The Use of Highly Validated Rabbit Monoclonal Antibodies to Analyze Epigenetic Marks and Mechanisms in Disease

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ABSTRACT

Research in the field of epigenetics has grown at a rapid pace since the discovery of the first histone acetyltransferase enzymes 19 years ago. Since then, significant advances have been made in our understanding of the basic epigenetic mechanisms regulating gene expression and genomic stability, and the impact of epigenetic deregulation on cancer, inflammation, metabolism, and neurological diseases. Much of our knowledge of the these mechanisms comes from the utilization of antibodies to probe the protein levels and localization of transcription factors, chromatin regulators, and histone modifications in different cell and tissue types, and across the genomes of a multitude of organisms. While antibodies have been a key reagent driving advancements in epigenetic research, there are increasing numbers of publications raising concerns about the quality of the antibodies being used in biomedical research. A very concerning large number of antibodies, both commercially available and those developed by individual laboratories, have not been completely validated, some showing a lack of specificity and sensitivity even in western blot (WB) or dot blot (DB) assays. Many additional antibodies show specificity in these assays, but fail to work in more demanding assays such as immunofluorescence (IF), flow cytometry (Flow), immunohistochemistry (IHC), chromatin IP (ChIP), and ChIP-sequencing (ChIP-seq). Even high quality, well-validated polyclonal antibodies have issues with reproducibility, as antibody attributes often change from lot to lot. Recent advancements in antibody technologies, specifically the development of rabbit monoclonal technologies, present solutions to many of these problems. We will demonstrate how the utilization of rabbit monoclonal technology combined with thorough antibody validation can lead to generation of high quality rabbit monoclonal antibodies that show exquisite specificity, sensitivity, and reproducibility across multiple applications, including IF, IHC, ChIP and ChIP-seq.

INTRODUCTION

An increasing number of publications in the literature have raised concerns about the implications of using incorrectly or insufficiently characterized antibodies in biomedical research. Several recent reports have underscored the magnitude of the problem and the need for better, more thorough antibody validation. Algenäs et al. (2014) tested the specificity and sensitivity of 13,000 antibodies against a range of proteins by WB. They found that only 45% of the antibodies showed appropriate staining, while 12% showed no staining and 43% stained inappropriate bands of the incorrect size **(1)**. Another report by the ENCyclopedia Of DNA Elements (ENCODE) Project (National Human Genome Research Institute) tested 227 commercially available antibodies against transcription factors and found that only 20% both passed specificity testing by DB or WB and also worked in ChIP-seq **(2)**. The lack of specificity of histone modification antibodies was addressed by Egelhofer et al. (2011), who analyzed the specificity of 246 antibodies against 57 different histone modifications and showed that 25% failed specificity testing by DB or WB, and of the 75% that passed, 22% failed in ChIP **(3)**. While it has become clear that there is an abundance of insufficiently characterized antibodies available for biomedical research, it is also clear that the use of "bad" antibodies can have significant ramifications on scientific research, resulting in non-reproducible results, loss of time and resources, and even retraction of publications, leading to loss of scientific reputation.

The many problems associated with the use of poorly characterized antibodies has led to a call for higher standards for antibody testing and validation, not just in WB and DB, but across multiple applications, including IF, Flow, IHC and ChIP **(1–5)**. In addition, many scientific journals have responded by increasing their requirements for clear demonstration of antibody specificity when manuscripts are submitted for publication **(6, 7)**. The ENCODE consortium recently published a set of guidelines for the validation of antibodies used for ChIP-seq experiments **(2)**. They recommend specific guidelines for the use of antibodies against transcription factors, epigenetic regulators, and antibodies against histones and histone modifications (see below), and they will not accept ChIP-seq data for publication unless the antibodies used have met these guidelines for specificity testing. This general push for higher standards for antibody validation and requirement for complete characterization of antibodies prior to publication will only increase the quality of research and publications coming out in the future.

Cell Signaling Technology (CST) specializes in the development of rabbit monoclonal antibodies for use in biomedical research. While both rabbit and mouse monoclonal antibodies have advantages over polyclonal antibodies, typically showing higher specificity and much less lot to lot variability than polyclonal antibodies, rabbit monoclonal technology has many advantages over mouse monoclonal technology. First, rabbits elicit a stronger immune response than mice and have 50 times more splenic lymphocytes, resulting in the generation of a wider repertoire of antibodies against epitopes with strong and weak immunogenicity **(8)**. Second, rabbit monoclonal antibodies have a simpler structure (no IgG subclasses) and show higher binding affinities (picomolar Kds), resulting in increased sensitivity without decreased specificity, an important aspect to consider when choosing an antibody for ChIP and ChIP-seq **(8)**. At CST, we have developed our own proprietary rabbit monoclonal antibody technology that we combine with strict antibody validation criteria that meet and even exceed the recommended ENCODE guidelines (see below). This allows us to generate highly validated rabbit monoclonal antibodies that show exquisite specificity and sensitivity in multiple applications, including WB, IF, Flow, IHC, ChIP, and ChIP-seq **(9)**.

ANTIBODY VALIDATION WORKFLOWS

CST Validation Summary:

Assays:
- -Western blot (WB)/Immunoprecipitation (IP)/Dot blot (DB)
- -Immunofluorescence (IF)
- -Immunohistochemistry (IHC)
- -Flow Cytometry (Flow)
- -Chromatin Immunoprecipitation (ChIP)
- -Peptide ELISA and Peptide Array

Tools:
- -Positive/Negative cell lines
- -Wild-type versus knockout cell lines
- -Treatments to induce expression, modification, or localization changes
- -siRNA/shRNA knockdown
- -Overexpression of epitope-tagged version of target
- -Peptide blocking

ENCODE Validation Guidelines:

Primary Characterization:
(one assay required)
- -WB/IP
- -IF

Secondary Characterization:
(one assay required)
- -siRNA knockdown
- -IP with antibody against different region of protein or complex subunit
- -IP with mass spectrometry
- -IP with epitope-tagged version of target
- -Motif enrichment

SUMMARY

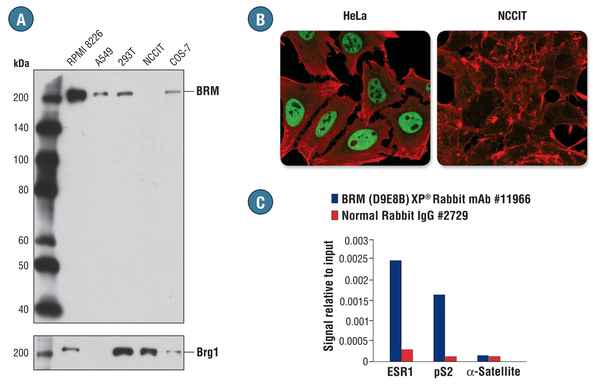
- CST provides rabbit mAbs that are thoroughly validated for specificity, sensitivity, and reproducibility across multiple applications using biologically relevant cell and tissue model systems. Our validation criteria meet and exceed ENCODE guidelines for antibody validation.
- Antibodies are optimized for every application and come with recommended dilutions and protocols.
- Rabbit mAbs provide better lot-to-lot consistency than polyclonal antibodies and typically show higher binding affinities than mouse mAbs, resulting in better sensitivity, without sacrificing specificity across multiple applications.
- CST has a large portfolio of over 225 ChIP-validated antibodies, including antibodies against 170 transcription factor and co-factor proteins that span multiple areas of research. For a complete list of ChIP products from CST, please visit our website at www.cellsignal.com/chip.

REFERENCES

1. Algenäs C. et al. (2014) *Biotechnol.* J. 9, 435–445.
2. Landt S.G. et al. (2012) *Genome Res.* 22, 1813–1831.
3. Egelhofer T. A. et al. (2011) *Nat. Struct. Mol. Biol.* 18, 91–93.
4. Howart W.J. et al. (2014) *Methods* 70, 34–38.
5. Perkel J.M. (2014) *Biotechniques* 56, 111–114.
6. Saper C.B. (2005) *J. Comp. Neurol.* 493, 477–478.
7. Gore A.C. (2013) *Endocrinology* 154, 579–580.
8. Feng L. et al. (2011) *Am. J. Transl. Res.* 15, 269–274.
9. Cheung W.C. et al. (2012) *Nat Biotechnol.* 30, 447–452.
10. Fuchs S.M. et al. (2011) *Curr. Biol.* 21, 53–58.

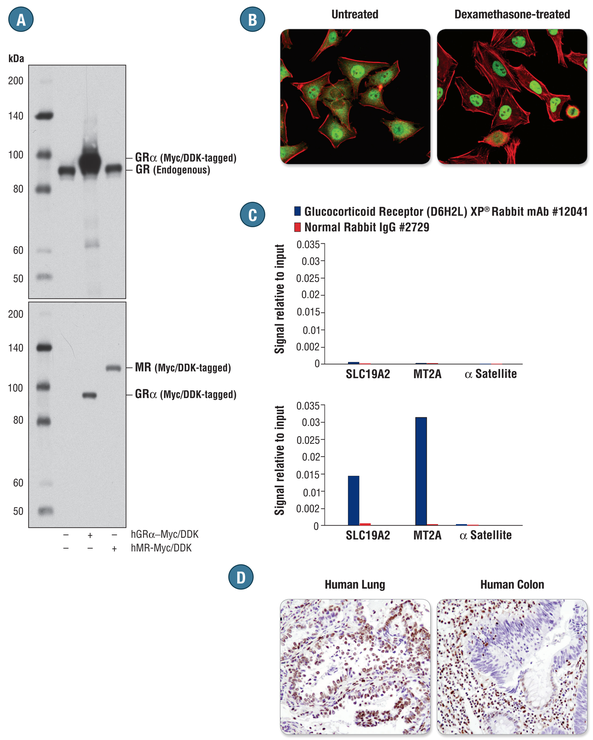
TRANSCRIPTION FACTOR ANTIBODY VALIDATION

BRM (D9E8B) XP® Rabbit mAb #11966



Validation of BRM rabbit mAb using positive and negative cells and cell treatments. (A) WB analysis of BRM on extracts from various cell lines, including NCCIT, which is known not to express BRM (top panel). Brg1, which is highly homologous to BRM is expressed in NCCIT cells and is shown as a reference (bottom panel). **(B)** IF analysis using the BRM antibody showing specific nuclear localization in BRM-positive HeLa cells (left panel) with no background staining in BRM-negative NCCIT cells (right panel). **(C)** ChIP was performed on MCF7 cells treated with estradiol showing recruitment of BRM to estrogen response elements but not to the non-specific alpha satellite repeat element using SimpleChIP® Plus Enzymatic Chromatin IP Kit #9005.

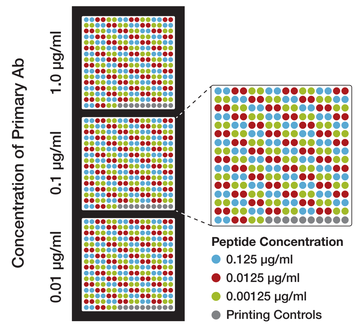
Glucocorticoid Receptor (D6H2L) XP® Rabbit mAb #12041



Validation of Glucocorticoid Receptor (GR) rabbit mAb using overexpression and cell treatments. (A) WB analysis of GR on extracts from 293T cells, either mock transfected or transfected with constructs expressing Myc/DDK-tagged full-length human GR-α, or Myc/DDK-tagged full-length human Mineralocorticoid Receptor (MR). In the upper panel, extracts are probed with Glucocorticoid Receptor (D6H2L) XP® Rabbit mAb #12041 showing increased signal with GR-α transfection, but no detectable increase with MR transfection. In the lower panel, extracts are probed with DYKDDDDK Tag Antibody #2368 showing appropriate expression of epitope-tagged GR-α and MR proteins. **(B)** IF with the GR antibody on HeLa cells either untreated (left panel) or dexamethasone-treated (right panel). Appropriate nuclear translocation is observed when cells are treated with dexamethasone. **(C)** ChIP was performed on A549 cells either left untreated (top panel) or treated with dexamethasone (bottom panel) using SimpleChIP® Plus Enzymatic Chromatin IP Kit #9005. Treatment with dexamethasone induces nuclear translocation and recruitment of GR to target genes, but not to the non-specific α Satellite repeat element. **(D)** IHC analysis of paraffin-embedded human lung carcinoma (left panel) and human colon carcinoma (right panel) showing appropriate staining of GR in each tissue.

HISTONE ANTIBODY VALIDATION

Histone Peptide Arrays



Three identical peptide arrays printed on a glass slide.

Validation of histone modification antibody specificity using histone peptide arrays developed at CST.
The specificity of all methyl-lysine, acetyl-lysine, and methyl-arginine histone antibodies is determined using histone peptide arrays and peptide competition ELISA.

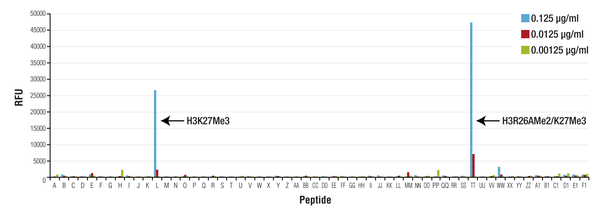
Background:
Our modification-specific histone antibodies are validated with a peptide array assay similar to the one described by Fuchs, S.M., et al. (2011). These arrays assess antibody reactivity against known modifications across all histone proteins in a single experiment. This method has the additional benefit of testing the effects of neighboring modifications on the ability of the antibody to detect a single modification site.

Method:
Antibodies are tested at three concentrations, as indicated in the diagram, which allows for a more thorough analysis of antibody reactivity.

Peptides containing a methyl-lysine (mono-, di-, or tri-methyl), acetyl-lysine or corresponding unmodified lysine, either alone or in combination with a known neighboring histone modification (e.g., histone H3K4Me3 and H3T3Phos) are spotted onto nitrocellulose as indicated in the diagram.

- **Primary Antibody:** Concentrations as indicated on the diagram.
- **Secondary Antibody:** According to manufacturer's recommendations (LI-COR, Inc.)
- **Detection:** LI-COR® Odyssey® Infrared Imager
- **Data Analysis:** ArrayVision Software (GE Healthcare)

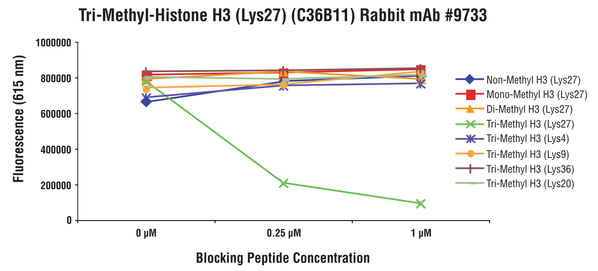
Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb #9733



A	H3 (Lys4) Non-methyl	V	H4 (Lys20) Mono-methyl	KK	H3 (Arg2) Asymmeric-di-methyl/(Lys4) Tri-Methyl	WW	H3 (Lys27) Tri-methyl/(Ser28) phospho
B	H3 (Lys4) Mono-methyl	W	H4 (Lys20) Di-methyl	LL	H3 (Arg8) Symmetric-di-methyl/(Lys9) Mono-methyl	XX	H3 (Lys9) Mono-Methyl/(Ser10/Thr11) phospho
C	H3 (Lys4) Di-methyl	X	H4 (Lys20) Tri-methyl	MM	H3 (Arg8) Symmetric-di-methyl/(Lys9) Di-methyl	YY	H3 (Lys9) Di-Methyl/(Ser10/Thr11) phospho
D	H3 (Lys4) Tri-methyl	Y	H2A (Lys5) Non-methyl	NN	H3 (Arg8) Symmetric-di-methyl/(Lys9) Tri-methyl	ZZ	H3 (Lys9) Tri-Methyl/(Ser10/Thr11) phospho
E	H3 (Lys9) Non-methyl	Z	H2A (Lys5) Mono-methyl	OO	H3 (Lys9) Mono-methyl/(Ser10) phospho	A1	H3 (Thr6) phospho/(Lys9) tri-methyl
F	H3 (Lys9) Mono-methyl	AA	H2A (Lys5) Di-methyl	PP	H3 (Lys9) Di-methyl/(Ser10) phospho	B1	H3 (Lys4) di-methyl/(Thr6) phospho
G	H3 (Lys9) Di-methyl	BB	H2A (Lys5) Tri-methyl	QQ	H3 (Lys9) Tri-methyl/(Ser10) phospho	C1	H3 (Lys4) mono-methyl/(Thr6) phospho
H	H3 (Lys9) Tri-methyl	CC	H3 (Thr3) Phospho/ (Lys4) Mono-Methyl	RR	H3 (Arg26) Asymmetric-di-methyl/(Lys27) Mono-methyl	D1	H3 (Lys4) tri-methyl/(Thr6) phospho
I	H3 (Lys27) Non-methyl	DD	H3 (Thr3) Phospho/ (Lys4) Di-Methyl	SS	H3 (Arg26) Asymmetric-di-methyl/(Lys27) di-methyl	E1	H3 (Thr6) phospho/(Lys9) di-methyl
J	H3 (Lys27) Mono-methyl	EE	H3 (Thr3) Phospho/ (Lys4) Tri-Methyl	TT	H3 (Arg26) Asymmetric-di-methyl/(Lys27) Tri-methyl	F1	H3 (Thr6) phospho/(Lys9) mono-methyl
K	H3 (Lys27) Di-methyl	FF	H3 (Arg2) Symmeric-di-methyl/(Lys4) Mono-Methyl	UU	H3 (Lys27) Mono-methyl/(Ser28) phospho		
L	H3 (Lys27) Tri-methyl	GG	H3 (Arg2) Symmeric-di-methyl/(Lys4) Di-Methyl	VV	H3 (Lys27) Di-methyl/(Ser28) phospho		
M	H3 (Lys36) Non-methyl	HH	H3 (Arg2) Symmeric-di-methyl/(Lys4) Tri-Methyl				
N	H3 (Lys36) Mono-methyl	I I	H3 (Arg2) Asymmeric-di-methyl/(Lys4) Mono-Methyl				
O	H3 (Lys36) Di-methyl	JJ	H3 (Arg2) Asymmeric-di-methyl/(Lys4) Di-Methyl				
P	H3 (Lys36) Tri-methyl						
Q	H3 (Lys79) Non-methyl						
R	H3 (Lys79) Mono-methyl						
S	H3 (Lys79) Di-methyl						
T	H3 (Lys79) Tri-methyl						
U	H4 (Lys20) Non-methyl						

Histone peptide array assay showing Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb is highly specific for tri-methyl histone H3 lysine 27 and is not affected by methylation of the neighboring Arg26 residue.

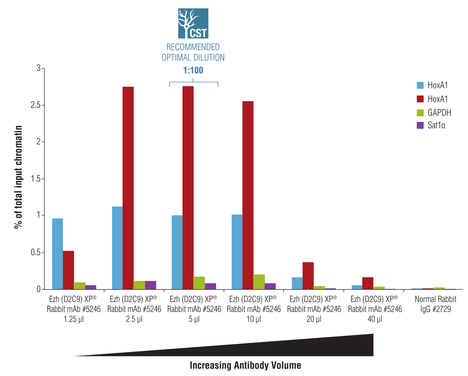
Peptide Competition ELISA



Histone peptide competition ELISA showing Tri-Methyl Histone H3 (Lys27) (C36B11) Rabbit mAb #9733 is highly specific for tri-methyl histone H3 lysine 27.
The graph depicts the binding of the antibody to pre-coated tri-methyl histone H3 (Lys27) peptide in the presence of increasing concentrations of various competitor peptides. As shown, only the tri-methyl histone H3 (Lys27) peptide competed away binding of the antibody.

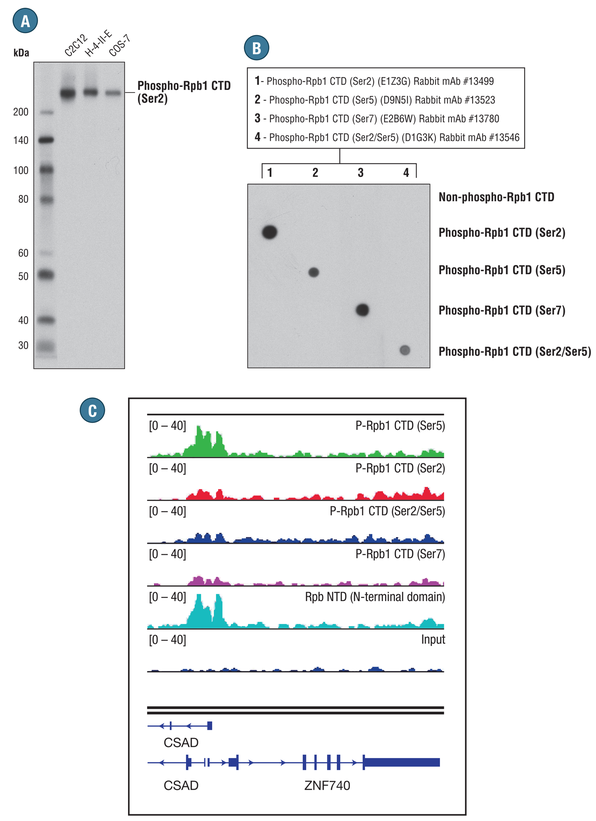
CHIP AND CHIP-SEQ VALIDATION

Optimization of CST Antibodies using the SimpleChIP Kit Protocol



A rabbit monoclonal antibody against Ezh2 was optimized in ChIP by titrating the antibody on cross-linked chromatin prepared from HCT116 cells treated with UV followed by a 3h recovery, using the SimpleChIP® Plus Enzymatic Chromatin IP Kit #9005. Ezh2 (D2C9) XP® Rabbit mAb #5246 was titrated at indicated amounts and compared to Normal Rabbit IgG #2729. The optimal dilution for this antibody in the SimpleChIP assay is 1:100, or 5.0 µl of antibody per 4 x 10^6 cells. The antibody: chromatin ratio is important to the success of a ChIP experiment, as adding too little or too much antibody can be detrimental to overall enrichment of target loci.

RNA Polymerase II C-terminal Domain (CTD) Rabbit mAbs



RNA Polymerase II CTD antibodies are validated using WB, DB, and ChIP-seq. (A) WB analysis of cell extracts using Phospho-Rbp1 CTD (Ser2) (E1Z3G) Rabbit mAb #13499. **(B)** Peptide DB analysis of various Phospho-Rpb1 CTD antibodies. Each antibody recognizes its specific phosphorylation site with no cross-reactivity with neighboring sites. **(C)** ChIP-seq tracks of the ZNF740 gene using a panel of Rpb1 antibodies. ChIP was performed on HeLa cells using SimpleChIP® Plus Enzymatic Chromatin IP Kit #9005. Libraries were constructed with the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (E7370S; New England Biolabs, Inc.) and sequenced using the Illumina NextSeq 500. Sequences were mapped to the UCSC Human Genome Assembly (hg19) and data is visualized using the Integrated Genomics Viewer (IGV). As shown, Rpb1 phosphorylated on Ser5 is enriched at the transcriptional start site and Rpb1 phosphorylated on Ser2 and Ser7 are found throughout the gene body. A total antibody against Rpb1 and input DNA tracks are shown as controls.

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