Cell Signaling Technology, Inc.

Immunohistochemistry

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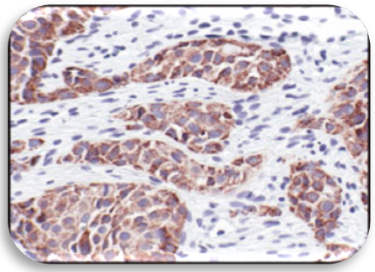
Product Specialist

Unparalleled Product Quality, Validation, and Technical Support

Outline

- Protocol and Companion products comparison
- Troubleshooting
- Automated Staining Platforms
- Miscellaneous Questions

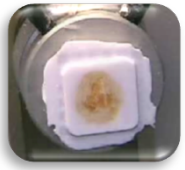
Just to remember



Immunohistochemistry (IHC)
Chromogenic detection of proteins in paraffin-embedded or frozen tissues or cells

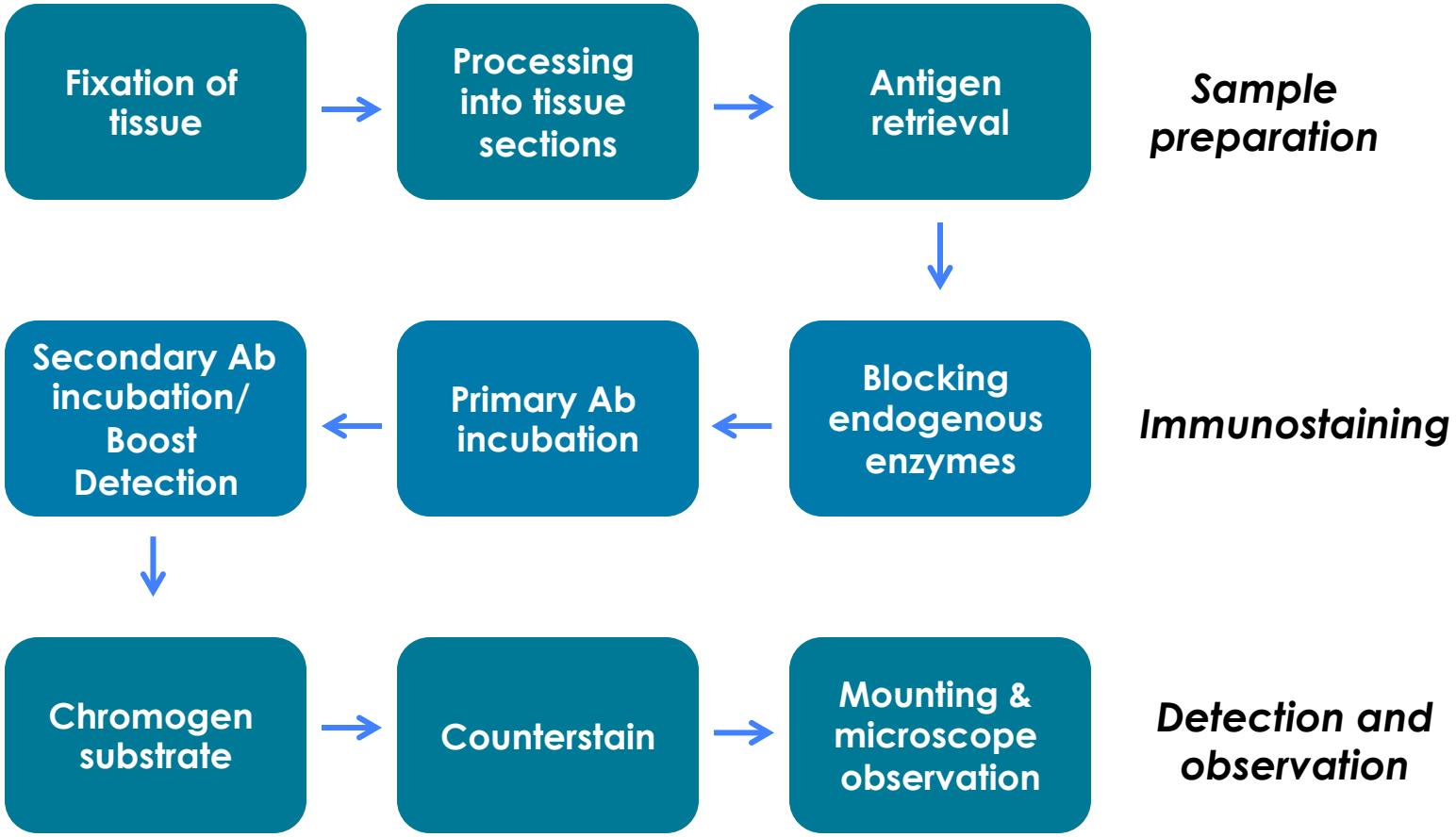
1. **Sample preparation**
   - Paraffin embedded (IHC-P)

   - Frozen (IHC-F)



2. **Immnunostaining**
3. **Observation**

IHC Protocol

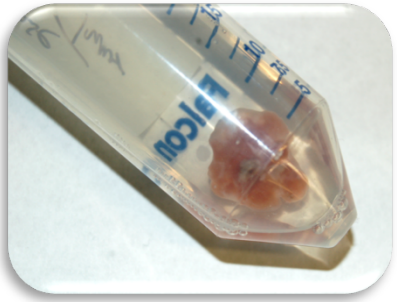


1. Fixation

Preservation and hardening of a tissue sample to retain its form and structure

- **10% Neutral buffered formalin (NBF) used in-house**
- 3-4% Paraformaldehyde (PFA)
- Alternative fixatives – Not recommended
  - Zinc formalin, Bouin's (picric acid, acetic acid, formaldehyde), Zamboni (paraformaldehyde and picric acid), B-5, Zenker's (Mercury), Carnoy's
- Frozen tissues do not need fixation (LN_2 frozen and OCT embedded)
- For optimal fixative refer to product datasheet

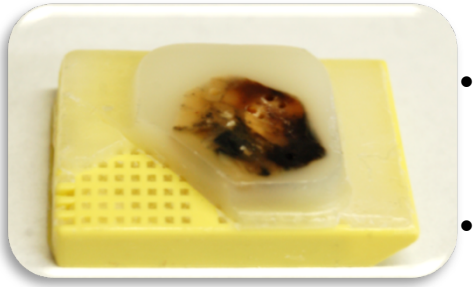
Things to take into account when fixing



- **Cross-linking fixatives**: 10% neutral buffered formalin (NBF) or 3-4% PFA
  - Over fixation can result in poor staining
  - Under fixation will result in edge effect, high background and/or poor resolution
  - Cross-linking can interfere with recognition of proteins
- **Fixative agents that don't cause cross-linking** (organic solvents: acetone, methanol, ethanol) **not recommended**
  - Inadequate cellular preservation
  - Variable tissue recognition

"The more fixatives you add to a laboratory, the more complicated you make it for everyone"

Paraffin embedding and deparaffination



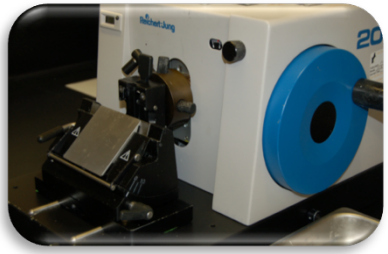
- Following fixation, tissue is dehydrated in sequential baths of ethanol and xylene
- Tissue is infiltrated with molten paraffin and then placed in fresh paraffin, forming a block
- These blocks can be stored and sectioned for long periods
- After sectioning, paraffin has to be removed by soaking slides in xylene and then rehydrated with an ethanol gradient
- Incorrect deparaffination - common cause of non-staining

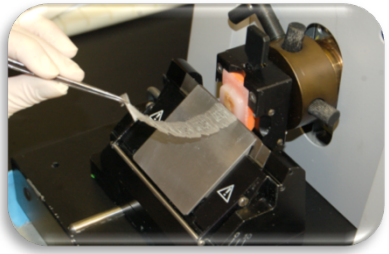
2. Processing into tissue sections

1. **Fresh frozen tissue** – LN_2 frozen and OCT embedded
2. **Fixed cryosections** – animal is perfused (quick fixation) then tissue is frozen and OCT embedded
3. **FFPE** – formalin-fixed paraffin-embedded

→ ***Cryostat***

→ ***Microtome***





IHC-P: Thin slices (4-6 μm) cut on a microtome, floated in a water bath, mounted on slides & dried overnight

IHC-F: 10-20 μm sections cut cold on a cryostat, mounted on slides and air dried

3. Antigen Retrieval

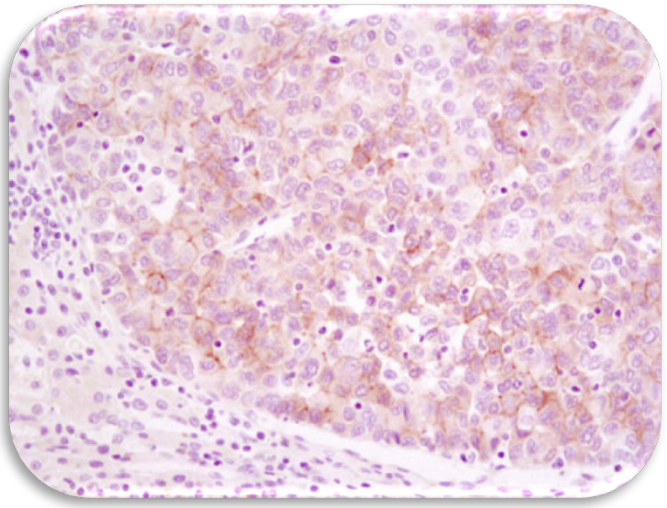
Removal of physical and chemical masks of epitopes as a result of fixation (IHC-P only)

- **Heat (HIER)**: Microwave, pressure cooker (Decloaking Chamber), water bath, autoclave, rice cooker, steamer
- **Enzyme**: Pepsin, Trypsin, Proteinase K, Enzyme cocktail

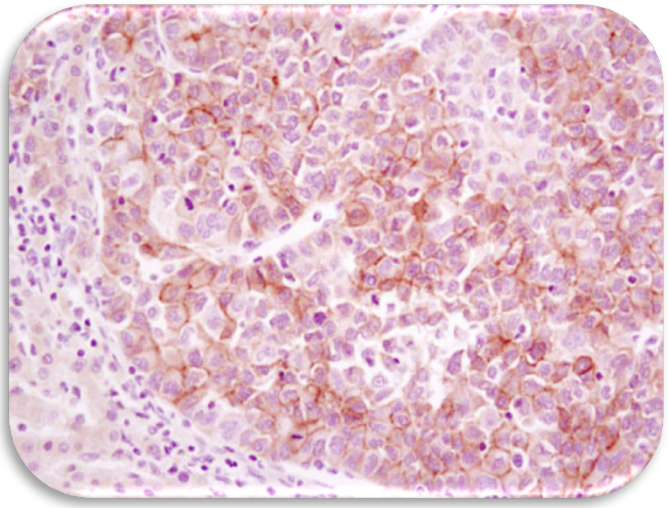
- The most uncontrolled step in IHC and most frequent cause of no-staining
- ***Our protocol: Heat (microwave) + Citrate, EDTA, TE or Pepsin***
  - Generally, phospho-antibodies perform better with EDTA
  - Antibodies that work with citrate likely to work with EDTA (may require titration)
  - Antibodies that work with EDTA not likely to perform well with citrate
  - EDTA tends to boost signal, both specific and non-specific

Impact of Antigen Retrieval

Citrate

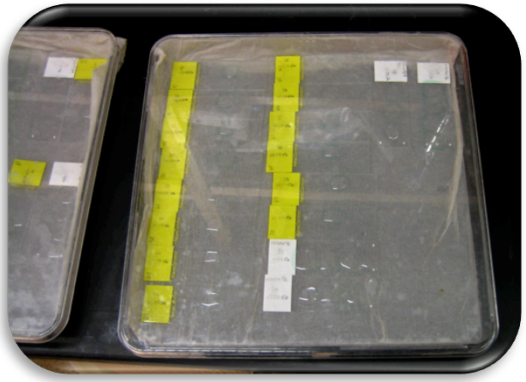


EDTA



EGF Receptor (D38B1) XP® Rabbit mAb #4267

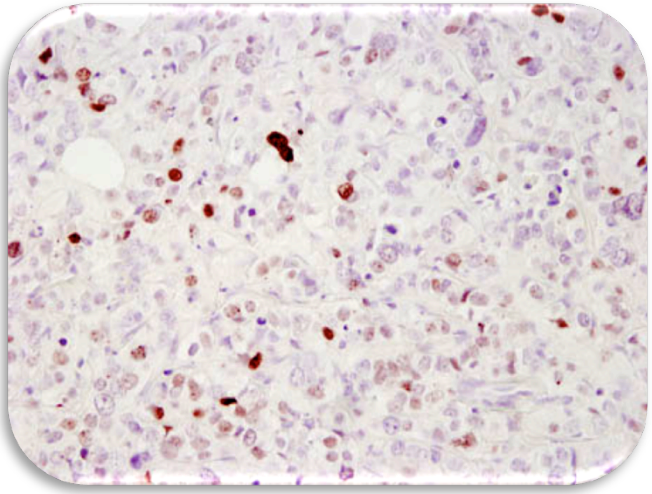
4. Blocking



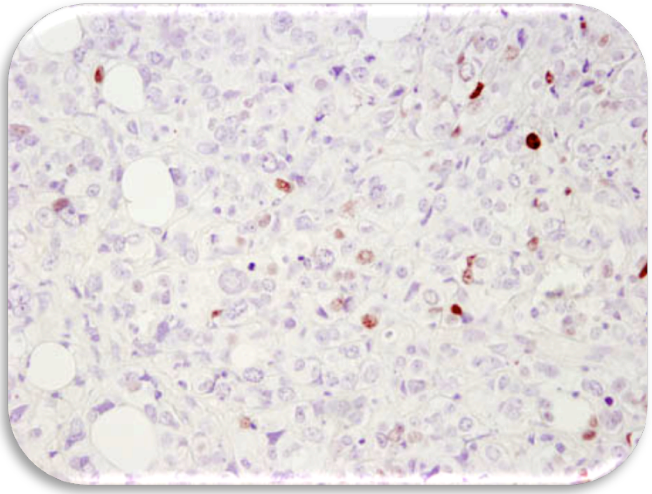
1. **Blocking of endogenous peroxidase** - Peroxidase quench
    - exhausts any endogenous peroxidase activity in samples
    - only needed when HRP detection is employed
2. **Blocking non-specific binding**
    - TBST/ 5% NGS (#5425)
    - Casein-containing blocking solutions may impact staining with phospho-specific antibodies

Impact of Blocking Agent

TBST/5% NGS



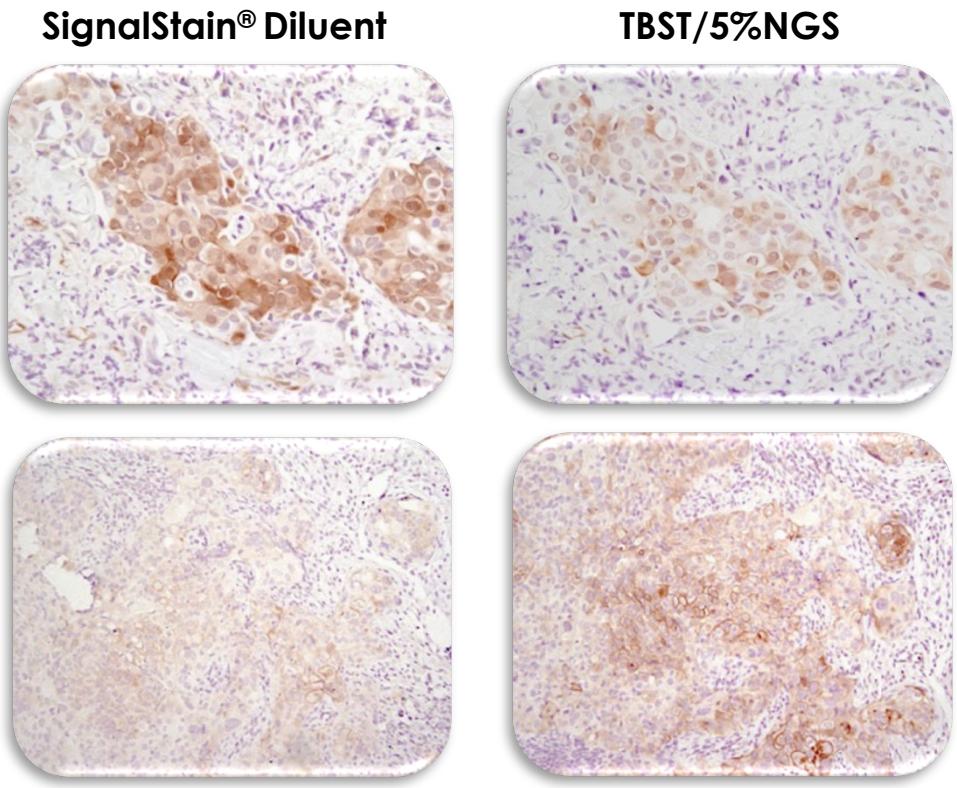
Casein block



Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb #9718

5. Primary Antibody incubation

- Dilution buffer will impact staining:
  - SignalStain® Antibody Diluent #8112
  - 5% NGS in TBST or PBST

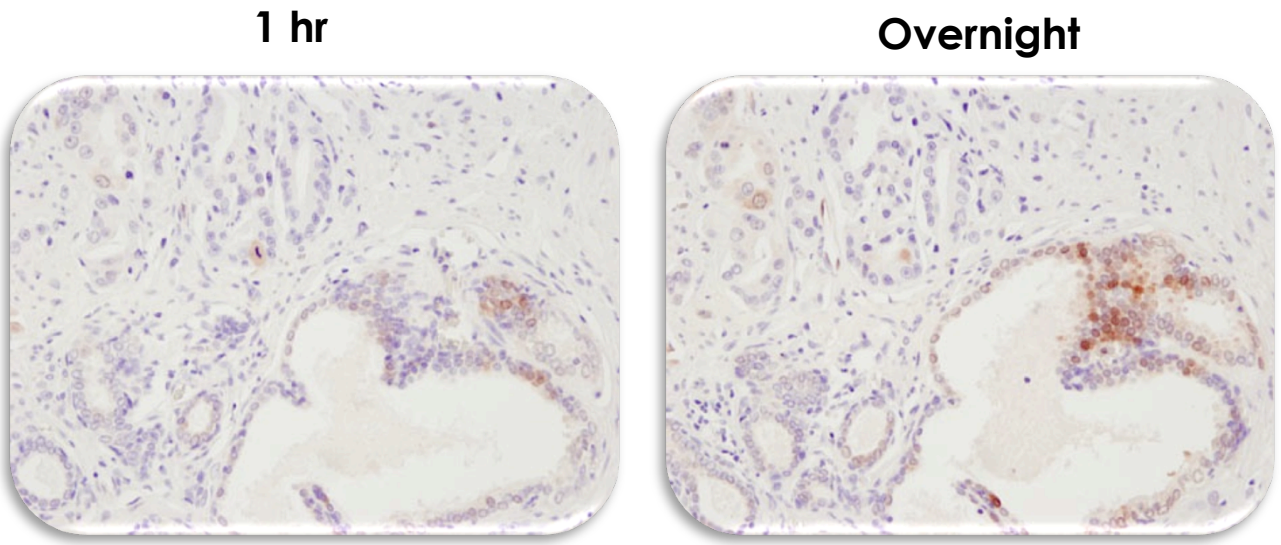


Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060

Phospho-EGF Receptor (Tyr1173) (53A5) Rabbit mAb #4407

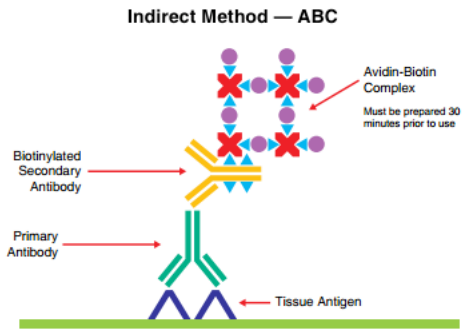
5. Primary Antibody incubation

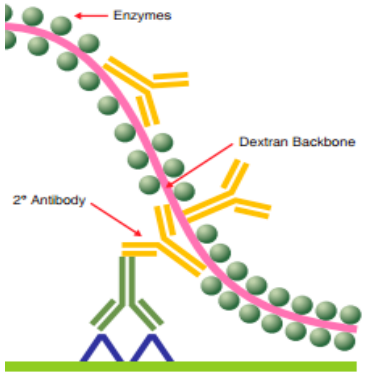
- Incubation time will also impact the staining: overnight in cold room
  - *Many antibodies are likely to work fine with a brief incubation. Always perform a side-by-side comparison to ensure optimal performance*



Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855

6. Detection

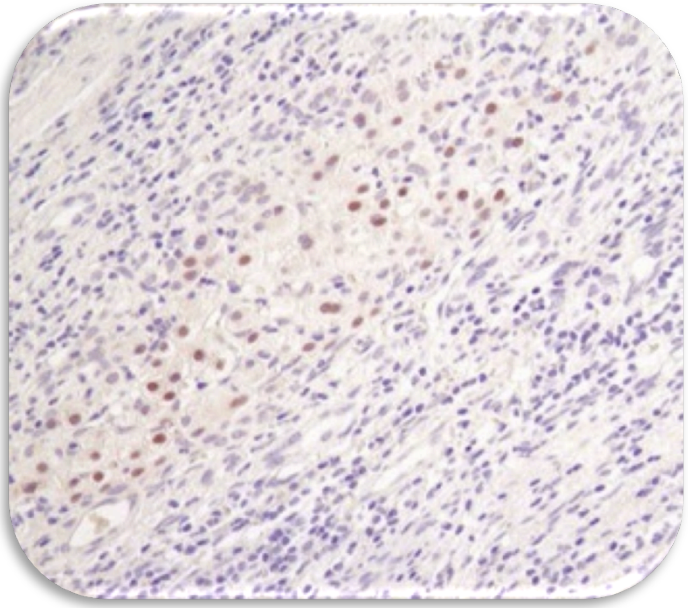




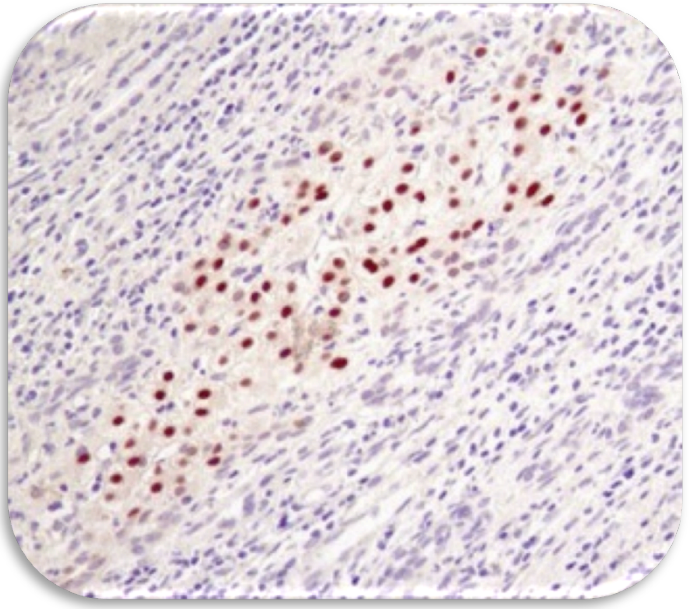
- **Avidin-biotin complex detection systems**
  - Rely on the strong affinity of avidin for biotin
  - 2ry antibody conjugated to biotin and avidin-peroxidase complex
  - Avidin binds non-specifically to lectin-like and negatively-charged tissue components
  - Background due to endogenous biotin
- **Polymer based detection system (SignalStain® Boost Detection Reagent)**
  - Does not rely on biotin: Backbone bound with secondary antibodies and peroxidase
  - One-step system and greater sensitivity
  - Many commercially available. Boost is among the most sensitive reagents we have evaluated

Impact of detection reagent

ABC detection



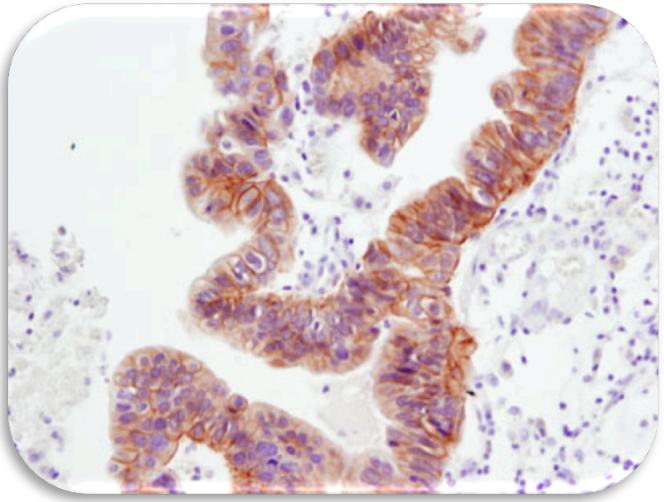
SignalStain® Boost Detection



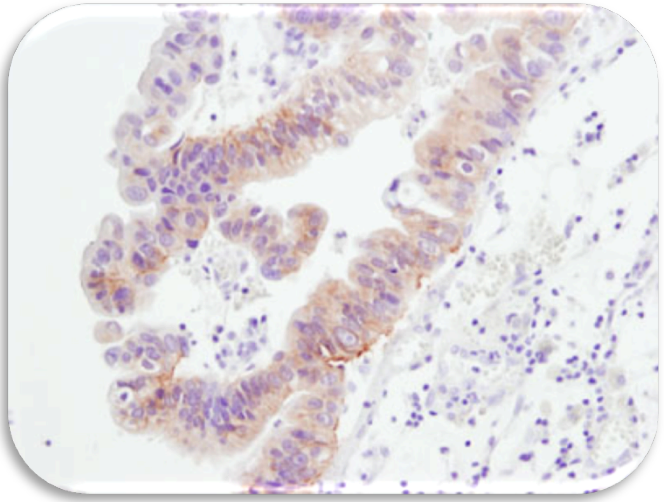
HNF4 α (C11F12) Rabbit mAb #3113

Impact of detection reagent

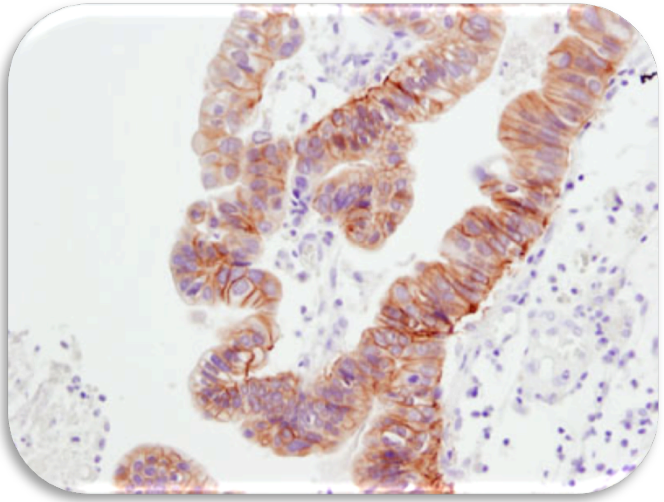
Boost #8114



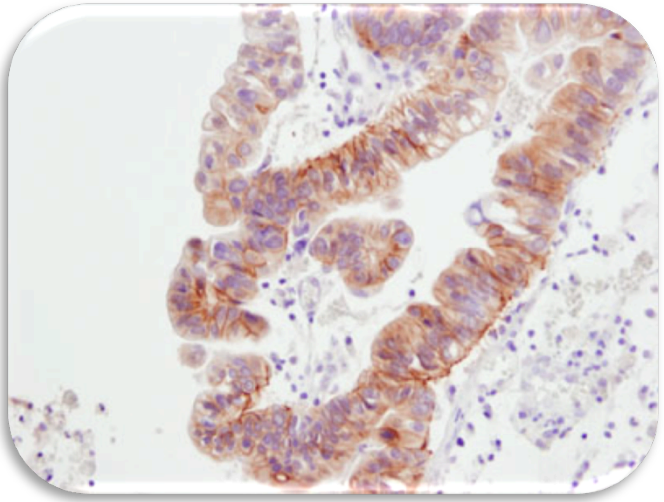
Competitor #1



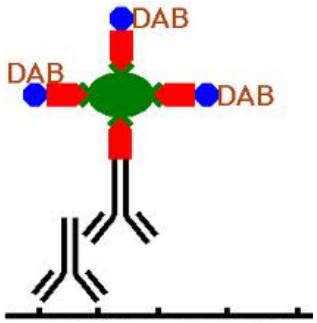
Competitor #2



Competitor #3

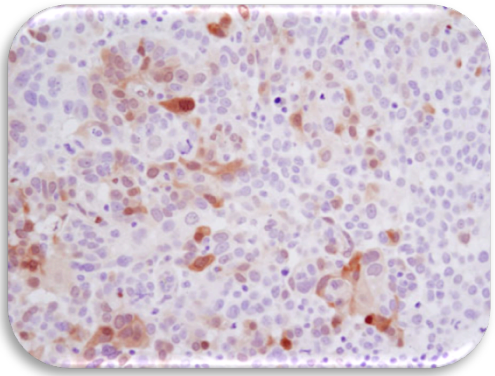


7. Chromogen substrate

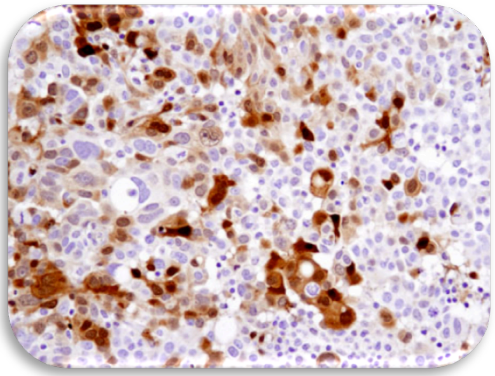


- Diaminobenzidine (DAB) reacts with peroxidase and forms a dark brown chromogen
- There are differences among commercially available DAB products
- #8059 DAB is universally superior to NovaRed
- One of commercially available DAB products that yield the strongest signal

NovaRed



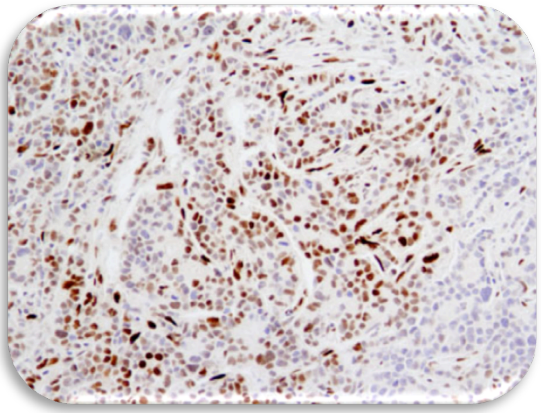
SignalStain® DAB #8059



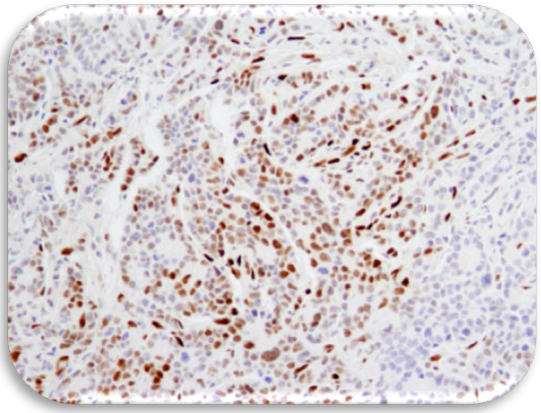
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370

7. Chromogen substrate

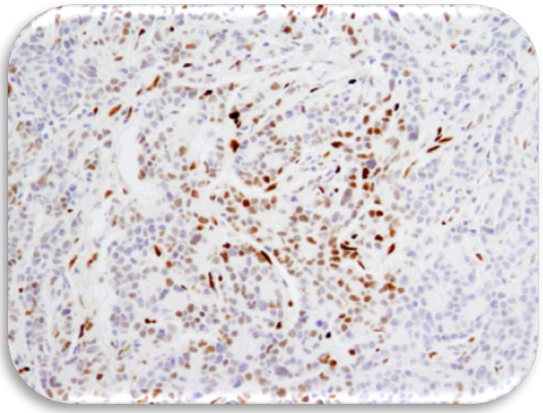
#8059 DAB



Competitor #1



Competitor #2



Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145

Outline

- Protocol and Companion products comparison
- Troubleshooting
- Automated Staining Platforms
- Miscellaneous Questions

Troubleshooting

Problem	Possible causes	Solutions
No staining	• Insufficient deparaffinization • Epitope modified by fixation • Protein not present/ low expression • Ab not suitable	• Deparaffinize for optimal period • Fixation for optimal period • Use antigen retrieval steps • Use positive controls
High background	• Insufficient washing • Insufficient blocking • Epitope modified by fixation • Too much enzymatic detection substrate	• Use appropriate blocking buffer and times • Wash with PBS between all steps • Use suitable Ag retrieval method • Use secondary control (no primary Ab)
Non-Specific staining	• Ab concentration too high • Active endogenous peroxidase	• Use negative controls • Block endogenous peroxidase

Outline

- Protocol and Companion products comparison
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Tips to use automated staining platforms

- CST antibodies validated for IHC can be used in automated platforms
- Control slides are needed and can be produced for the customer to assist with optimization
- Antibody dilution:
  - Some antibodies will work fine at recommended dilution
  - Most will require a higher concentration to work optimally – titration up to optimize
  - SignalStain® antibody diluent (#8112) can be used in all instruments but requires manual application of the primary antibody

Outline

- Protocol and Companion products comparison
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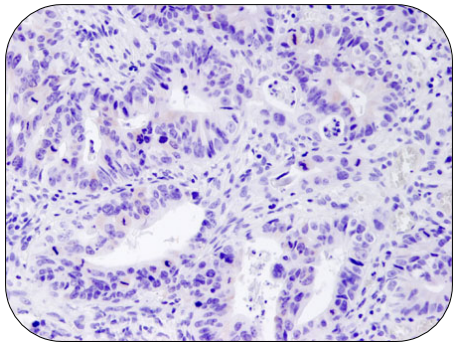
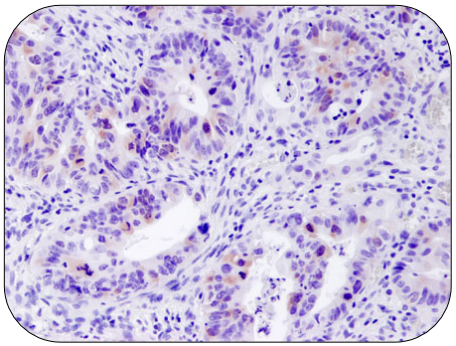
Benefits of using CST companion reagents

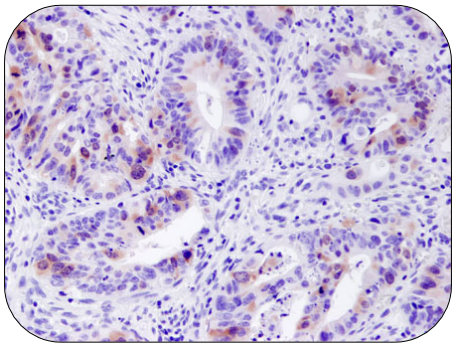
PLK1 (208G4) Rabbit mAb #4513



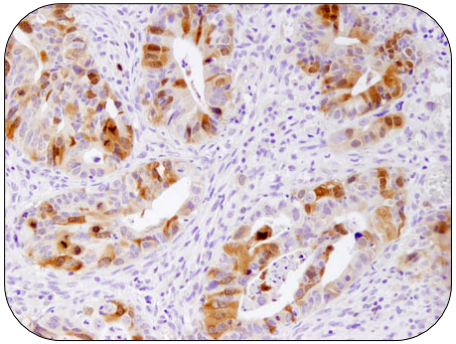
- Diluent: TBST-5% NGS
- Detection: Biotin-based
- Chromogen: NovaRed



- Diluent: **SignalStain® Antibody Diluent #8112**
- Detection: Biotin-based
- Chromogen: NovaRed



- Diluent: **SignalStain® Antibody Diluent #8112**
- Detection: **SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114**
- Chromogen: NovaRed



- Diluent: **SignalStain® Antibody Diluent #8112**
- Detection: **SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114**
- Chromogen: **SignalStain® DAB Substrate Kit #8059**

IHC – Mouse on Mouse

The mouse research community has experience difficulties for years as there were only mouse monoclonal Abs available

"Antigen detection with mouse primary Ab on mouse tissues is complicated by high levels of background staining due to the binding of secondary anti-mouse Ab to endogenous mouse tissue IgG and other components"

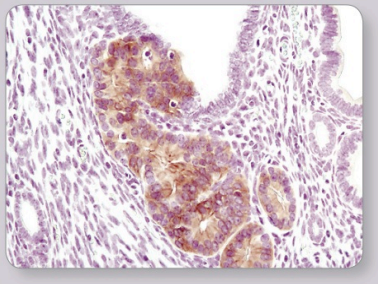
CST solution = rabbit monoclonal and XP antibodies validated on mouse

Antibodies Validated for IHC on Mouse Tissue

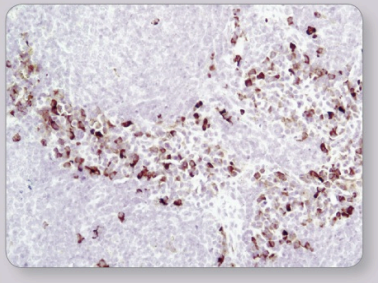
The development and characterization of mouse models are critical for drug discovery and the advancement of our understanding of basic cancer biology.

A growing number of antibodies from Cell Signaling Technology (CST) have been validated by our IHC group for use on mouse tissue. IHC-validated CST™ rabbit monoclonal antibodies demonstrate the exceptional specificity, sensitivity, and performance required for credible results in challenging applications such as IHC, while eliminating problems seen with mouse-on-mouse staining.

For the most up-to-date list of antibodies validated for IHC on mouse tissue, please visit our website.



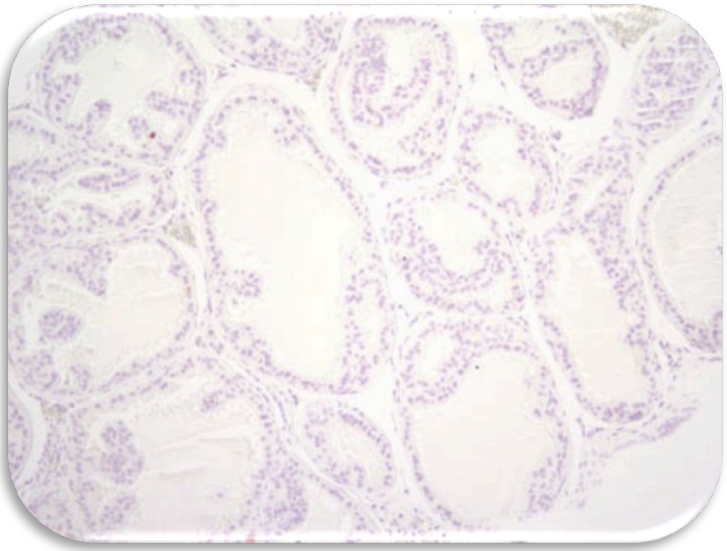
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060: *IHC analysis of paraffin-embedded PTEN heterozygous mutant mouse endometrium using #4060. (Tissue section courtesy of Dr. Sabina Signoretti, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.)*



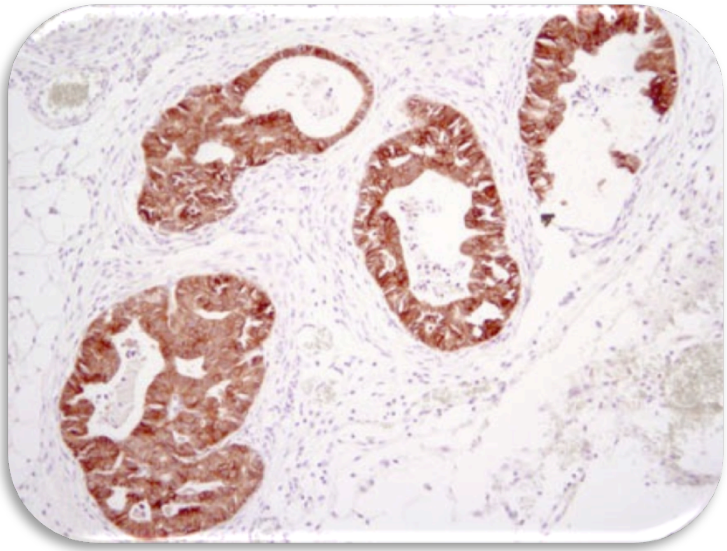
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858: *IHC analysis of paraffin-embedded mouse spleen using #4858.*

Mouse Model of Cancer

Wild type (mouse prostate)



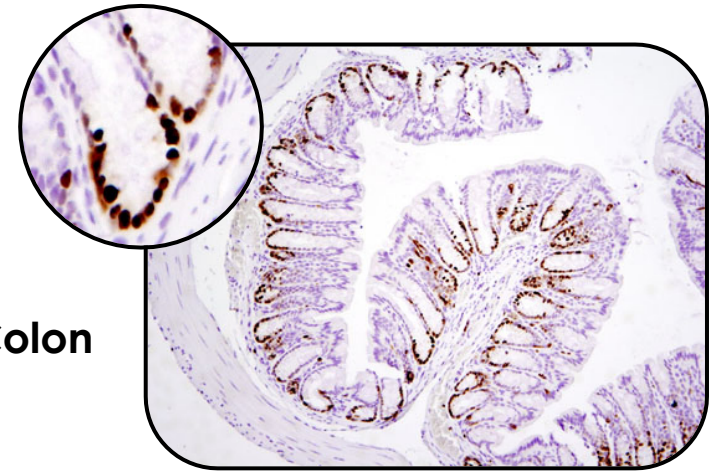
PTEN -/- (mouse prostate)

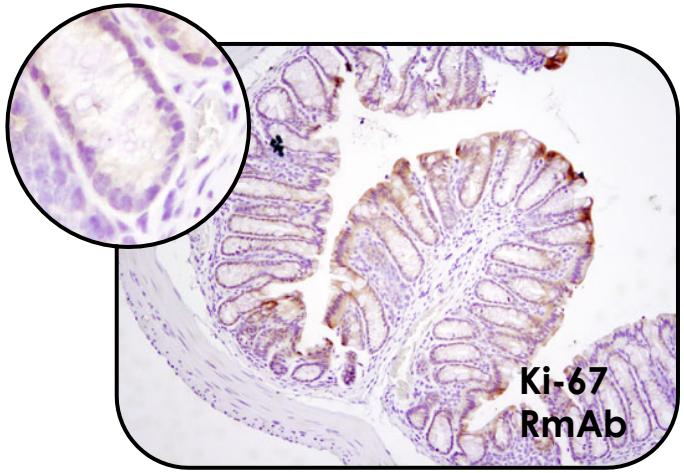


Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060

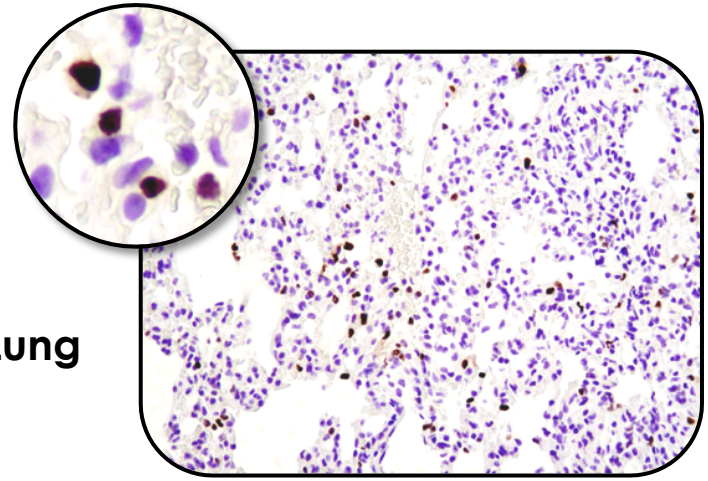
Mouse IHC and competition

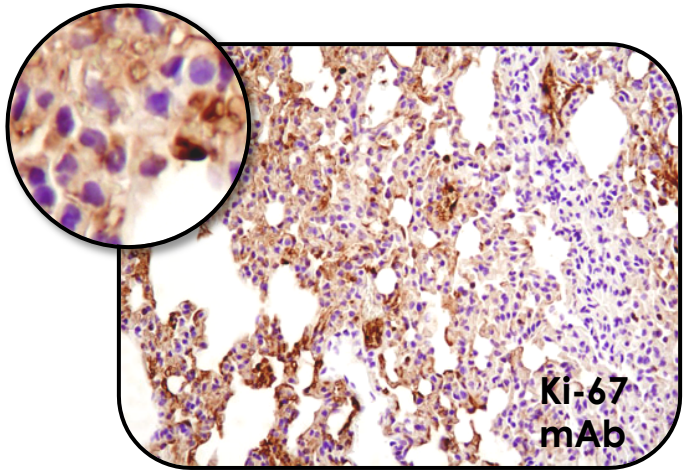
Colon





Lung

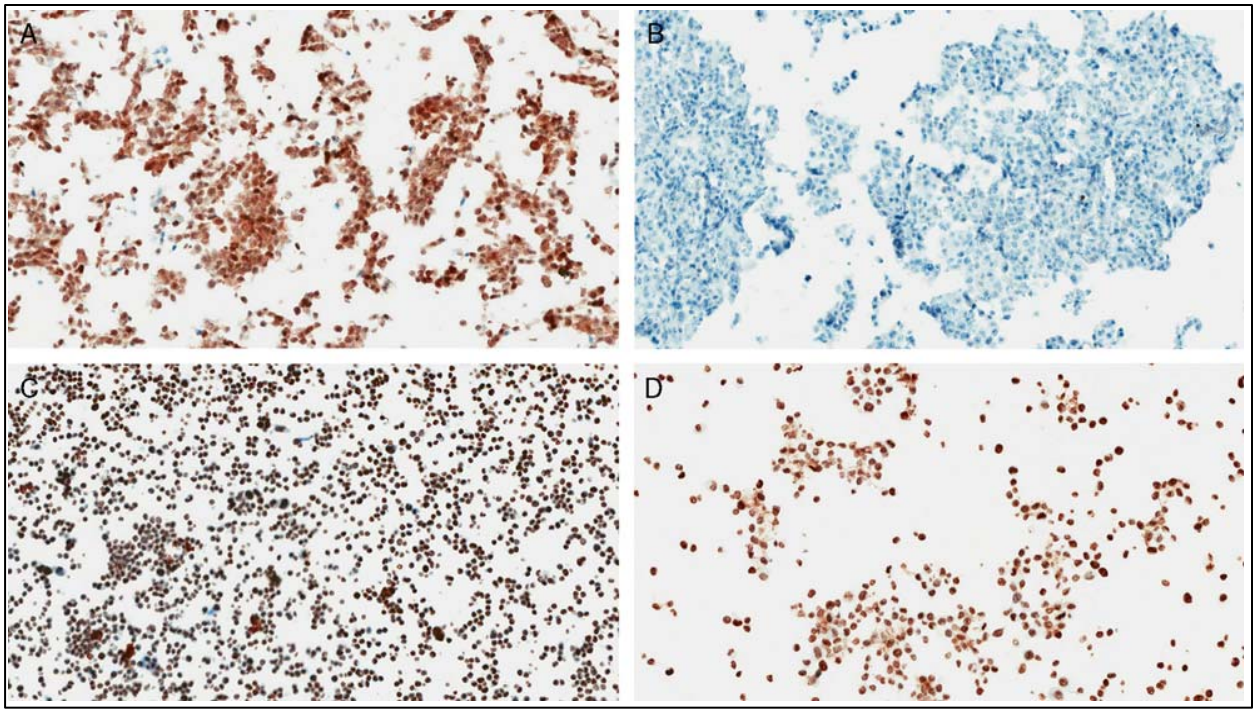




Ki-67 (D3B5) Rabbit mAb
(Mouse Specific; IHC Formulated) #12202

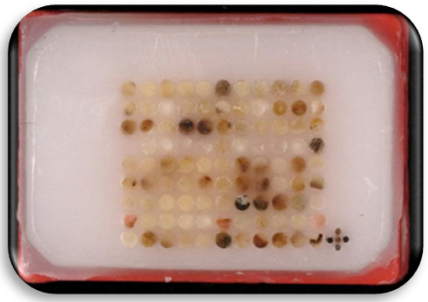
Competitor monoclonal

What sets us apart from the competition?



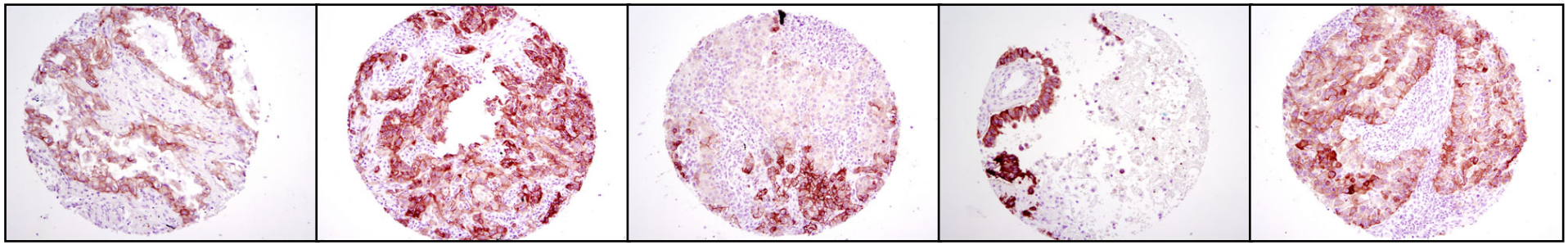
Sangale *et al*, 2011. Appl Immunohistochem Mol Morphol, 19: 173

Validation sets us apart

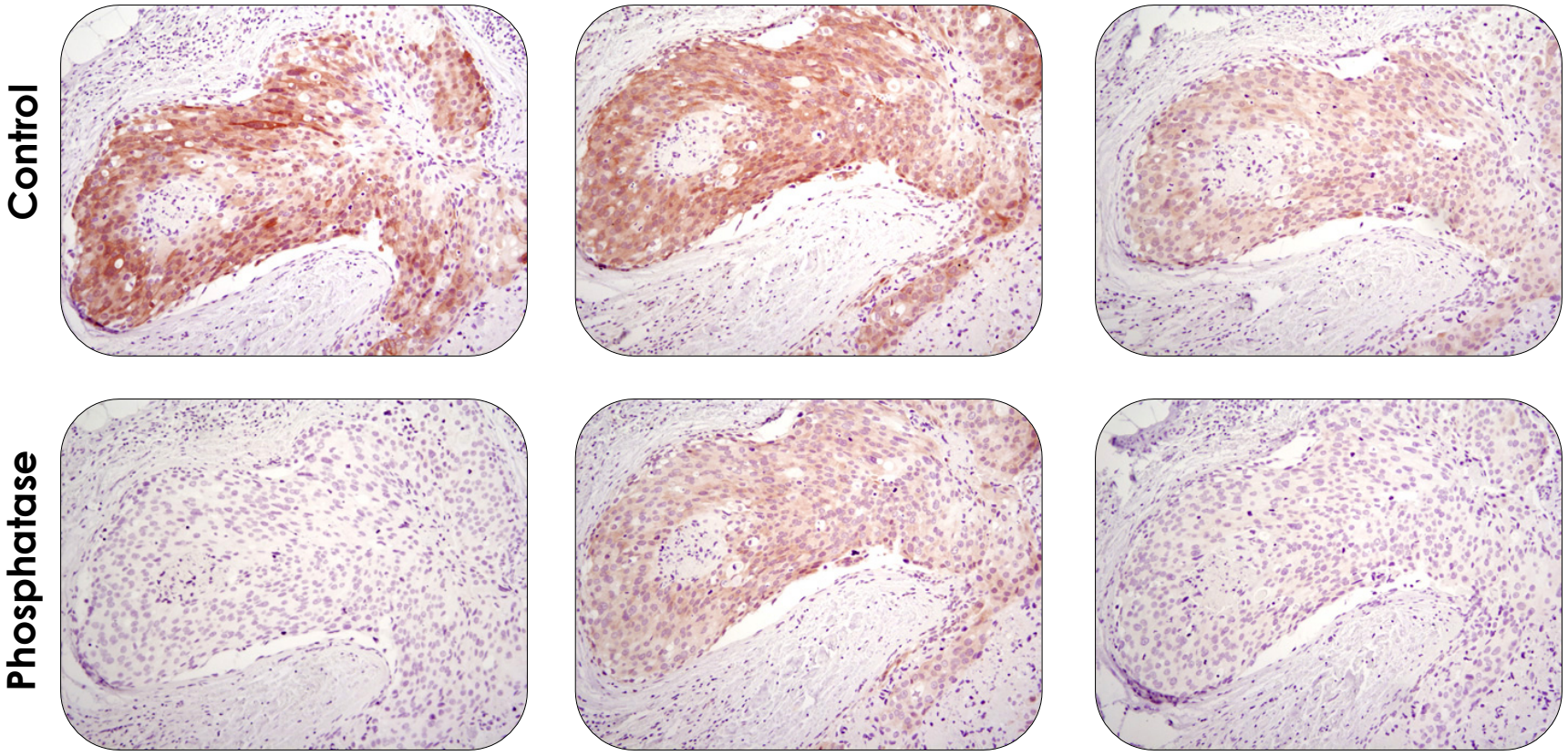


Tissue Microarray:

Allows for the assessment of performance over a large number of tissues or a wide range of tissue types



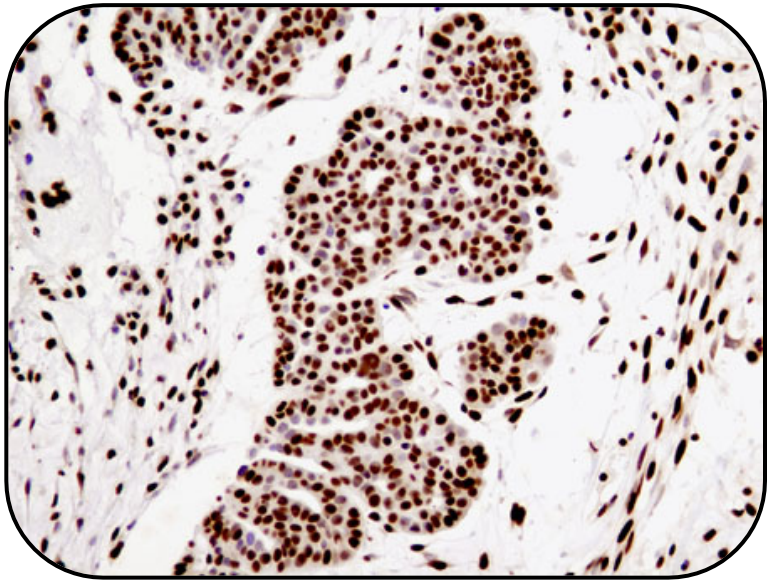
Validation sets us apart



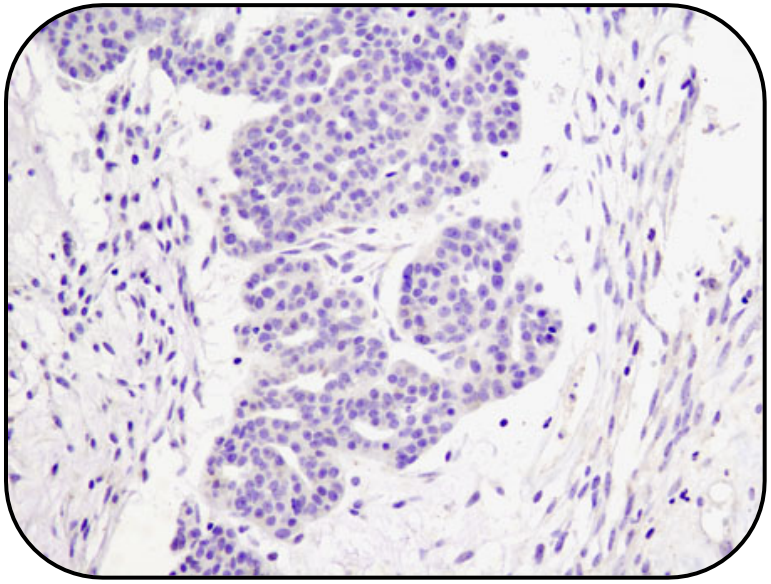
Other unique validation steps

Verification of Acetyl-specificity

Non-acetyl peptide



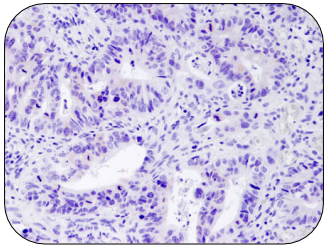
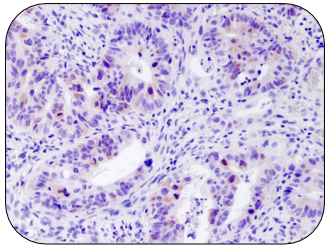
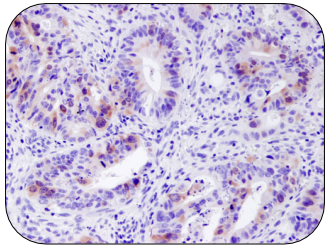
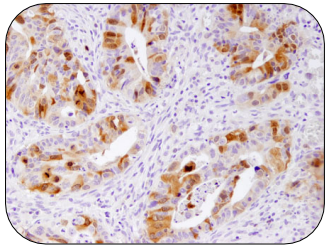
Acetyl peptide



Acetyl-Histone H3 (Lys9/Lys14) Antibody #9677

Summary

- Complex technique that requires highly specific antibodies
- The advent of polymer detection systems and automated stainers has elevated the role of IHC in diagnostics and biomarker research
- Antibody-antigen interactions are complex, and one standard protocol is not the answer for every product
- Deviation from these conditions is one of the most common themes in tech support

Cell Signaling Technology, Inc.

Questions?
Thank you!

Unparalleled Product Quality, Validation, and Technical Support