Optimizing IHC for challenging tissues

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Outline

- Introduction
- Antibody selection
- Antigen retrieval
- Blocking
- Controls

Immunohistochemistry

To determine **the localization of antigens** (expression) in tissue sections using **labeled antibody** as specific reagent through antigen-antibody interactions that are visualized by marker such as **fluorescent dye or enzyme**

Selection of the proper antibody

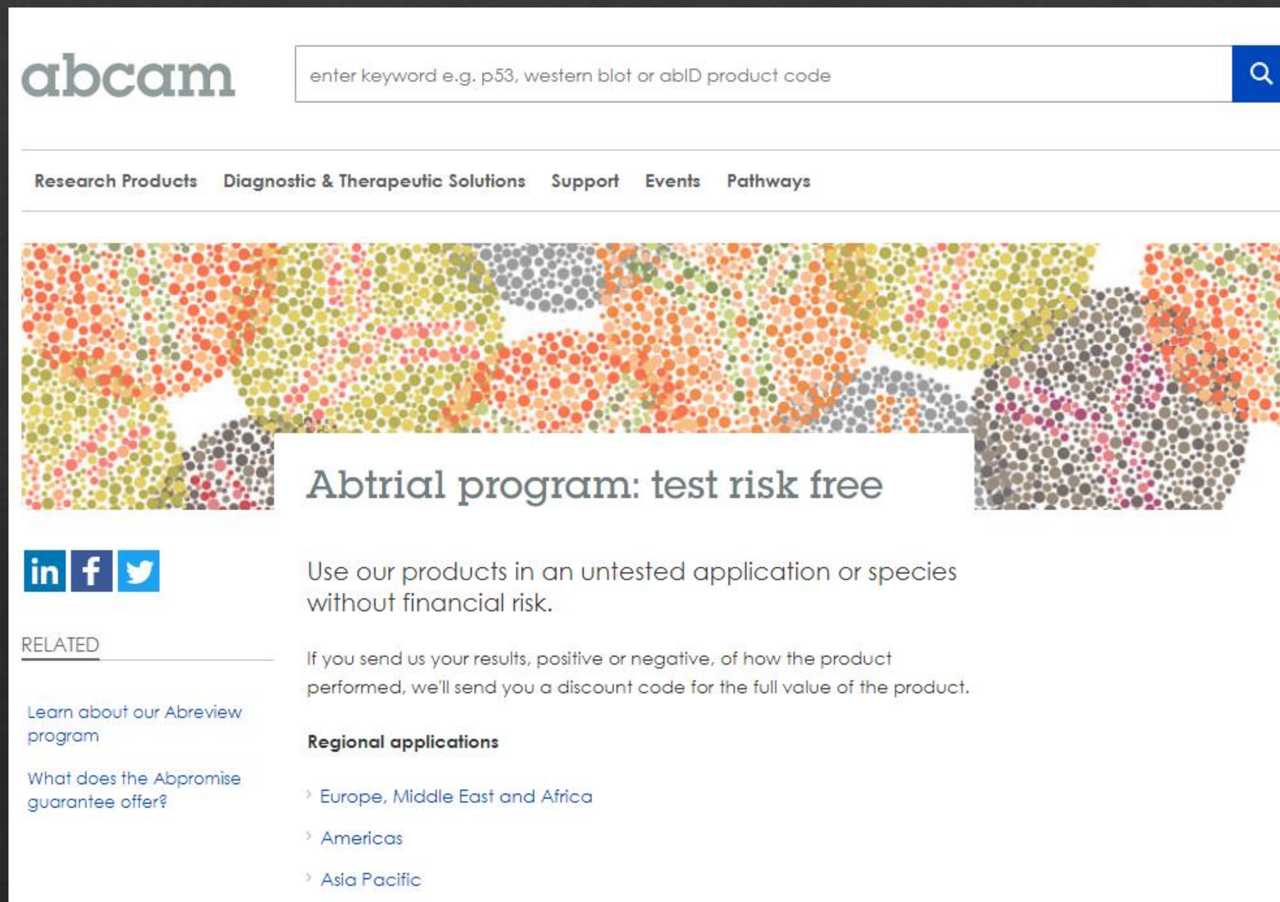
- **Antibody type:**

Monoclonal	Polyclonal
Produced by the single B cell clone	Derived from multiple B-cells
Harvested from culture vessels	Harvested directly from animal serum
High specificity	High affinity
Less cross reactivity	High cross reactivity
High reproducibility	More robust detection

- **Reactivity: dependent on the species of your sample**
  - Searching database: https://www.antibodypedia.com/; https://www.uniprot.org/
  - BLAST to test cross reactivity
  - Abtrial program
- **Antibody dilution and incubation time**

IHC on specimen of special species: Abtrial program

- https://www.abcam.com/content/abtrial-program-test-risk-free



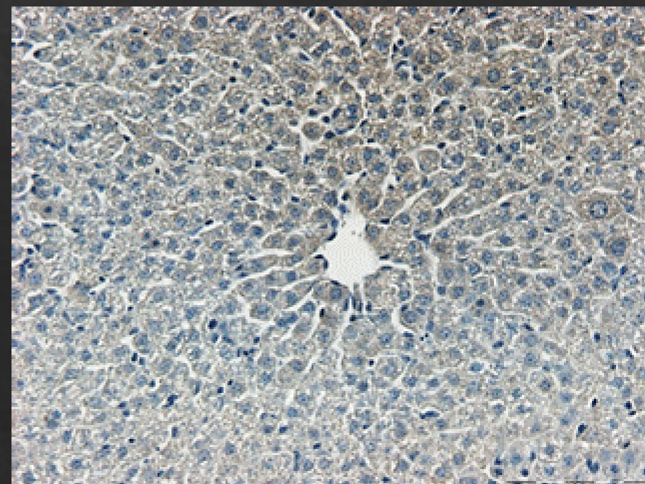
1. Email and ask about the antibody, application and species you are interested in
2. Technical team check the cross reactivity of the antibody
3. Submit order and forward order number to Abcam
4. Receive and test your antibody
5. Submit your result on Abreview
6. Get approval and activate your discount code

Why antigen retrieval is need

- Introduced by Shi et al in 1991
- 4% PFA or 10% NBF fixed paraffin sections (FFPE)
  - Molecular modification of antigens may result in loss of the ability of the antibody to react with the antigen (protein cross linking)
  - Can be corrected by retrieval
- Commonly refer to the datasheet of your antibody

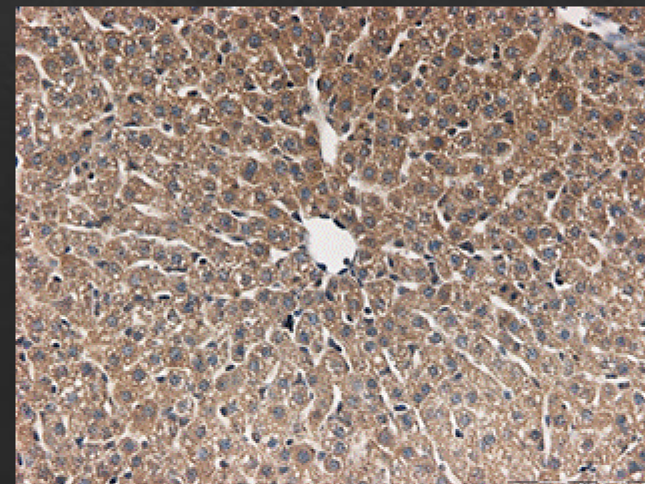
α-phos-STAT5
Mouse liver sections

Weak staining



No antigen retrieval

Optimal signal



+Citrate pH 6.0 microwave 3 min

Heat-induced vs proteolytic-induced epitope retrieval

HIER **80°C-100°C (20min water bath) or microwave** 60**°C (overnight) (good for fragile sections)**	**PIER** **37°C (3min - 30min)**
Citrate buffer, pH 6.0 (10mM sodium citrate)	Pepsin (1mg/mL) in Tris-HCl, pH 2.0
Tris-EDTA buffer, pH 9.0 (10mMTris/1mM EDTA)	Proteinase K (20μg/ml) in TE buffer, pH 8.0* (good for membrane antigens)
EDTA buffer, pH 8.0 (1mM EDTA)	Trypsin (0.05%) in $CaCl_2$, pH 7.8*
	Combination of 0.1U/mL chondroitinase ABC and 2 mg/mL hyaluronidase in PBS with 0.02% BSA (goof for GAG rich tissue)

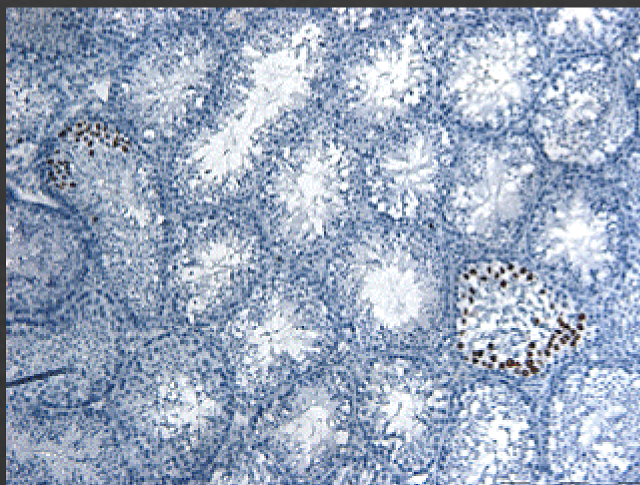
* May damage sections if incubated too long

HIER vs PIER

α-phos-histone H3

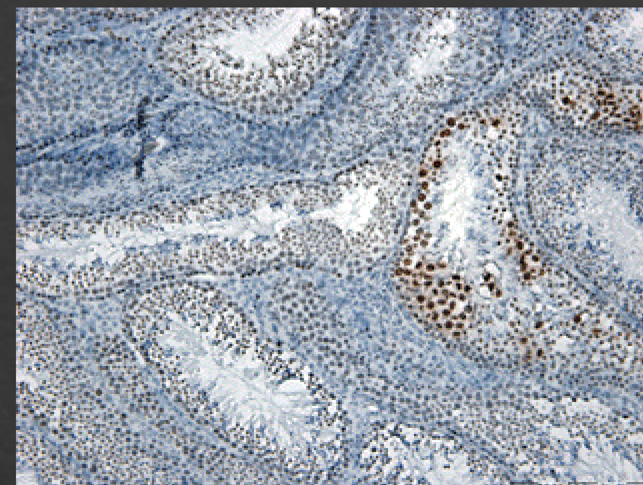
Mouse testis sections

Weak staining



+pepsin
37°C 30 min

Optimal signal

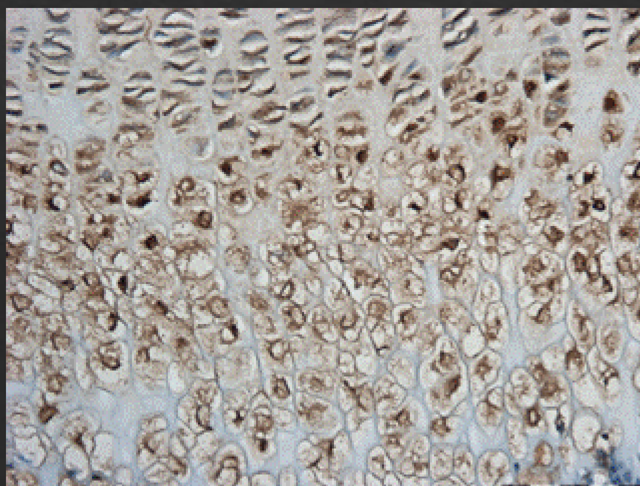


+Citrate pH 6.0
microwave 3 min

α-p57

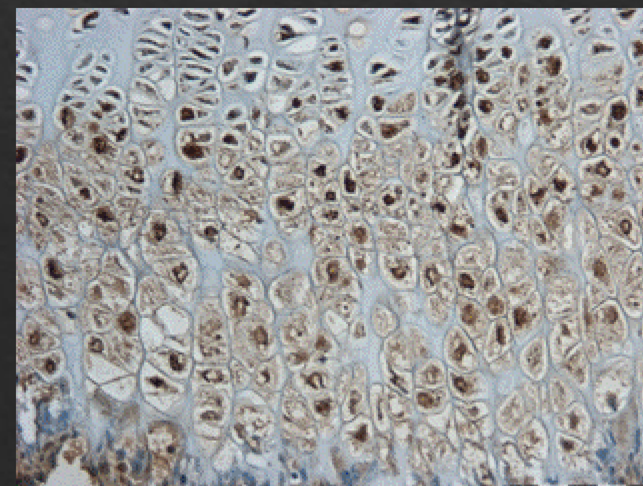
Mouse tibia growth plate sections

Background staining



+chondrotinase

Optimal signal



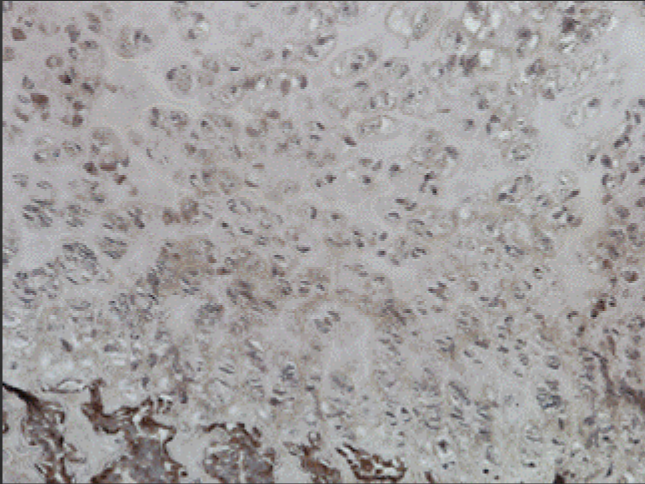
+Citrate pH 6.0

HIER: temperature, pH and time

α-RUNX2

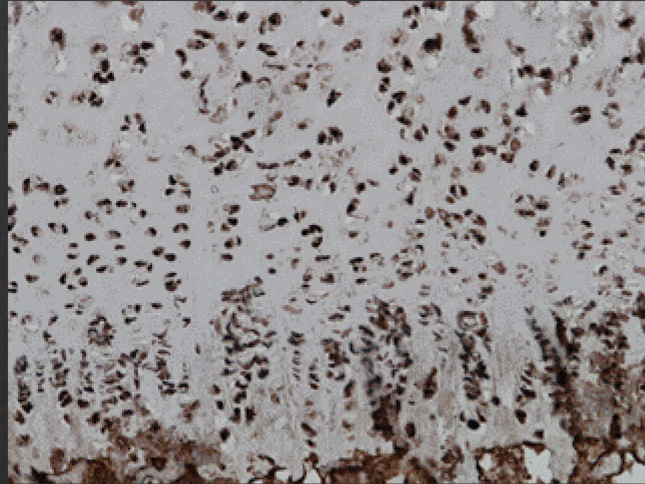
Dog vertebrae sections

Weak signal



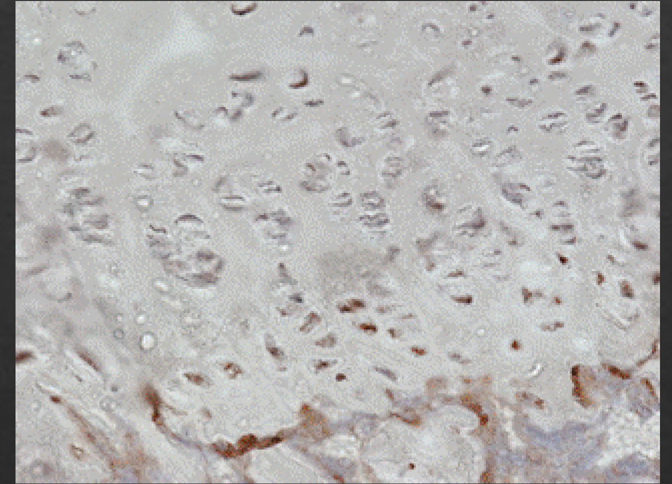
+Citrate pH 6.0
60°C O/N

Overstained signal



+EDTA pH 8.0
60°C O/N

Optimal signal



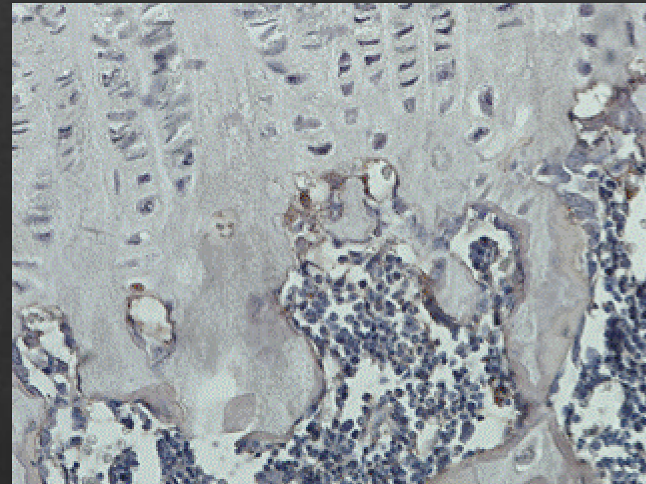
+Citrate pH 8.0
80°C 20 min

PIER combination

α-MMP9

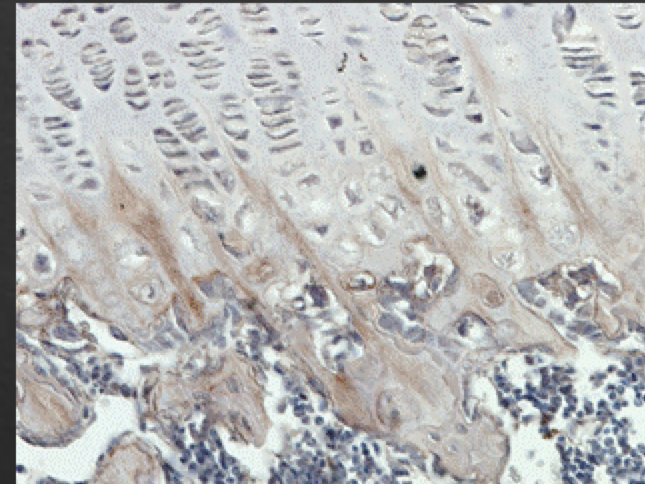
Dog vertebrae sections

Weak signal



+chondrotinase
37°C 30 min

Optimal signal



+chondrotinase
+hyaluronidase
37°C 30 min

Blocking - reduce background and unspecific staining

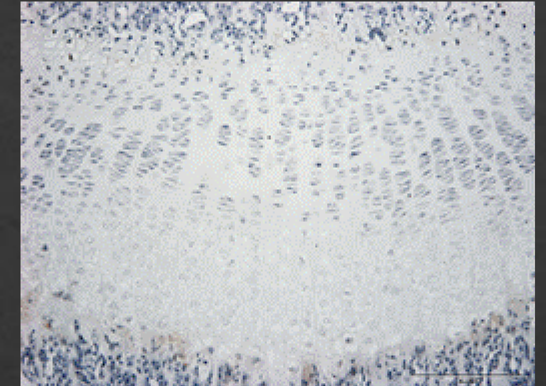
- Endogenous peroxidase blocking

  3% H_2O_2, room temperature, 10-15 min in methanol (peroxidase-rich tissue) or PBS (cell surface or membrane markers)

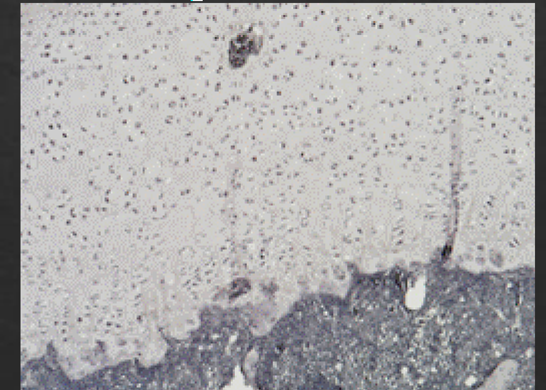
- Serum blocking solution
  - 5% Goat serum
  - 5% Horse serum
  - 1% BSA (or higher w/v)
- Commercial blocking buffer

Which samples can be used as controls?

- **Positive control:**
  - High expression structure
  - Tissue from species with known reactivity
- **Negative control:**
  - No primary antibody control: simple necessary
  - Isotype control: good for monoclonal primary antibodies
    - Incubate with a non-immune immunoglobulin of the same isotype and concentration as the primary primary antibody
  - Absorption control: antibody binds specifically to the antigen of interest
  - Tissue type control: tissue known to not express the antigen of interest



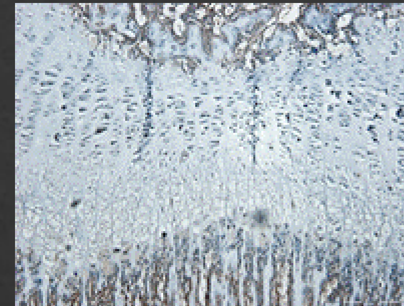
+citrate pH 6.0 60°C O/N
Mouse tibia growth plate
Rabbit IgG control



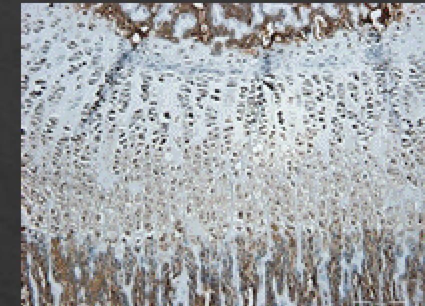
+chondrotinase
+hyaluronidase 37°C 30 min
Dog vertebrae growth plate
Rabbit IgG control

Absorption control (inactivated antibody)

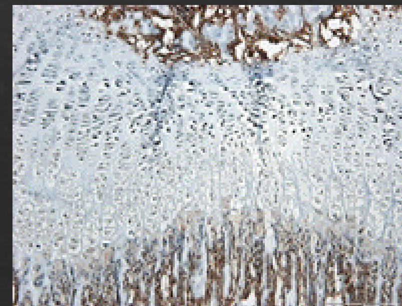
1. Find the antigen of interest (recombinant peptide) used to raise the antibody
2. Find optimal dilution of primary antibody
3. Find the optimal ratio of the antigen to antibody mixture (little or no staining at least in the area of interest)
4. Incubate the antigen and the primary antibody overnight at 4°C, agitate
5. Apply inactivated antibody instead of primary antibody as control



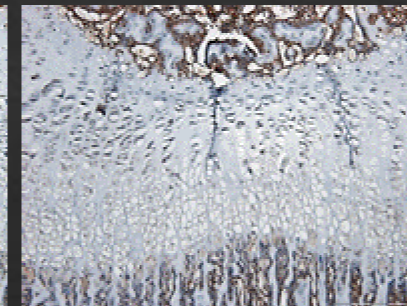
+citrate
+Ihh Ab (5ug/ml)



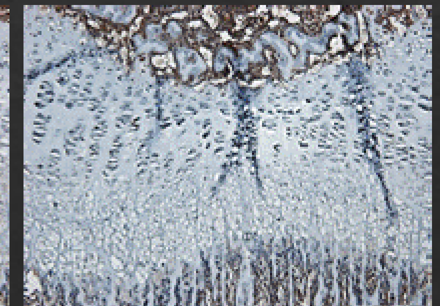
+citrate
+Ihh Ab (10ug/ml)



+citrate
+Ihh(10ug/ml)
+Ihh Ab (10ug/ml)



+citrate
+Ihh(20ug/ml)
+Ihh Ab (10ug/ml)



+citrate
+Ihh(40ug/ml)
+Ihh Ab (10ug/ml)