



A GUIDE TO SUCCESSFUL IMMUNOHISTOCHEMISTRY

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IHC



Immunohistochemistry (IHC) is a technique commonly used for morphological characterization of tumors or other tissue malignancies. This guide highlights critical steps in the IHC protocol and demonstrates how protocol changes can affect the final outcome of your experiment.



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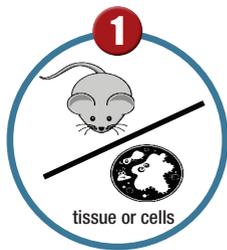
Introduction

Immunohistochemistry (IHC) is a technique commonly used for morphological characterization of tumors or other tissue malignancies. IHC uses antibodies to detect and analyze protein expression while maintaining the composition, cellular characteristics, and structure of native tissue. Chemical fixation, often with neutral buffered formalin (NBF) or formaldehyde, locks into place molecular interactions within and between cells. Tissue samples may be embedded in paraffin wax or frozen in a cryogenic solution for preservation before being cut into thin slices and mounted onto slides for analysis. Tissue collection, preservation, and fixation can vary greatly depending on the sample or the target of interest.

IHC identifies the presence and pattern of expression of proteins in a biological sample through specific antibody binding. The precise binding that occurs between an antibody and its target protein epitope enables the detection of highly specific amino acid sequences within a protein. Antibodies can also detect specific post-translational modifications (PTM) of a protein. Phospho-specific antibodies have been used to identify components of specific signaling pathways and to study changes in phosphorylation events in various biological contexts. Antibodies specific to other PTMs have been developed recently, allowing researchers to monitor changes in acetylation, methylation, or ubiquitination status of a protein.

At Cell Signaling Technology (CST), scientists specializing in IHC test a large number of antibodies and only recommend those best suited for the application. Our scientists test multiple methods of antigen retrieval and immunostaining to determine the optimal conditions for use of each antibody in IHC. In addition, our scientists develop companion reagents for IHC to enhance antigen detection and improve the efficiency of IHC protocols. The IHC protocols we have been optimizing for over a decade can be seen on pages 12 & 13 and are also available online so you can replicate the procedure and produce reproducible and reliable results.

Here, we will highlight critical steps in the IHC protocol and provide data to support and explain the recommendations found throughout our procedure. We will also discuss the critical importance of using a well-validated antibody in your IHC experiments. Finally, we will provide a list of commonly used IHC reagents that are utilized in-house by CST scientists and work optimally with our antibodies.



1

tissue or cells

Slide Preparation

Use freshly prepared slides for best results

11 Step Protocol for Successful IHC

Here we give an overview of our recommended protocol and discuss which steps we believe are key to a successful experiment. We provide recommendations on reagents and procedures based upon our extensive experience with IHC as part of our antibody validation and technical support process. IHC is a challenging application and problems often occur. This guide aims to help you improve your IHC analysis by providing suggestions to allow you to achieve the expected results with minimal end-user optimization.

PLEASE NOTE: The following steps reflect the protocol for paraffin-embedded samples.



2

Deparaffinization/Rehydrating

Completely remove paraffin for even staining



3

Antigen Retrieval

Use freshly prepared buffer* and boil in the microwave

*Recommended retrieval buffer for each antibody is indicated on the product datasheet



4

Quenching

Eliminate endogenous peroxidase activity using 3% H₂O₂ (for HRP-based detection)



5

Blocking

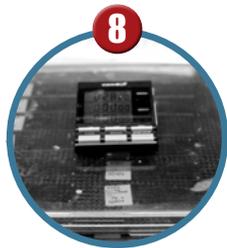
1 hour at room temperature in TBST/5% normal goat serum (NGS)



6

Washing

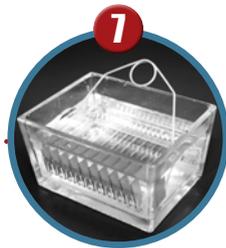
3 x 5 min in TBST



7

Detection

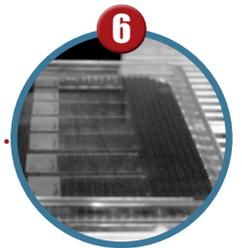
Use SignalStain® Boost IHC Detection Reagent and SignalStain® DAB Substrate Kit



8

Washing

3 x 5 min in TBST



9

Primary Antibody Incubation

Overnight at 4°C using SignalStain® Antibody Diluent or TBST/5% NGS as recommended on the product datasheet



10

Dehydrating

Remove all water before applying nonaqueous mounting media



11

Mounting & Examination

Add mounting media and coverslips for specimen protection and optimal viewing

The importance of a well-validated antibody.

The primary antibody is a critical component of any IHC assay and has a direct effect upon data quality. A poor primary antibody can result in dirty, uninterpretable, or misleading results. It takes more than just positive staining in a single tissue sample for an antibody to be approved for use in IHC. Antibodies should undergo a stringent validation procedure to ensure that the antibody detects the target accurately.

IHC validation includes:

- Western blot analysis to assess cross-reactive bands.
- Specificity testing on paraffin-embedded cell pellets using cell lines with known target expression levels, including treatments to verify modification specificity (e.g. phosphorylation, acetylation, cleavage, etc.).
- Phosphatase treatment to verify phospho-specificity.
- The use of blocking peptides to verify specificity and rule out Fc-mediated binding, biotin background, and other nonspecific staining.
- Specificity testing on relevant mouse models of cancer.
- Specificity testing on xenografts generated from cell lines with known target expression levels, including modulation of target expression in response to drug treatment.
- Antibody performance testing over a broad spectrum of tissue types using human tissue microarrays.
- Antibody performance testing on fresh frozen tissues when appropriate.

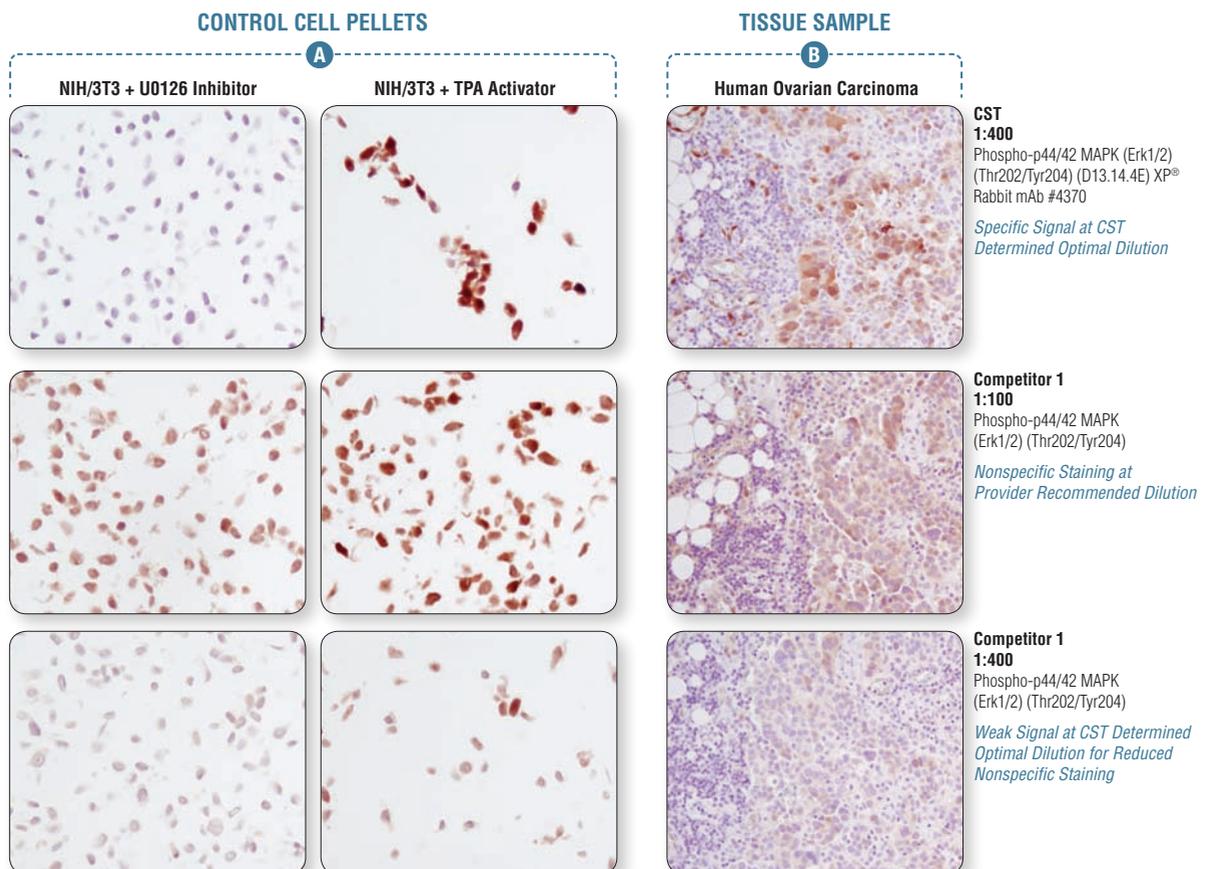
Be in Control of Your IHC Analysis

It's easy to apply an antibody to tissue and obtain a signal. But is it a specific signal? An important consideration in any experiment is the inclusion of appropriate controls. Positive and negative controls instill confidence that your antibody is detecting its intended target.

Prior to testing on tissue, antibody performance can be evaluated at the cellular level using a variety of cell lines and treatment conditions. For example, total protein specificity can be assessed through the use of positively and negatively expressing cell lines. Likewise, cells can be treated with biological or chemical modulators known to induce signaling changes to verify modification specificity, such as phosphorylation, acetylation, cleavage, etc. Phospho-specific antibodies can be further evaluated with phosphatase treatment. In addition, isotype control antibodies help rule out nonspecific staining of primary antibodies due to Fc receptor binding or other protein-protein interactions and should have the same immunoglobulin type as the test antibody.

In the figure below, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibodies were evaluated at their recommended dilutions in negatively (U0126) and positively (TPA) stimulated paraffin-embedded NIH/3T3 cell pellets (**A**) and human ovarian carcinoma (**B**). Even though both antibodies adequately stain tissue at their recommended dilutions, the alternate provider's antibody also stains the negative cell pellet. When the alternate provider's antibody dilution was optimized to eliminate this non-specific staining, there was only minimal staining observed in the tissue. This demonstrates the importance of always using a well-validated antibody and appropriate controls.

The use of proper controls provides experimental validity and is necessary for accurate data analysis, ultimately providing confidence in your results. In this example, the use of positive and negative control cell pellets reveal important differences in nonspecific staining.



Appropriate controls are necessary to ensure specific signal. Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370 was compared to a competitor's IHC-approved phospho-p44/42 (Tyr202/Tyr204) antibody. The optimal dilution of each antibody was individually evaluated to minimize nonspecific staining in NIH/3T3 cells (**A**) treated with the inhibitor U0126 (left) and to maximize specific signal in NIH/3T3 cells treated with the activator TPA #4174 (right). The determined optimal dilution for each antibody was utilized in IHC analysis of paraffin-embedded human ovarian carcinoma (**B**). Note that at the recommended dilution, the competitor's antibody stains the inhibitor-treated cells (NIH/3T3 + U0126). At the CST determined optimal dilution, the competitor's antibody no longer stains the inhibitor-treated cells, but also fails to stain tissue significantly. The CST antibody accurately stains both the cells and tissue.

See our list of available paraffin-embedded cell pellet control slides appropriate for many of our IHC-approved antibodies.

www.cellsignal.com/ihccontrols

Critical steps in the recommended protocol—how critical are they?

Protocol changes can have a significant effect on IHC results. We have tested common variations in antigen retrieval buffers, boiling devices, antibody diluents, and detection systems, and we have identified those that yield optimal results. In the pages that follow, you will find the data used to support and influence our protocol recommendations.

Slide Preparation

PLEASE NOTE: Always consult your product datasheet to determine if a product is validated for paraffin-embedded (IHC-P) or frozen (IHC-F) samples. Because most of the IHC-approved antibodies from CST are intended for paraffin-embedded samples, our recommendations herein will primarily focus on the IHC-P protocol. Variations in procedure will be called out as appropriate. Consult our protocols for IHC-P and IHC-F on pages 12 & 13 for full details.

IHC-P: Paraffin-embedded Cell Pellets and Tissue

Prior to immunostaining, cell pellet samples must be harvested and fixed in 10% neutral buffered formalin (NBF) to preserve cell morphology and target epitopes. Use an autoproccessor to dehydrate the samples and to infiltrate the samples with paraffin wax. Place the paraffin-infiltrated samples in a mold with a small volume of liquid paraffin and allow them to cool. Section the sample using a microtome to a 4–6 μm thickness and place on positively charged slides, which help the sample to adhere. Complete details on how to prepare paraffin-embedded cell pellets from both adherent and suspension cells can be found in our Cell Pellet Preparation Protocol on page 12. For tissue samples, requirements for the harvesting, fixation, and paraffin-embedding steps can vary based on the individual tissue type.

IHC-F: Frozen Tissue

Frozen tissue should be stored at -80°C and then embedded in OCT prior to sectioning. When ready to stain, equilibrate tissue at -20°C for 15 minutes before attempting to section. Section the tissue to a 6–8 μm thickness using a microtome and place on a positively charged slide. To further help the sample adhere to the slide, we recommend air drying the slides at room temperature for a few minutes before fixation in order to remove any residual water. Consult your product datasheet for optimal fixative solution and conditions.

Slide Storage

The following section pertains to paraffin-embedded samples only.

For best results, use freshly cut slides. Slides may lose antigenic potential over time in storage. This process is variable and dependent upon the protein target. The effect of slide storage on staining has not been established for every protein; therefore, it is best practice for slides to be freshly cut before use. If slides must be stored, do so unbaked at 4°C .

Deparaffinization/Rehydration

The following section pertains to paraffin-embedded samples only.

In order for antibody binding and staining to occur, the paraffin wax must be completely removed. This is done through a series of sequential xylene/ethanol/water washes that remove the wax and rehydrate the tissue for subsequent antibody binding. Insufficient paraffin removal can lead to spotty, uneven background staining. If this occurs, repeat the experiment with new sections using fresh xylene. Once the deparaffinization and rehydration steps have been completed, it is important that the slides not be allowed to dry out at any point throughout the remaining protocol.

Antigen Retrieval

The following section pertains to paraffin-embedded samples only.

The crosslinks created during the fixation step can prevent antibody binding by inhibiting access to the antigen; therefore, it is important to reverse crosslinks using a procedure called antigen retrieval (also known as antigen unmasking or epitope retrieval). Antigen retrieval can be achieved through either a heat-induced method (heat-induced epitope retrieval; HIER) or through enzymatic digestion. The recommended method for your CST™ antibody will be clearly indicated on the product datasheet.

Heat-induced Epitope Retrieval (HIER)

Buffer

There are multiple buffers available for HIER. The two commonly recommended by CST are a 10 mM citrate buffer, pH 6, and a 1 mM EDTA buffer, pH 8. The appropriate buffer for your experiment will depend on the primary antibody you are using. Always refer to your product datasheet for the recommended retrieval buffer for your specific antibody. In general, most phospho-tyrosine-specific antibodies require the EDTA buffer, whereas the citrate buffer can be used for most other antibodies. Always prepare fresh 1X solutions daily.

As shown, using the appropriate retrieval buffer can have significant effects on the quality of your final staining. **(C)**

Boiling Device

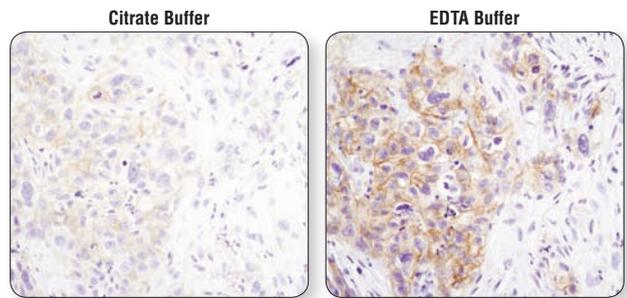
Antigen retrieval occurs when the slides are heated to boiling in the recommended buffer for a specific period of time. This step is typically performed using a microwave oven or a pressure cooker. Some researchers also use a water bath. However, the device you choose for this step can have a positive or negative impact on your staining. We recommend using a microwave oven or a pressure cooker for optimal antigen retrieval. **(D)**

Enzymatic Antigen Retrieval

Antigen retrieval can also be achieved through enzymatic digestion with pepsin, trypsin, or proteinase K. For those antibodies that require enzymatic retrieval rather than HIER, the recommended enzyme and digesting conditions will be clearly indicated on the product datasheet.

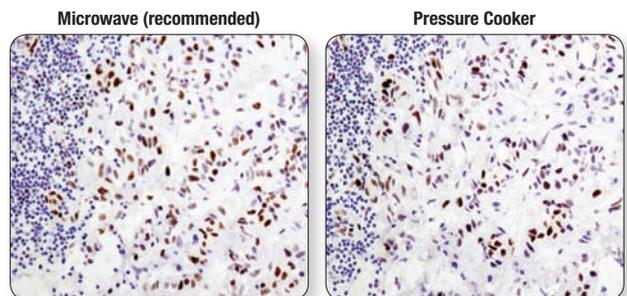
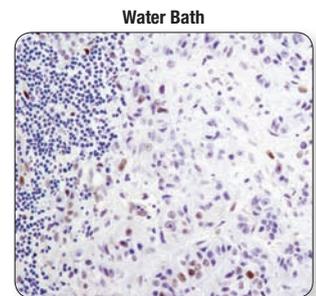
Antigen Retrieval is an Antibody/Antigen Phenomenon

It is important to note that antigen retrieval requirements are specific for the unique antibody/antigen and not for the protein itself. Therefore, if you use more than one antibody against a particular protein target, the optimal retrieval conditions for each antibody must be determined individually. **(E)**

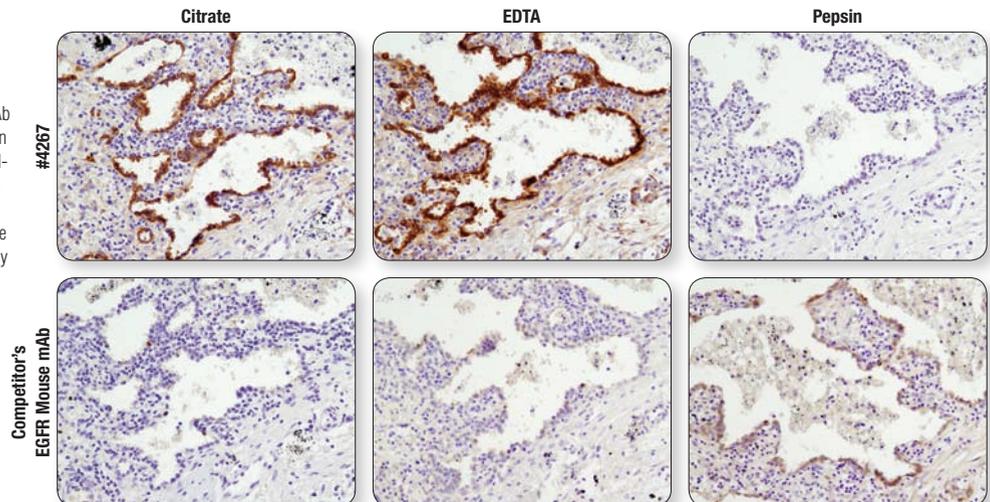


(C) Using the CST-recommended retrieval buffer can enhance your final staining results. IHC analysis of paraffin-embedded human lung carcinoma using Phospho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb #2842 after antigen retrieval using a citrate buffer (left) or EDTA buffer (right). As shown, using EDTA buffer with #2842 results in a more robust signal with increased staining observed at the cell membrane. Always check the product datasheet for the appropriate retrieval buffer for the antibody you are using.

(D) A microwave oven is recommended for antigen retrieval. IHC analysis of paraffin-embedded human lung carcinoma using Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145 after antigen retrieval using a water bath (right), microwave oven (bottom left), or pressure cooker (bottom right). A clear difference in performance is seen when using a microwave as compared with a water bath. For some antibodies, using a pressure cooker may enhance signals beyond those obtained with a microwave.



(E) Antibodies targeting the same protein may have different preferred methods of antigen retrieval. IHC analysis of paraffin-embedded human lung carcinoma using EGF Receptor (D38B1) XP® Rabbit mAb #4267 and an EGFR mouse mAb after antigen retrieval by boiling in citrate buffer (left), boiling in EDTA buffer (center), or digestion with pepsin (right). For #4267, superior signal is obtained with EDTA retrieval. However, for the competitor's EGFR mouse mAb, signal is only achieved with pepsin digestion.



Immunostaining

Quench

If you are using an HRP-based detection system, it is necessary to block activity from endogenous peroxidases that can interfere with your signal. Quench slides in a 3% hydrogen peroxide (H₂O₂) solution, diluted in distilled water (dilute H₂O₂ in methanol when performing IHC-F), for 10 minutes prior to incubation with the primary antibody.

Block

For IHC-P, we recommend blocking the samples in Tris buffered saline with Tween® 20 (TBST) plus 5% normal goat serum (NGS) for 1 hour at room temperature to prevent nonspecific background staining. Block samples in 1X TBS with 0.3% Triton™ X-100 and 5% NGS when performing IHC-F.

Commercially available blocking solutions that contain casein tend to diminish signal when used in conjunction with phospho-specific primary antibodies; therefore, we recommend that you do not use casein containing blocking reagents with phospho-specific antibodies. **(F)**

The above recommendations for the blocking step were developed using SignalStain® Boost IHC Detection Reagent as the intended detection reagent. If you will be using an alternate detection reagent, we recommend blocking in a serum of the same species as your secondary antibody.

Primary Antibody

Antibody Diluent

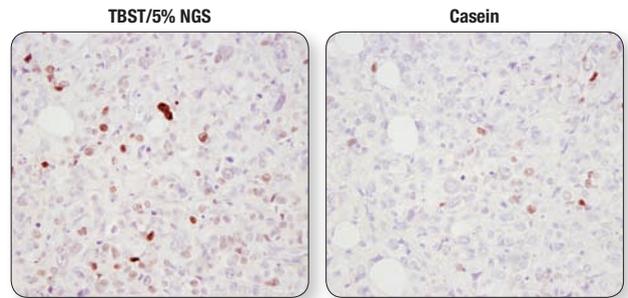
In IHC-P, there are a number of options when it comes to antibody diluent, and the one you choose can have a significant impact on your staining. At CST, we dilute the primary antibody using SignalStain® Antibody Diluent, TBST/5% NGS, or PBST/5% NGS. The proper diluent is antibody specific; therefore, always check the product datasheet for the recommended diluent for the antibody you are using **(G)**. When performing IHC-F, the primary antibody should be diluted in blocking buffer (TBS/0.3% Triton™ X-100/5% NGS).

Antibody Incubation

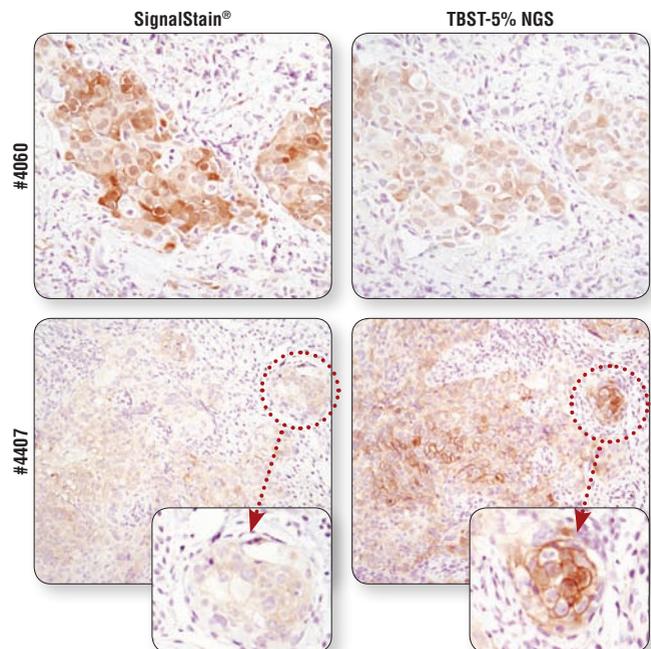
We recommend an overnight primary antibody incubation at 4°C, and all our recommended dilutions are based upon overnight incubation. This does not mean that CST antibodies will not work with a brief incubation period, which is often used with automated platforms. It simply means that optimization will likely be required in order to achieve an optimal signal.

Washing

Adequate washing is critical for contrasting low background and high signal. Wash slides three times for 5 minutes with either TBST for IHC-P or with TBS for IHC-F after primary and secondary antibody incubations.



(F) Do not block with casein containing reagents when using phospho-specific antibodies. IHC analysis of paraffin-embedded human breast carcinoma using Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb #9718 after blocking with TBST/5% NGS (left) or a casein-based blocking solution (right). As shown, casein block produces a lower overall signal as compared with TBST/5% NGS.



(G) For optimal results, always use the recommended primary antibody diluent, as indicated on the product datasheet. IHC analysis of paraffin-embedded human breast carcinoma (top) and HCC827 xenograft (bottom) using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060 or Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb #4407 after dilution in either SignalStain® Antibody Diluent (left) or TBST/5% NGS (right). As shown, a superior signal is achieved when #4060 is diluted in SignalStain® Antibody Diluent as compared with TBST/5% NGS. In contrast, #4407 performs better when diluted in TBST/5% NGS. Always check the product datasheet for the recommended diluent for your specific antibody.

Detection

Detection Systems

Traditional IHC detection methods take advantage of the natural affinity between avidin and biotin. These avidin-biotin-complex (ABC) systems require a 2-step process of detection involving biotinylated secondary antibody followed by exposure to an avidin-HRP complex prior to chromogenic detection. Systems that rely on biotin are prone to background staining, particularly in tissues such as liver and kidney that possess high levels of endogenous biotin. Therefore, we recommend using polymer-based detection systems such as SignalStain® Boost IHC Detection Reagents (**H**). These biotin-free, polymer-based systems consist of enzymes and secondary antibodies directly conjugated to a polymer backbone. In addition to superior sensitivity and elimination of false-positive staining due to endogenous biotin, SignalStain® Boost IHC Detection Reagents save time by eliminating one step in the detection procedure and are compatible with all peroxidase-based substrates.

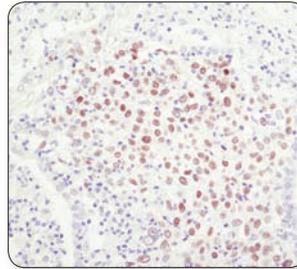
Chromogens

Diaminobenzidine (DAB) substrate is one of the most commonly used chromogens in peroxidase-based detection systems. DAB reacts with HRP to form a brown precipitate at the site of antibody binding. Always use a high quality DAB substrate in your experiments. CST scientists in the IHC group use SignalStain® DAB Substrate Kit for all in-house IHC antibody validation (**I**). The SignalStain® DAB Substrate Kit offers high levels of sensitivity and works optimally with our primary antibodies. After eliminating unbound secondary antibody with 3 x 5 minutes washes in TBST (wash for 3 x 5 minutes in TBS for IHC-F), apply 100–400 µl SignalStain® DAB to each tissue section and monitor closely for appropriate color development. Generally, 1–10 minutes provides an acceptable staining intensity. Once an appropriate staining level is reached, immerse slides in dH₂O to stop further color development.

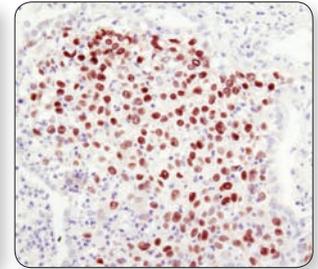
Counterstains

After antibody detection but before coverslip mounting, most researchers prefer to counterstain the tissue to visualize cellular anatomy and orient the viewer in respect to the specific staining. Many commercially available counterstains are available (**J**). At CST, we counterstain our samples using hematoxylin, which colors the nucleus blue. Counterstain according to the manufacturer's instructions. When choosing a counterstain, it is important that the counterstain be compatible with your chromogen. For example, if the counterstain color is too similar to the chromogen color, the antibody signal will be difficult to recognize.

Biotin-based Detection



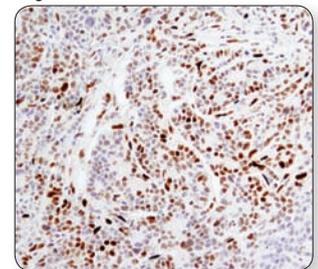
Polymer-based Detection



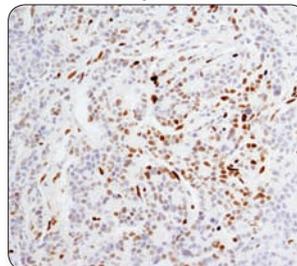
(H) Polymer-based detection is more sensitive than biotin-based systems. IHC analysis of paraffin-embedded human lung carcinoma using Sox2 (D6D9) XP® Rabbit mAb #3579 and either biotin-based detection (left) or polymer-based detection (SignalStain® Boost IHC Detection Reagent #8114; right). As shown, polymer-based detection offers enhanced sensitivity and results in more robust staining.

(I) Not all DAB substrate performs equally. IHC analysis of paraffin-embedded human breast carcinoma using Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145. Chromogenic detection was performed using SignalStain® DAB Substrate Kit #8059 (right), DAB supplied by competitor 1 (bottom left), or DAB supplied by competitor 2 (bottom right). Although competitor 2 DAB produces a signal comparable to the SignalStain® DAB Substrate Kit, competitor 1 DAB results in a much weaker signal.

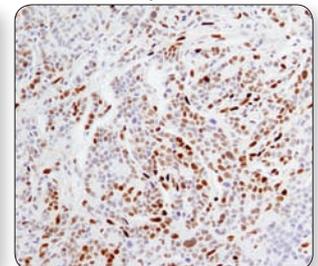
SignalStain® DAB Substrate Kit #8059



Competitor 1



Competitor 2



The CST recommended protocol is based upon a peroxidase-based detection system and a peroxidase-based chromogen. Other enzyme-based systems are available, such as alkaline phosphatase (AP). If you choose an alternate enzyme system, be sure to use a compatible chromogen.

(J) Commonly used Counterstains

Counterstain	Target	Color
Hematoxylin	Nuclei	Blue
Methyl Green	Nuclei	Green
Nuclear Fast Red	Nuclei	Pink

TROUBLESHOOTING TIP

When the primary antibody is from the same species as the sample being tested, the secondary antibody may bind endogenous IgG in some tissues, causing high background (mouse-on-mouse staining, for example). Include a control slide stained without the primary antibody to confirm whether the secondary antibody is the source of the background.

Dehydration, Mounting & Examination

Slides should be mounted with coverslips for specimen preservation and optimal viewing. Aqueous and nonaqueous (permanent) mounting media are available. The mounting media you choose depends on the chromogen used during the detection step and its solubility in organic solvents or water; alcohol- or xylene-soluble chromogens should not be used with nonaqueous mounting media and water-soluble chromogens should not be used with aqueous media (**K**). Using the incorrect mounting media will erode the signal. We recommend using DAB substrate and a nonaqueous mounting medium. Nonaqueous mounting media is not compatible with water; therefore, the samples must be first dehydrated with a series of ethanol and xylene washes:

- 2 x 10 sec each in **95% ethanol**
- 2 x 10 sec each in **100% ethanol**
- 2 x 10 sec each in **xylene**

Some researchers, however, require an aqueous mounting media. This is especially true in multiplex analysis when multiple antibodies and chromogens are used on the same sample. If aqueous mounting media is used, you do not need to perform the dehydration steps. Mounting media can be directly applied to one end of the slide before adding coverslips. Subject slides to microscopic evaluation.

(K) Commonly used Peroxidase-based Chromogens and Corresponding Mounting Media

Chromogen	Color	Mounting Media
DAB	Brown	Nonaqueous or Aqueous
AEC	Red	Aqueous
NovaRed™	Red	Nonaqueous

There are many other commercially available peroxidase-based chromogens. Always consult the manufacturer for the recommended mounting media.

TROUBLESHOOTING TIP

If using a chromogen other than DAB, be sure to check for compatibility with mounting media. Some chromogens are soluble in organic solvents and require an aqueous mounting medium. When aqueous mounting medium is used, the dehydration steps should be omitted.

CST Cell Pellet Preparation

1. Grow cells in 15 cm plates (adherent cell lines) or in T-175 flasks (suspension cell lines). When the plates are approximately 80% confluent or the flask is sufficiently cloudy, the cells are dense enough to make cell pellets.

NOTE: It takes approximately 2 confluent plates to make a good-sized cell pellet.

2. For adherent cells, aspirate the growth medium, wash once in sterile 1X PBS then aspirate. Scrape the cells in 5 ml–1X PBS, centrifuge and aspirate. For suspension cells, centrifuge in the growth medium and aspirate. Wash cells in sterile 1X PBS then centrifuge and aspirate.
3. Fix cells in 10% neutral buffered formalin (NBF) for 30 min, then centrifuge and aspirate. Wash cells again in 1X PBS, centrifuge and aspirate.
4. Mix an equal amount of liquefied HistoGel™ to the cell pellet. Allow the cell/HistoGel™ mixture to solidify.

NOTE: Eppendorf tubes may be used as a mold for the pellets.

5. Place cell pellets in a biopsy cassette and fix overnight in 10% NBF. Cassettes containing cell pellets can be stored in 70% ethanol until processing.
6. Process pellets in an auto-processor. The recommended program is: 30 min 70% ethanol (2x), 45 min 95% ethanol (2x), 30 min 100% ethanol (2x), 45 min 100% ethanol, 30 min Clear-Rite™ 3 or Xylene (3x), 60 min Paraffin (2x).
7. Embed processed cell pellets in melted paraffin wax onto the bottom half of a biopsy cassette. Allow paraffin to solidify.
8. Section paraffin blocks in 4 µm slices and place on positively charged slides.

Immunohistochemistry Protocol (Frozen)

using SignalStain® Boost Detection Reagent

***IMPORTANT:** Please refer to the APPLICATIONS section on the front page of the product datasheet or product webpage to determine whether a product is validated and approved for use frozen tissue sections. Please see product datasheet or product webpage for appropriate antibody dilution. **NOTE:** Please see product datasheet and website for product-specific protocol recommendations.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. Xylene
2. Ethanol (anhydrous denatured, histological grade 100% and 95%)
3. Hematoxylin (optional)
4. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH₂O, mix.
5. **Fixative Options:** For optimal fixative, please refer to the product datasheet.
 - a. 10% Neutral buffered formalin
 - b. Acetone
 - c. Methanol
 - d. **3% Formaldehyde:** To prepare 100 ml, add 18.75 ml 16% formaldehyde to 81.25 ml 1X PBS.
6. **Wash Buffer:**
 - a. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997)
To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH₂O, mix.
7. **Methanol/Peroxidase:** To prepare, add 10 ml 30% H₂O₂ to 90 ml methanol. Store at -20°C.
8. **Blocking Solution:** 1X TBS/0.3% Triton™ X-100/5% Normal Goat Serum (#5425). To prepare, add 500 µl normal goat serum and 30 µl Triton™ X-100 to 9.5 ml 1X TBS.
9. **Detection System:** SignalStain® Boost IHC Detection Reagents (HRP, Mouse #8125; HRP, Rabbit #8114)
10. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

B. Sectioning

1. **For tissue stored at -80°C:** Remove from freezer and equilibrate at -20°C for approximately 15 min before attempting to section. This may prevent cracking of the block when sectioning.
2. Section tissue at a range of 6–8 µm and place on positively charged slides.
3. Allow sections to air dry on bench for a few min before fixing (this helps sections adhere to slides).

C. Fixation Options

NOTE: Consult product datasheet to determine the optimal fixative.

1. After sections have dried on the slide, fix in optimal fixative as directed below.
 - a. **10% Neutral buffered formalin:** 10 min at room temperature. Proceed with staining procedure immediately (Section D).
 - b. **Cold acetone:** 10 min at -20°C. Air dry. Proceed with staining immediately (Section D).
 - c. **Methanol:** 10 min at -20°C. Proceed with staining immediately (Section D).
 - d. **3% Formaldehyde:** 15 min at room temperature. Proceed with staining immediately (Section D).
 - e. **3% Formaldehyde/methanol:** 15 min at room temperature in 3% formaldehyde, followed by 5 min in methanol at -20°C (do not rinse in between). Proceed with staining immediately (Section D).

D. Staining

1. Wash sections in wash buffer two times for 5 min.
2. Incubate for 10 min at room temperature in methanol/peroxidase.
3. Wash sections in wash buffer two times for 5 min.
4. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
5. Remove blocking solution and add 100–400 µl primary antibody diluted in blocking solution to each section.
6. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections in wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100–400 µl SignalStain® DAB to each section and monitor closely. 1–10 min generally provides an acceptable staining intensity.
13. Immerse slides in dH₂O.
14. If desired, counterstain sections with hematoxylin per manufacturer's instructions.
15. Wash sections in dH₂O two times for 5 min each.
16. **Dehydrate sections:**
 - a. Incubate sections in 95% ethanol two times for 10 sec each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.
 - c. Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount sections with coverslips.

Immunohistochemistry Protocol (Paraffin)

using SignalStain® Boost Detection Reagent

***IMPORTANT:** Please refer to the APPLICATIONS section on the front page of product datasheet or product webpage to determine whether a product is validated and approved for use on paraffin-embedded (IHC-P) tissue sections. Please see product datasheet or product webpage for appropriate antibody dilution, diluent and unmasking solution.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)
5. **Wash Buffer:**
 - a. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997)
To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH₂O, mix.
6. ***Antibody Diluent Options:**
 - a. **SignalStain® Antibody Diluent:** (#8112)
 - b. **TBST/5% Normal Goat Serum:** To 5 ml 1X TBST, add 250 µl Normal Goat Serum (#5425).
 - c. **PBST/5% Normal Goat Serum:** To 5 ml 1X PBST, add 250 µl Normal Goat Serum (#5425).
20X Phosphate Buffered Saline with Tween® 20 (PBST): (#9809) To prepare 1 L 1X PBST: add 50 ml 20X PBST to 950 ml dH₂O, mix.
7. ***Antigen Unmasking Options:**
 - a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
 - b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
 - c. **TE:** 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Tris base (C₄H₁₁NO₃) and 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 950 ml dH₂O. Adjust pH to 9.0, then adjust final volume to 1 L with dH₂O.
 - d. **Pepsin:** 1 mg/ml in Tris-HCl, pH 2.0.
8. **3% Hydrogen Peroxide:** To prepare 100 ml, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
9. **Blocking Solution:** TBST/5% Normal Goat Serum: to 5 ml 1X TBST, add 250 µl Normal Goat Serum (#5425).
10. **Detection System:** SignalStain® Boost IHC Detection Reagents (HRP, Mouse #8125; HRP, Rabbit #8114)
11. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**
 - a. Incubate sections in three washes of xylene for 5 min each.
 - b. Incubate sections in two washes of 100% ethanol for 10 min each.
 - c. Incubate sections in two washes of 95% ethanol for 10 min each.
2. Wash sections two times in dH₂O for 5 min each.

C. Antigen Unmasking*

NOTE: Consult product datasheet for specific recommendation for the unmasking solution/protocol.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA, pH 8.0; follow with 15 min at a sub-boiling temperature. No cooling is necessary.
3. **For TE:** Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0; then maintain at a sub-boiling temperature for 18 min. Cool at room temperature for 30 min.
4. **For Pepsin:** Digest for 10 min at 37°C.

D. Staining

NOTE: Consult product datasheet for recommended antibody diluent.

1. Wash sections in dH₂O three times for 5 min each.
2. Incubate sections in 3% hydrogen peroxide for 10 min.
3. Wash sections in dH₂O two times for 5 min each.
4. Wash sections in wash buffer for 5 min.
5. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
6. Remove blocking solution and add 100–400 µl primary antibody diluted in recommended antibody diluent to each section*. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections with wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100–400 µl SignalStain® DAB to each section and monitor closely. 1–10 min generally provides an acceptable staining intensity.
13. Immerse slides in dH₂O.
14. If desired, counterstain sections with hematoxylin per manufacturer's instructions.
15. Wash sections in dH₂O two times for 5 min each.
16. **Dehydrate sections:**
 - a. Incubate sections in 95% ethanol two times for 10 sec each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.
 - c. Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount sections with coverslips.



Recommended Application Solutions

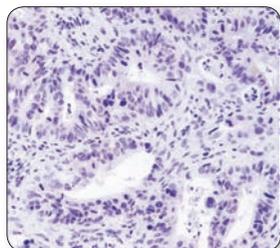
Cell Signaling Technology offers a wide selection of companion reagents, kits, and controls to support your IHC experiments. These products are used in-house for antibody validation in IHC and, therefore, work optimally with our primary antibodies.

Reagents Do Make A Difference!

Using the optimal reagents and protocols for your antibody can greatly improve your results.

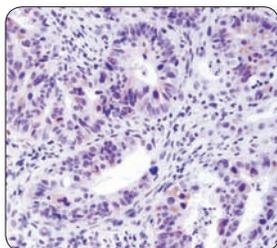
IHC analysis of paraffin-embedded human colon carcinoma using PLK1 (208G4) Rabbit mAb #4513 and various IHC reagents, as indicated.

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:



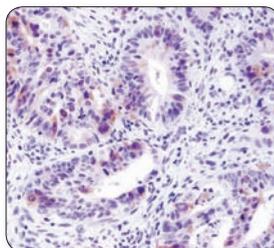
Diluent: TBST/5% NGS
Detection: biotin-based
Chromogen: NovaRed™

When our **SignalStain® Antibody Diluent #8112** was later developed, we observed a slightly improved signal as compared with our original conditions.



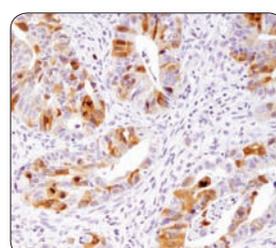
Diluent: #8112
Detection: biotin-based
Chromogen: NovaRed™

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



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

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: #8112
Detection: #8114
Chromogen: #8059

Reagents available from CST

Buffer Reagents

Normal Goat Serum #5425
Tris Buffered Saline with Tween® 20 (TBST-10X) #9997
Tris Buffered Saline (TBS-10X) #12498
Phosphate Buffered Saline with Tween® 20 (PBST-20X) #9809
Phosphate Buffered Saline (PBS-20X) #9808

Antibody Diluent

SignalStain® Antibody Diluent #8112

Detection Systems and Substrate

SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125
SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114
SignalStain® DAB Substrate Kit #8059

Controls

Rabbit (DA1E) mAb IgG XP® Isotype Control #3900
Mouse (G3A1) mAb IgG1 Isotype Control #5415
SignalSlide® Phospho-Akt (Ser473) IHC Controls #8101
SignalSlide® Cleaved Caspase-3 (Asp175) IHC Controls #8104
SignalSlide® Phospho-EGF Receptor IHC Controls #8102
SignalSlide® Phospho-ErbB Family IHC Controls #8117
SignalSlide® HER3/ErbB3 IHC Controls #12128
SignalSlide® Phospho-Met (Tyr1234/1235) IHC Controls #8118
SignalSlide® NF-κB p65 IHC Controls #12873
SignalSlide® Phospho-p44/42 MAPK (Thr202/Tyr204) IHC Controls #8103
SignalSlide® PTEN IHC Controls #8106
SignalSlide® Phospho-Stat1/3/5 IHC Controls #8105

IHC Kits

SignalStain® Akt Pathway IHC Sampler Kit #8107
SignalStain® Phospho-ErbB Family IHC Sampler Kit #8111
SignalStain® Proliferation/Apoptosis IHC Sampler Kit #8109
SignalStain® Phospho-Stat IHC Sampler Kit #8113
SignalStain® Apoptosis (Cleaved Caspase-3) IHC Detection Kit #12692

Blocking Peptides

CST offers an extensive list of blocking peptides for target specificity analysis. Visit www.cellsignal.com/blockingpeptides for a complete listing.

Other Suppliers

Solutions

Harleco® Dehydration Alcohol 100 from VWR, cat# 34172-020
Harleco® Dehydration Alcohol 95 from VWR, cat# 34172-022
Clearant Xylene from VWR, cat# 66004-950
Hydrogen peroxide solution 30% (w/w) H₂O, from Sigma, cat# H1009-100M

Counterstain

Vector® Hematoxylin from Vector Laboratories, Inc., cat# H-3401

Mounting Medium

VectraMount™ Mounting Medium from Vector Laboratories, Inc., cat# H-5000

Coverslips

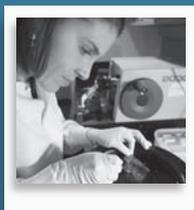
25 X 50 mm (1" X 2") from VWR, cat# 48393-241

CST offers companion product application solutions to aid in your IHC analysis.

www.cellsignal.com/ihccompanions

Technical Support

We hope this paper is a helpful resource for performing Immunohistochemistry in your own lab. Cell Signaling Technology prides itself in providing you with exceptional customer service and support, and we are happy to share our experience with you. Since all of our antibodies are produced in-house, the same scientists who develop and assay these reagents are available as technical resources for our customers. These scientists can be contacted directly and will personally provide technical assistance to you, our customer.



FRONT COVER PHOTO:
Jes, IHC Group Leader, has been with CST for over 6 years.

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