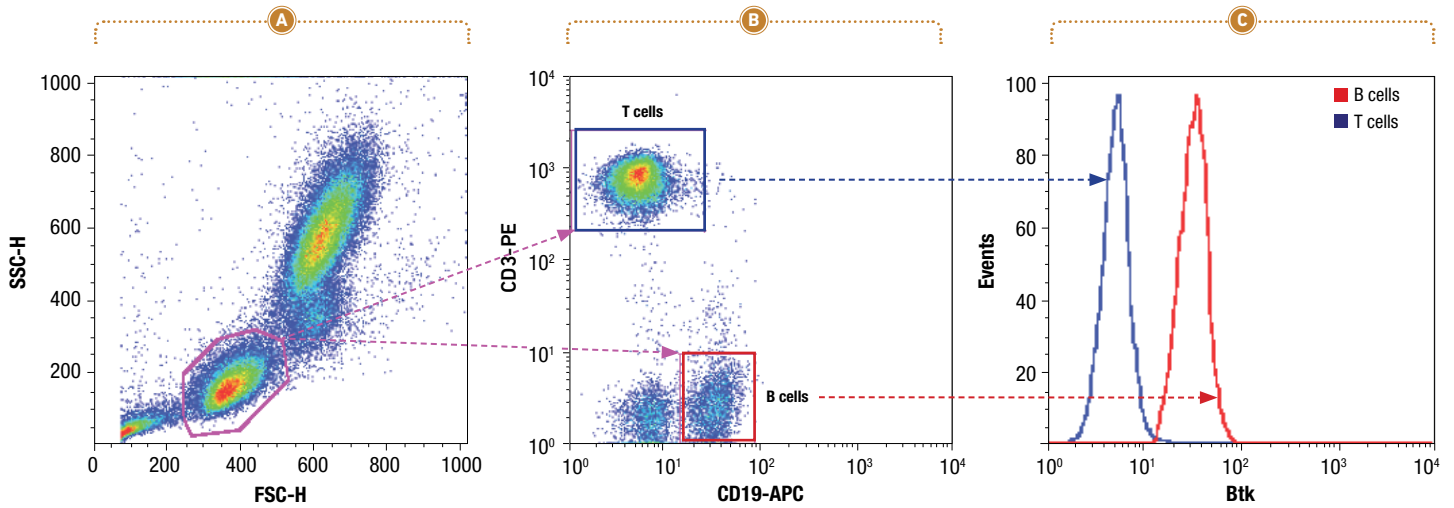

Btk (D3H5) Rabbit mAb #8547: Human whole blood was fixed, lysed, and permeabilized as per the CST Flow Alternate Protocol and stained using #8547. Cells were gated as shown in (**A**). Samples were co-stained using CD3-PE and CD19-APC to distinguish T and B cell subpopulations, respectively (**B**). B (red) and T (blue) cell population gates were applied to a histogram depicting the mean fluorescence intensity of Btk (**C**). Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate) #4412 was used as a secondary antibody.

Validation across multiple applications

When comparing CST's Phospho-STAT5 (Tyr694) (D47E7) XP® Rabbit mAb with one from another competitor, our flow team found that the competitor antibody yielded much more robust signal (A). However, upon further investigation, we found that the Phospho-STAT5 antibody from the other supplier demonstrated significant cross-reactivity with RTK's by IF/IC (B) and western blot (not shown). This illustrates the importance of using multiple methods to validate the specificity of any antibody.

By flow cytometry analysis alone, both antibodies show appropriate signal
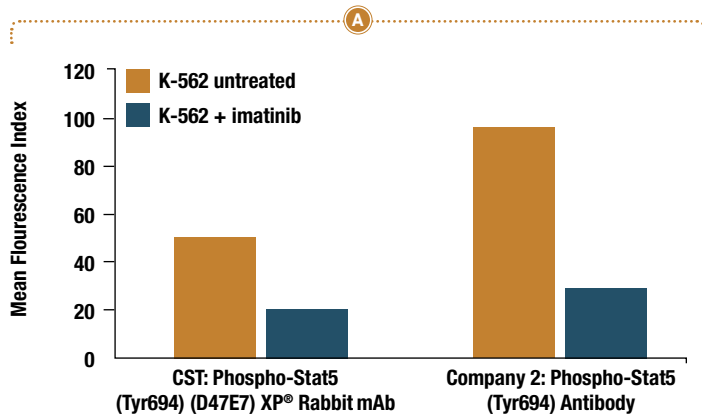
Additional IF testing proves only CST antibody is specific with appropriate cellular staining
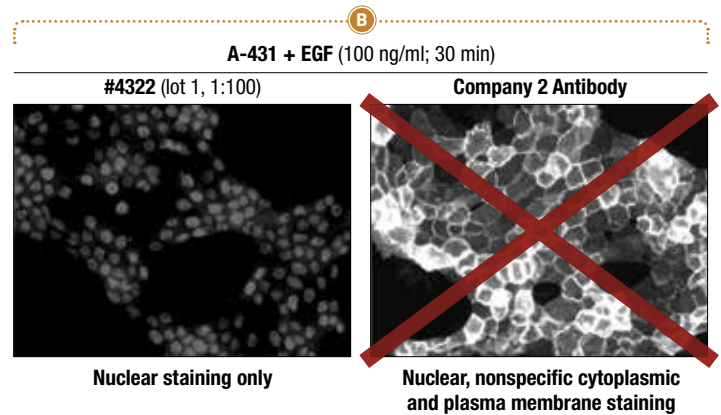
Flow cytometric analysis suggests a brighter signal from another company's Phospho-Stat5 (Tyr694) antibody compared to a lower fold induction with Phospho-Stat5 (Tyr694) (D47E7) XP® Rabbit mAb #4322 (**A**). However, immunofluorescent analysis reveals that the competitor antibody inappropriately stains the cytoplasm and plasma membrane, while #4322 demonstrates only the appropriate nuclear staining (**B**).
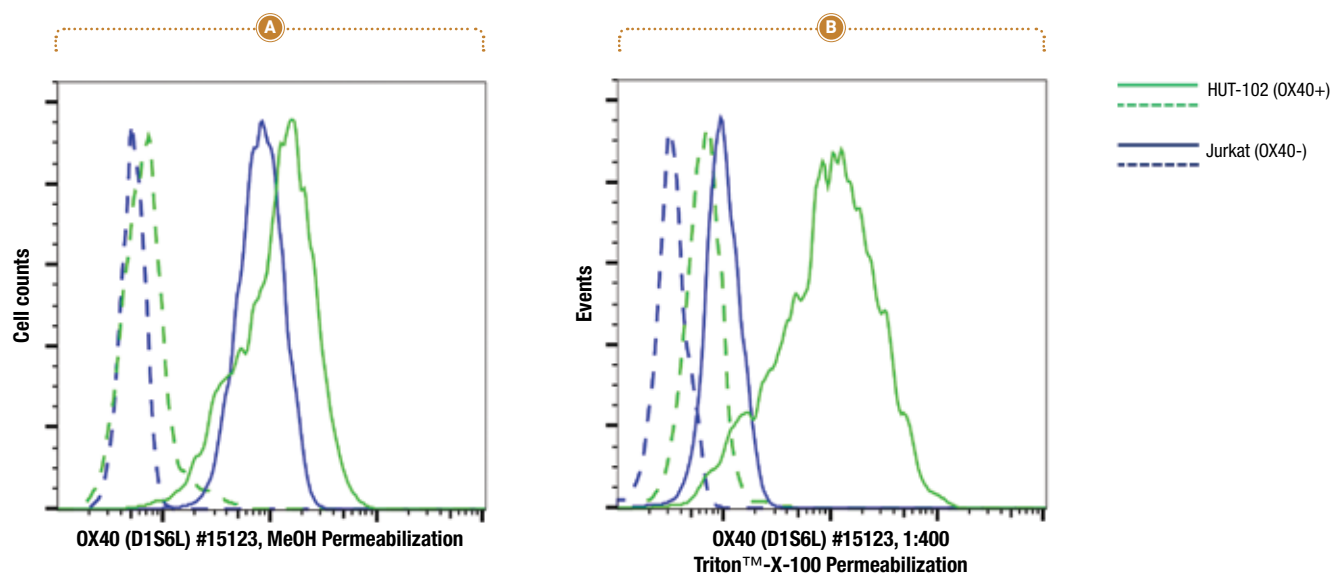
# Is your antibody supported by optimized reagent and protocols?

As part of validation, CST determines the optimal dilution and staining protocols for each antibody and provides this information to you.

Protocol optimization for antibody performance

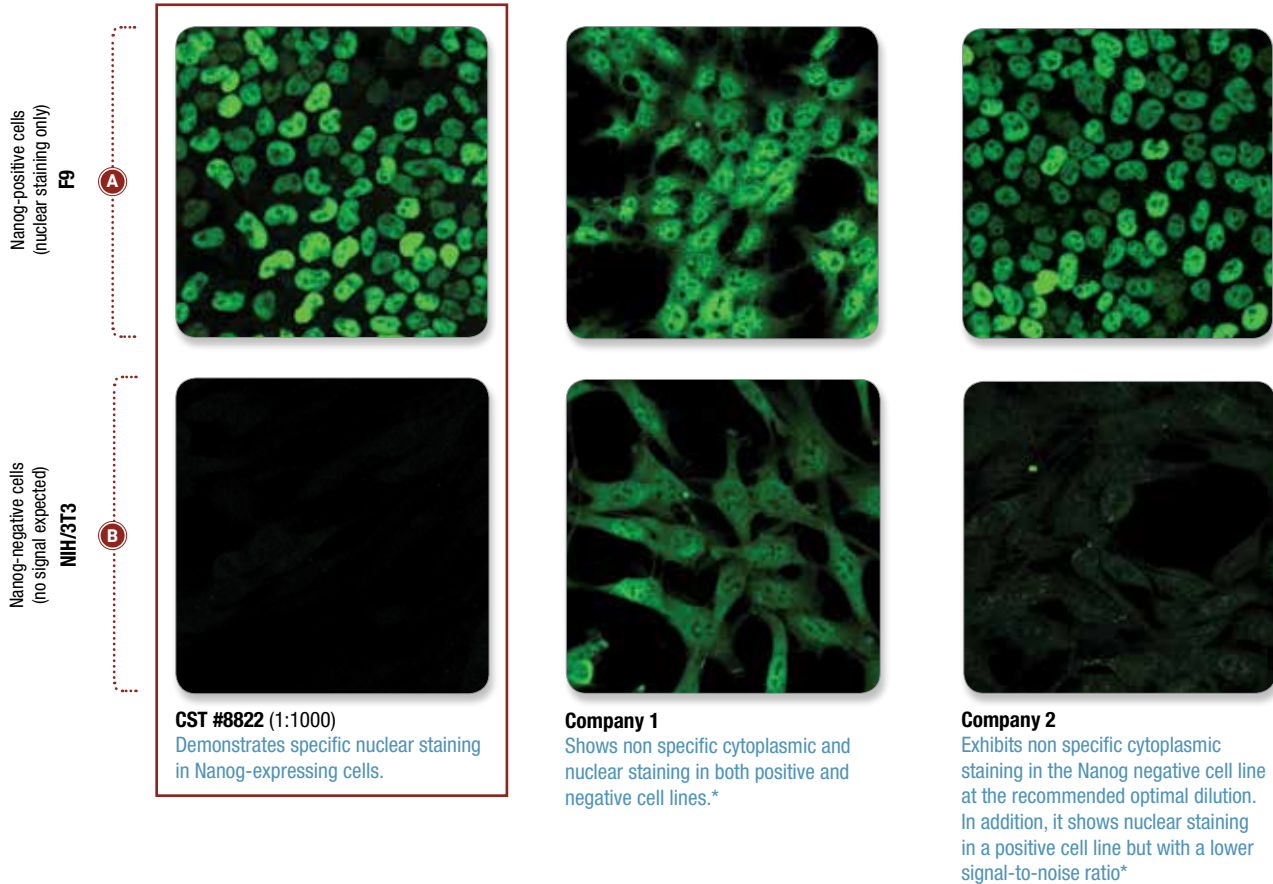Multiple protocol variables are tested to maximize signal-to-noise ratio.

OX40 (D1S6L) #15123, MeOH Permeabilization

OX40 (D1S6L) #15123, 1:400 Triton™-X-100 Permeabilization

— HUT-102 (OX40+)
-- Jurkat (OX40-)

When using methanol permeabilization, OX40 (D1S6L) Rabbit mAb #15123, exhibits nonspecific binding in Jurkat cells (0x40 negative) as compared to HUT-102 (0x40 positive) (**A**). However, nonspecific binding is significantly reduced when Triton™ X-100 is used for permeabilization, generating a much higher S/N ratio for the same concentration of the antibody (**B**).
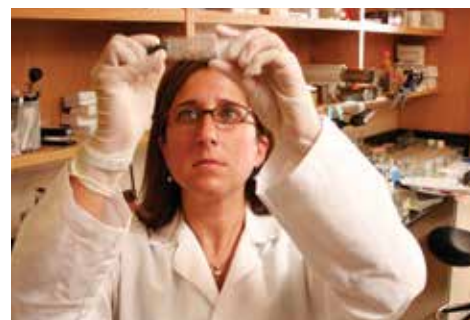
Is your antibody specific?

CST's Nanog (D2A3) XP® Rabbit mAb #8822 in Nanog-positive F9 cells compared to Nanog-negative NIH/3T3 cells demonstrate superior antibody specificity, compared to antibodies from other suppliers.

Nanog-positive cells (nuclear staining only) **F9** — A

Nanog-negative cells (no signal expected) **NIH/3T3** — B

CST #8822 (1:1000)
Demonstrates specific nuclear staining in Nanog-expressing cells.

Company 1
Shows non specific cytoplasmic and nuclear staining in both positive and negative cell lines.*

Company 2
Exhibits non specific cytoplasmic staining in the Nanog negative cell line at the recommended optimal dilution. In addition, it shows nuclear staining in a positive cell line but with a lower signal-to-noise ratio*

Nanog (D2A3) XP® Rabbit mAb (Mouse specific) #8822: Confocal IF analysis of F9 cells (Nanog positive) (**upper**) and NIH/3T3 cells (Nanog negative) (**lower**) was performed using #8822 and antibodies from 2 other companies. *All antibodies were used in accordance with manufacturers' recommendations.

Specificity of GSK3a (D80D1) XP® Rabbit mAb #8418 in murine embryonic fibroblasts (MEFs)

| WT MEFGSK3α/β (WT) | GSK3β(-/-) | GSK3α(-/-) |
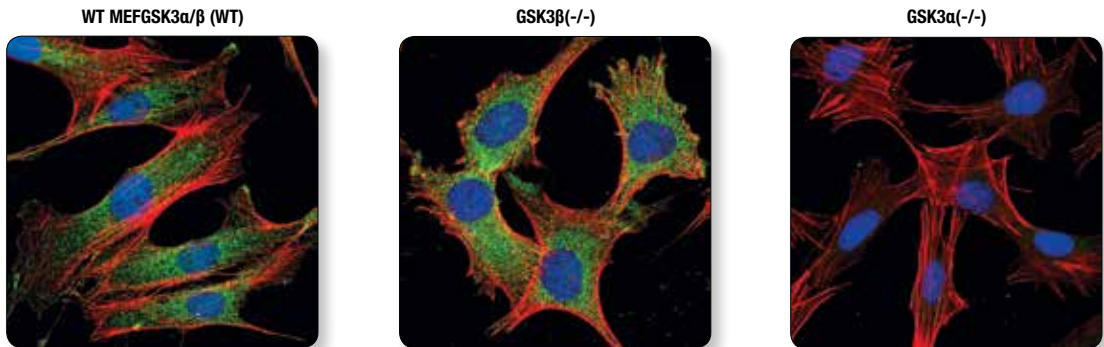
GSK3a (D80D1) XP® Rabbit mAb #8418: Confocal immunofluorescent analysis of MEF/GSK-3 wildtype cells (left), MEF/GSK-3β (-/-) cells (center) and MEF/GSK-3α (-/-) cells (right), using #8418 (green). Actin filaments have been labeled with DY-554 phalloidin (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye). (MEF/wildtype, GSK-3α (-/-) and GSK-3β (-/-) cells were kindly provided by Dr. Jim Woodgett, University of Toronto, Canada).
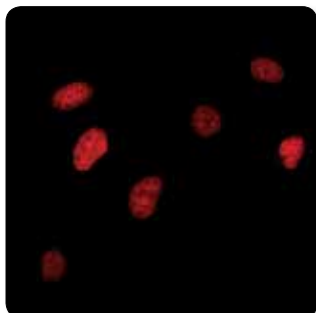
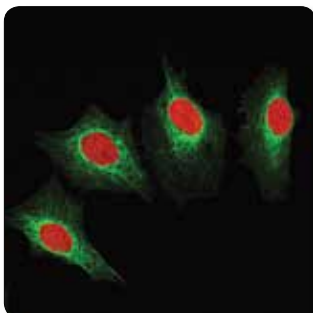# Is your antibody supported by an optimized IF protocol?

Save yourself from spending precious time and reagents on efforts to optimize a protocol that works. At CST, we test our antibodies using a variety of fixation and permeabilization conditions to determine the protocols that achieve optimal results.
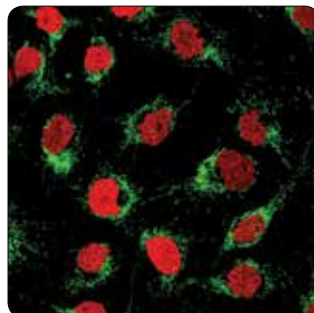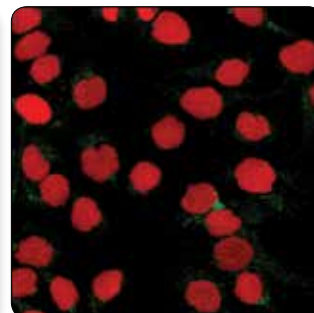
Formaldehyde   **Methanol**

Best with methanol fixation

Keratin 8/18 (C51) Mouse mAb #4546: IF analysis of HeLa cells, fixed with formaldehyde (left) or methanol (right), using #4546 (green). Red = Propidium Iodide (PI)/RNase Staining Solution #4087.
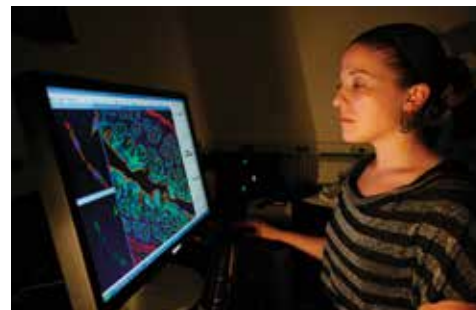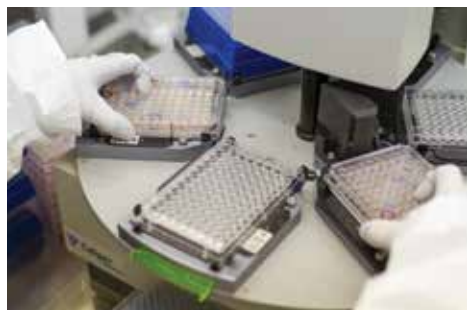
Formaldehyde   **Methanol**

Best with formaldehyde fixation

AIF (D39D2) XP® Rabbit mAb #5318: IF analysis of HeLa cells, fixed with formaldehyde (left) or methanol (right), using #5318 (green). Red = Propidium Iodide (PI)/RNase Staining Solution #4087.

Resources and references for more information on scientific reproducibility and antibody validation.

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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.

Technical Support:
www.cellsignal.com/support

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

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