

# Optimizing IHC for challenging tissues

Zhirui Jiang

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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

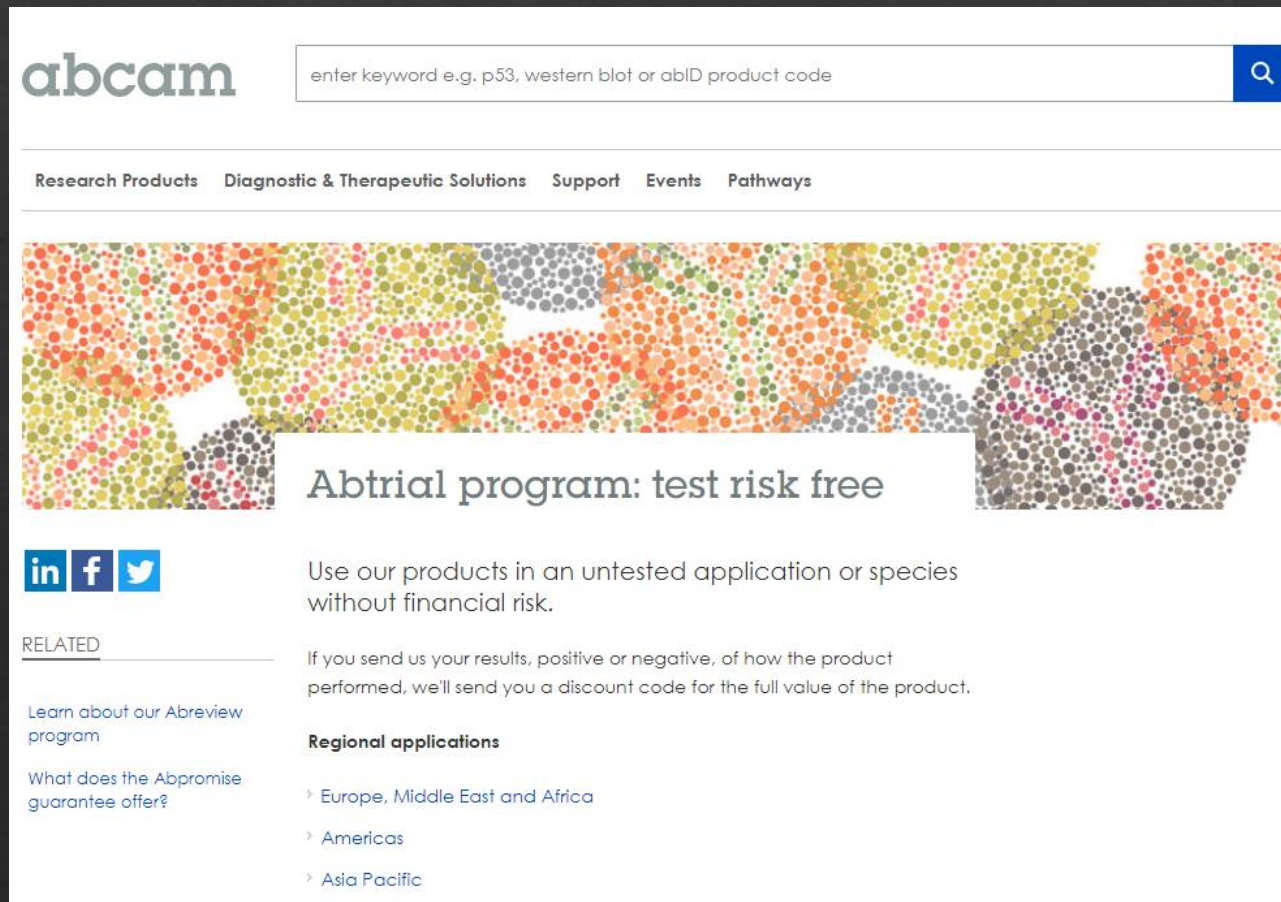
- Searching database: <https://www.antibodypedia.com/>; <https://www.uniprot.org/>
- BLAST to test cross reactivity
- Abtrial program

## ◇ Antibody dilution and incubation time



# IHC on specimen of special species: Abtrial program

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



The screenshot shows the Abcam website's 'Abtrial program: test risk free' page. At the top, there is a search bar with the text 'enter keyword e.g. p53, western blot or abID product code' and a magnifying glass icon. Below the search bar is a navigation menu with links for 'Research Products', 'Diagnostic & Therapeutic Solutions', 'Support', 'Events', and 'Pathways'. The main content area features a colorful, abstract graphic of dots in various colors (red, orange, yellow, green, blue, grey) arranged in a pattern that resembles a molecular structure or a network. Below the graphic, the text reads 'Abtrial program: test risk free'. To the left of this text are social media icons for LinkedIn, Facebook, and Twitter. The main text states: 'Use our products in an untested application or species without financial risk. If you send us your results, positive or negative, of how the product performed, we'll send you a discount code for the full value of the product.' Below this, there is a section titled 'Regional applications' with three bullet points: 'Europe, Middle East and Africa', 'Americas', and 'Asia Pacific'. On the left side of the page, there is a 'RELATED' section with two links: 'Learn about our Abreview program' and 'What does the Abpromise guarantee offer?'.

Email and ask about the antibody, application and species you are interested in

Technical team check the cross reactivity of the antibody

Submit order and forward order number to Abcam

Receive and test your antibody

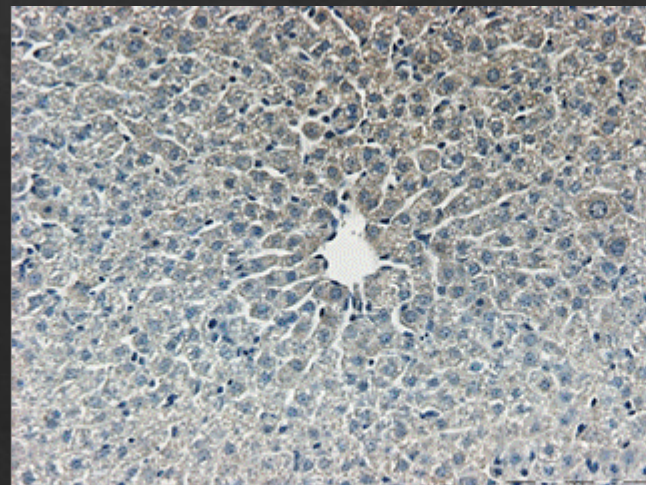
Submit your result on Abreview

Get approval and activate your discount code

# Why antigen retrieval is need

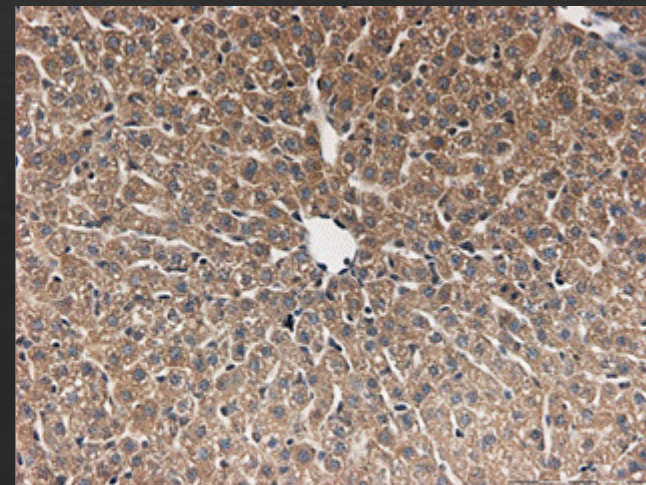
- ◇ Introduced by Shi et al in 1991
- ◇ 4% PFA or 10% NBF fixed paraffin sections (FFPE)
  - Molecular modification of antigens may result in loss of the ability of the antibody to react with the antigen (protein cross linking)
  - Can be corrected by retrieval
- ◇ Commonly refer to the datasheet of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

**Weak staining**



**No antigen retrieval**

**Optimal signal**



**+Citrate pH 6.0 microwave 3 min**

**α-phos-STAT5**  
**Mouse liver sections**

# Heat-induced vs proteolytic-induced epitope retrieval

<b>HIER</b> 80°C-100°C (20min water bath) or microwave 60°C (overnight) (good for fragile sections)	<b>PIER</b> 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 (10mM Tris/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 <sub>2</sub> , pH 7.8*
	Combination of 0.1U/mL chondroitinase ABC and 2 mg/mL hyaluronidase in PBS with 0.02% BSA (good for GAG rich tissue)

\* May damage sections if incubated too long

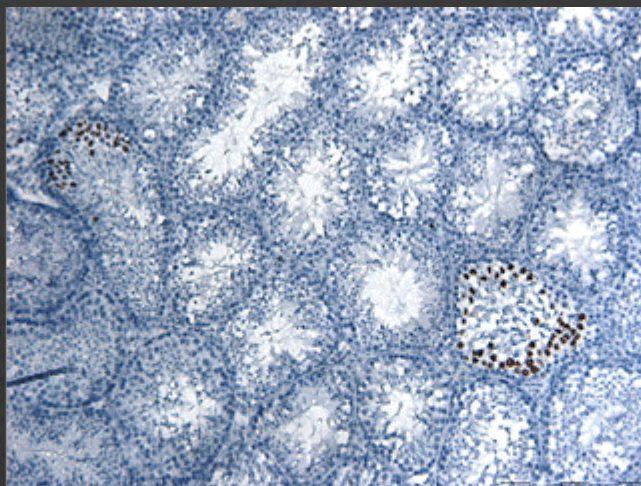


# HIER vs PIER

$\alpha$ -phos-histone H3

Mouse testis sections

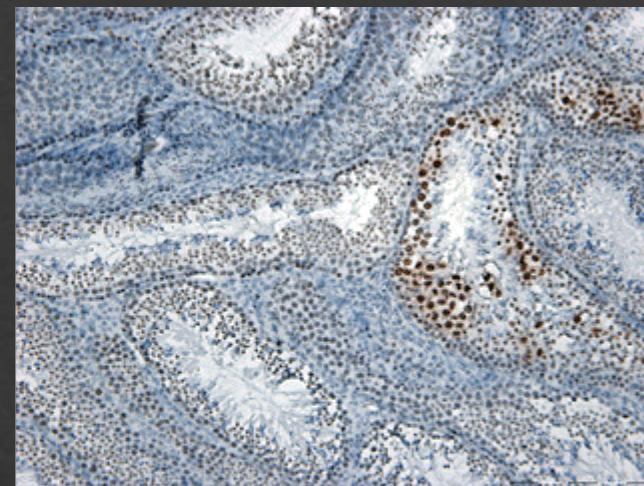
Weak staining



+pepsin  
37°C 30 min

Background staining

Optimal signal

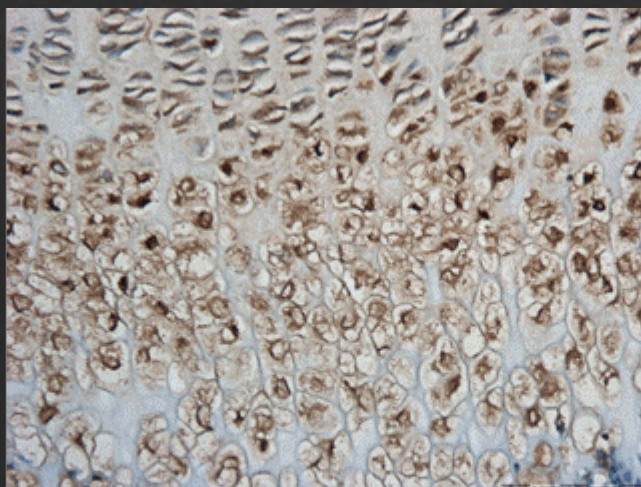


+Citrate pH 6.0  
microwave 3 min

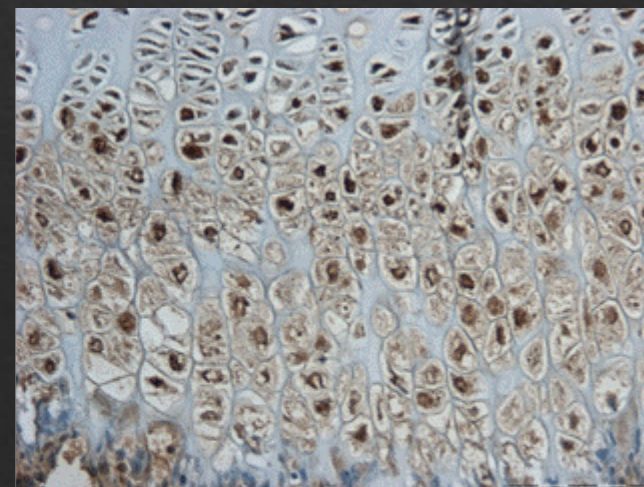
Optimal signal

$\alpha$ -p57

Mouse tibia growth  
plate sections



+chondrotinase



+Citrate pH 6.0



# HIER: temperature, pH and time

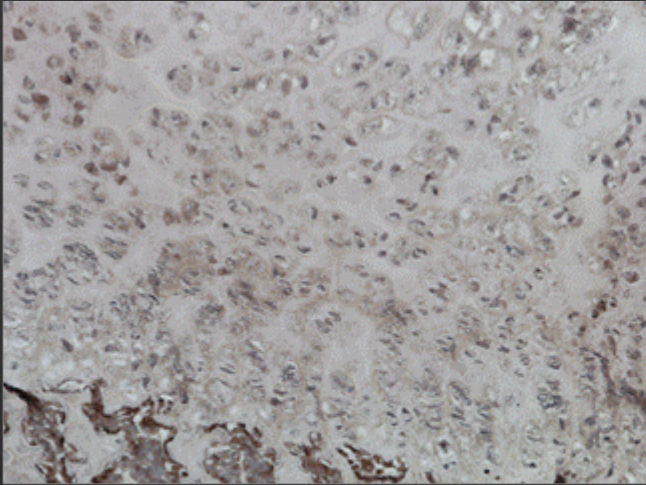
**Weak signal**

**Overstained signal**

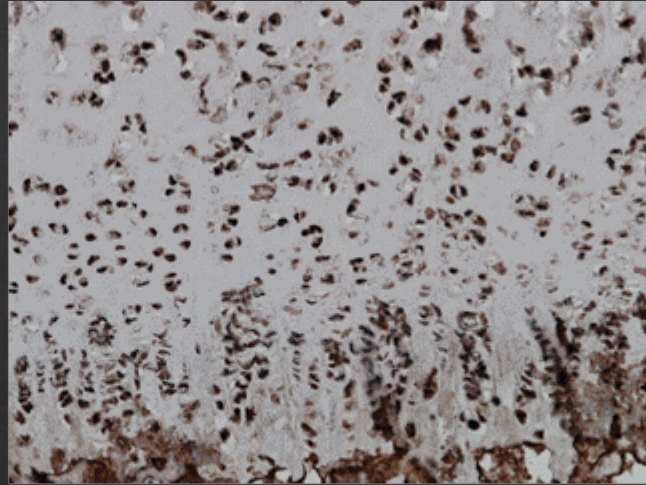
**Optimal signal**

$\alpha$ -RUNX2

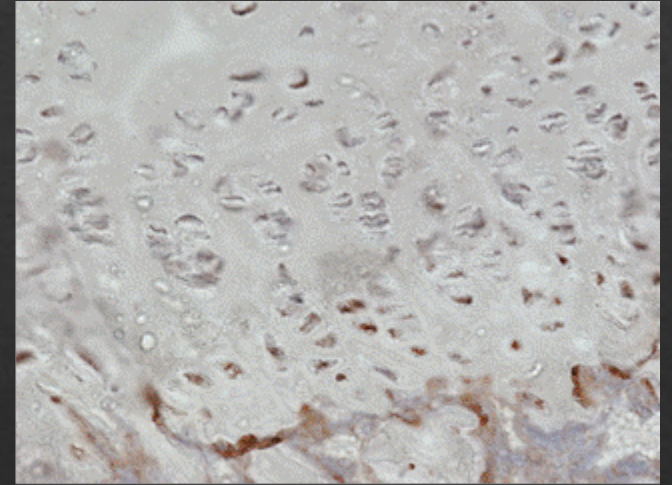
Dog vertebrae  
sections



+Citrate pH 6.0  
60°C O/N



+EDTA pH 8.0  
60°C O/N



+Citrate pH 8.0  
80°C 20 min

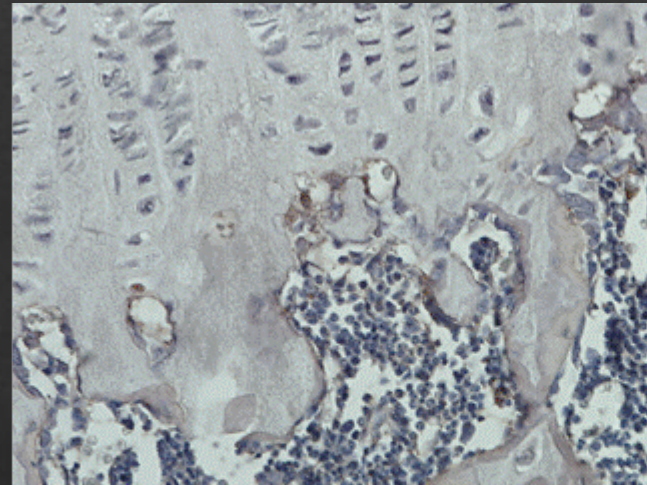
# PIER combination

**Weak signal**

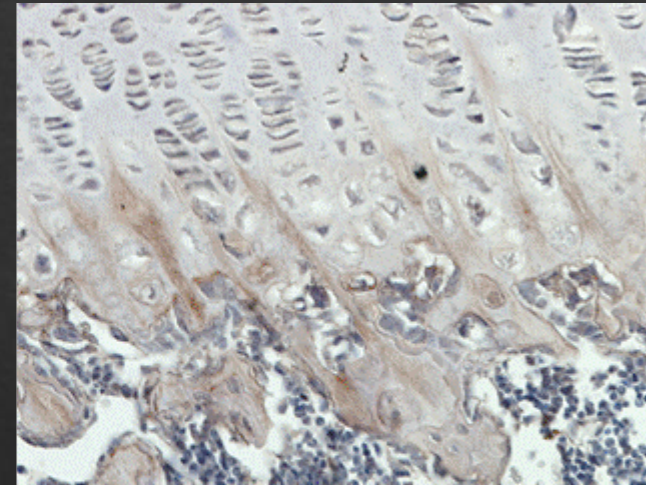
**Optimal signal**

$\alpha$ -MMP9

Dog vertebrae  
sections



+chondrotinase  
37°C 30 min



+chondrotinase  
+hyaluronidase  
37°C 30 min



# Blocking - reduce background and unspecific staining

## ◆ Endogenous peroxidase blocking

3% H<sub>2</sub>O<sub>2</sub>, 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



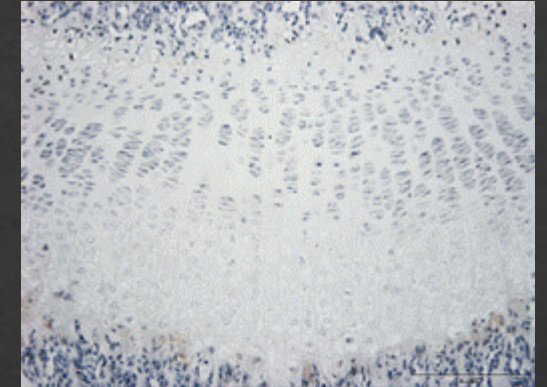
# Which samples can be used as controls?

## ◆ Positive control:

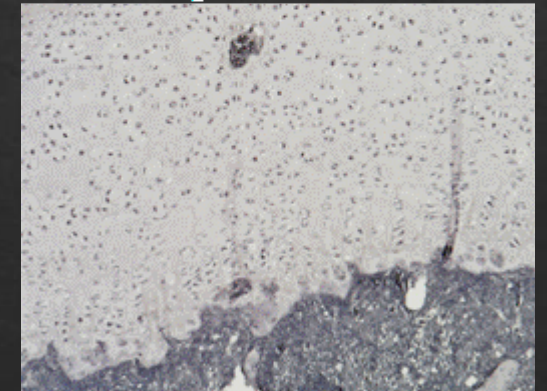
- High expression structure
- Tissue from species with known reactivity

## ◆ 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



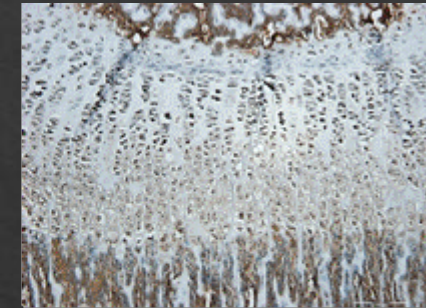
+chondrotinase  
+hyaluronidase 37°C 30 min  
Dog vertebrae growth plate  
Rabbit IgG control

# 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



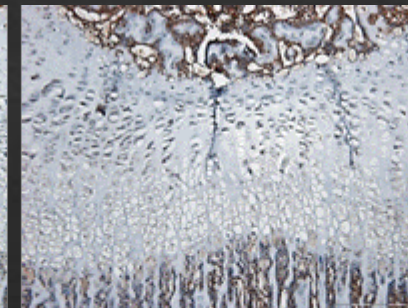
+citrate  
+Ihh Ab (5ug/ml)



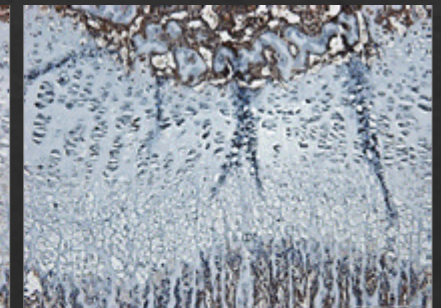
+citrate  
+Ihh Ab (10ug/ml)



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



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



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