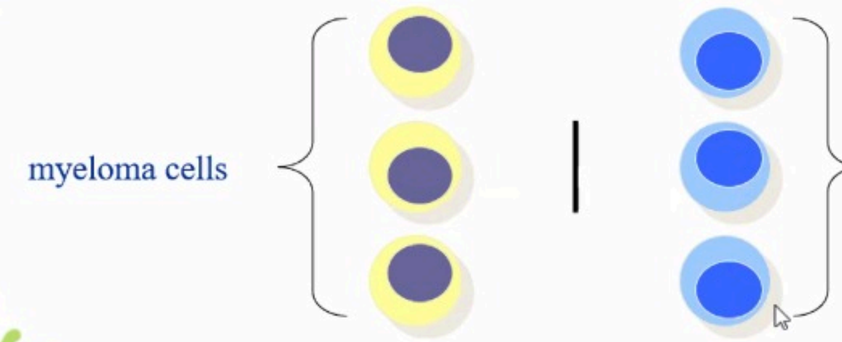
Clinical Uses for Monoclonal Antibodies

- Very useful as diagnostic, imaging, and therapeutic reagents in clinical medicine
  - Monoclonal antibodies were used primarily as in vitro diagnostic reagents
  - Radiolabeled monoclonal antibodies can also be used in vivo detecting or locating
- Immunotoxins
  - To compose of tumor-specific monoclonal antibodies coupled to lethal toxin
  - Valuable therapeutic reagent

Kusmardi | Sabrina Rizki Andriani | Risti Khafidah | Devita Friska Santy | Tri Adinda | PA_Fairi

Monoclonal Antibody Production



The B-cells are fused with mouse myeloma cells forming immortal hybrid cells or hybridomas.

Tania Parasit: Saya ikut sampai jam 09.30 krn setelah itu a...

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Polyclonal and Monoclonal Antibody

Immunohistochemistry

Staining Methods

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Immunohistochemistry

- Histochemistry is a science that combines the techniques of biochemistry and histology in the study of the chemical constitution of tissues and cells.
- Immunology is a science that deals with the immune system, cell-mediated and humoral aspects of immunity and immune responses.
- Immunohistochemistry (IHC) is the integration of the above mentioned disciplines.

Immunohistochemistry

- IHC utilizes antibodies and antibody based technology to detect and localize specific tissue antigens.
- The basic principle of any IHC procedures is that an antibody will specifically bind with an antigen to produce an exclusive antibody-antigen complex.
- This bonding is used to visualize both normal and diseased states of tissues, infectious agents and other components that may not be demonstrated by histochemical or special stains.

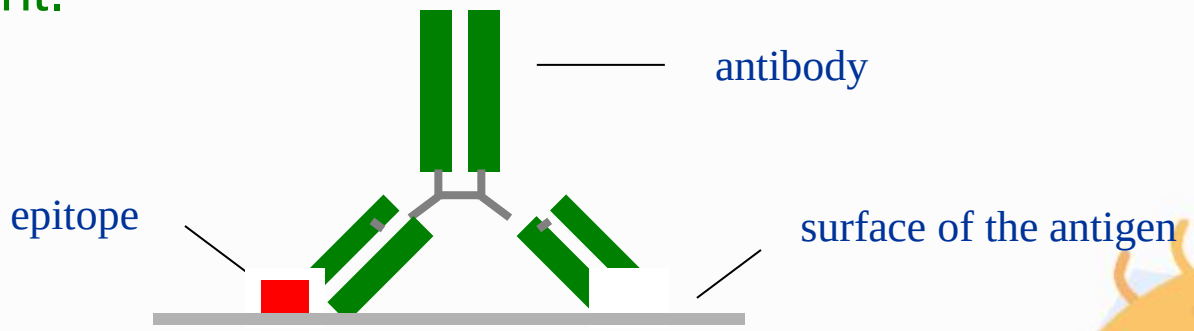
Antigen and Antibody

Antigen

- An antigen is a foreign substance that stimulates antibody formation and has the ability to bind to an antibody.
- Antigens or “antibody generators” are commonly proteins or glycoproteins of high molecular weight.
- An antigen that elicits a strong immune response is said to be strongly immunogenic.

Antigen

- The site on an antigen to which a complementary antibody may specifically react is called an epitope or antigenic determinant.

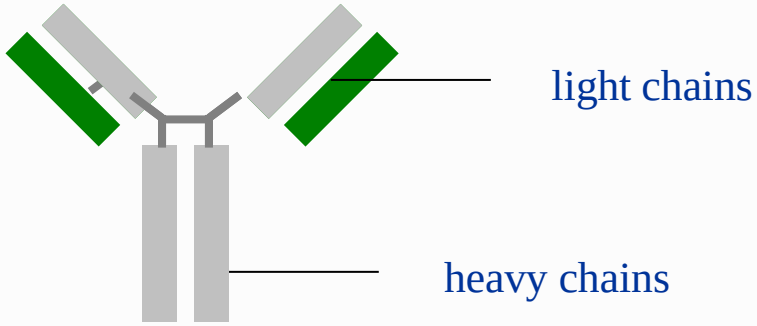


Antibody

- An antibody is an immunoglobulin (Ig) capable of specific combination with the antigen that caused its production.
- They are produced by B-cells (B-lymphocytes) as a protection from invasion of foreign substances such as bacteria, viruses, etc.
- Each B-cell produces antibodies with a single specificity
- This B-cell is then called a Plasma Cell

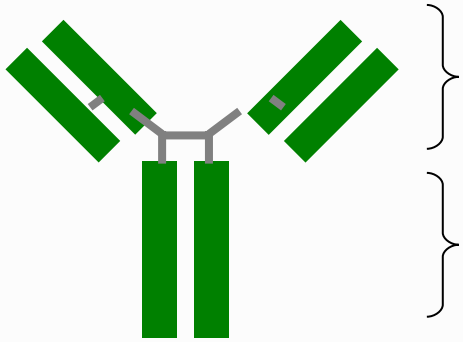
Antibody

- Each antibody consists of four polypeptides – two identical heavy chains and two identical light chains joined to form a "Y" shaped molecule.



Antibody Structure

- The classic Y shape of the antibody is composed of two variable antigen-specific Fab “arms” and the constant Fc “tail”.



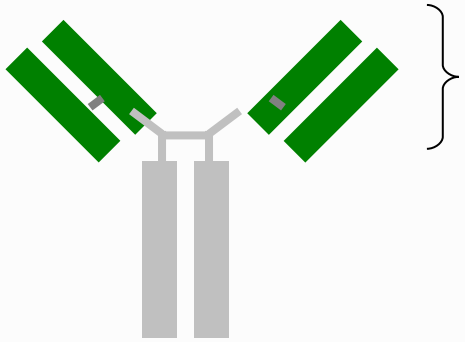
Fab

Fc

Menentukan respon imun

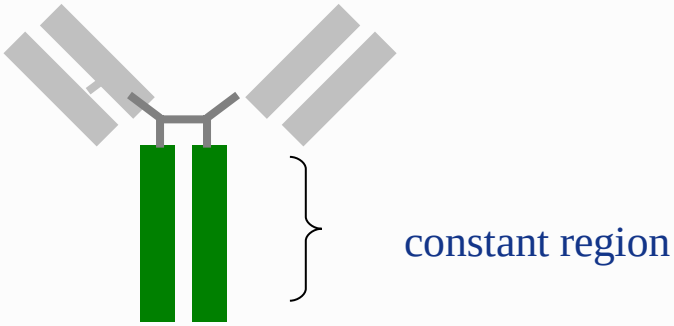
Antibody Structure

- The amino acid sequence in the tips of the “arms" varies among different antibodies. This variable region gives the antibody its specificity for binding antigen.



Antibody Structure

- The constant region determines the mechanism used to destroy antigen. Antibodies are divided into five major classes based on this region's structure and immune function.



Antibody

- Antibodies can be divided into five major classes: IgG, IgM, IgA, IgD and IgE.
- The heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as gamma, mu, alpha, delta and epsilon, respectively.
- The light chains of any antibody can be classified as either a kappa or lambda type based on small polypeptide structural differences.

Antibody

- The most commonly used antibody in IHC is of the IgG class because it is the major class of immunoglobulin released in serum.

Antibody-Antigen Reaction

Antibody-Antigen Reaction

- Antibodies bind to antigen through the variable regions of the antibody. This bonding may be hydrophobic, ionic, Van der Waals or hydrogen bonding.
- The strength of the binding of an antibody to a specific antigen is called affinity.
- High affinity antibodies will bind larger amounts of antigen in a given period of time, and can be used at higher dilutions.

Antibody-Antigen Reaction

- The rate of antigen-antibody reaction is affected by temperature and pH of buffers and diluents used in IHC procedures.
- Higher incubation temperature permits rapid antigen-antibody binding.
- The buffer’s pH and ionic content can affect the charge of amino acids in both the antibody and antigen.

Poly- and Mono- Clonal Antibodies

- Polyclonal antibody
  - Antigens possess multiple epitopes
  - Serum antibodies are heterogeneous,
    - To increase immune protection in vivo
  - To response facilitates the localization, phagocytosis, and complement-mediated lysis of antigen
  - To have clear advantages for the organism in vivo
- Monoclonal antibody
  - Derived from a single clone, specific for a single epitope
  - For most research, diagnostic, and therapeutic purposes

Antibody Production

Antibody Production

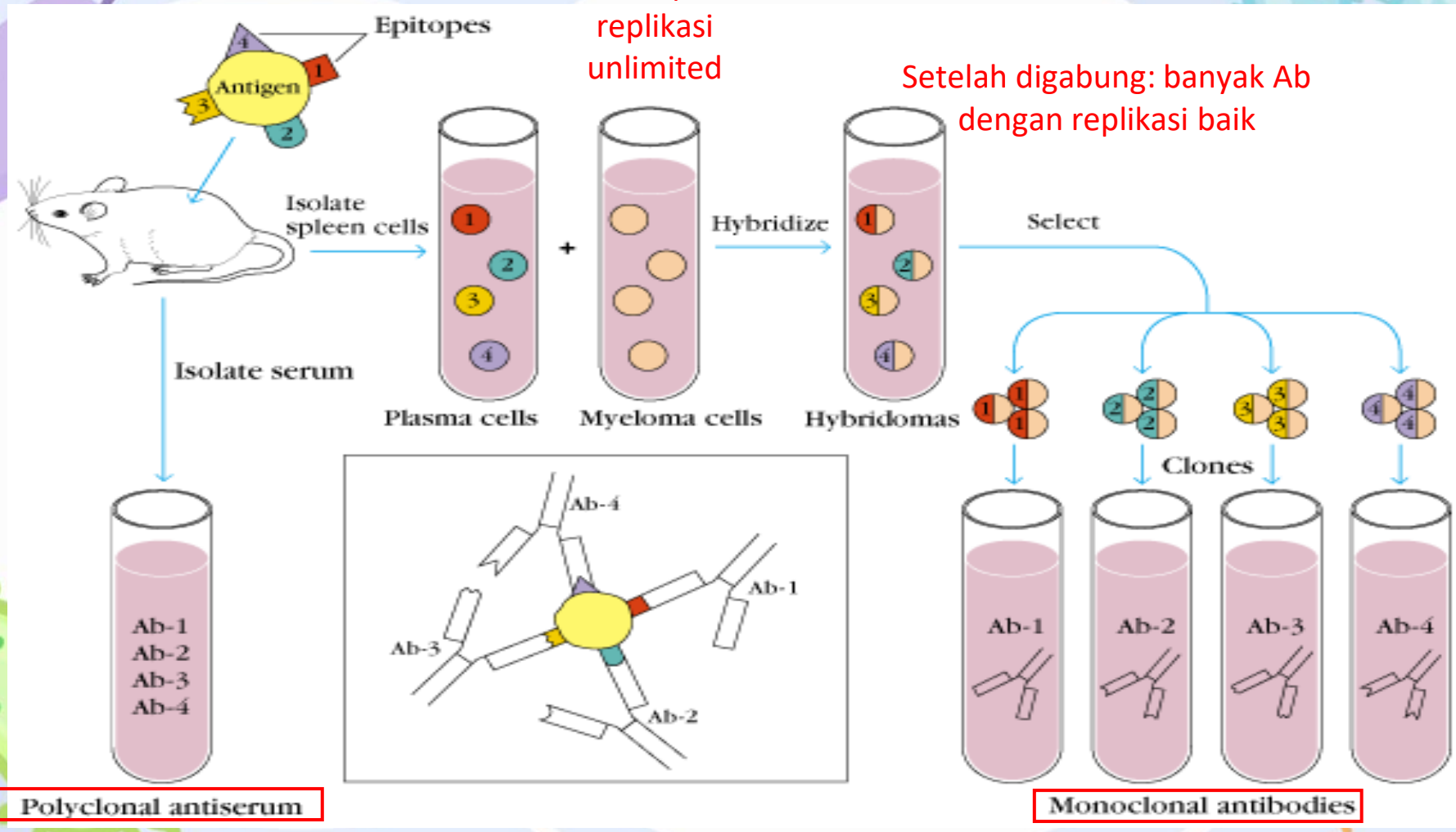
- Antibodies are generated by immunizing (injecting) animals with purified antigen. The animal responds by producing antibodies that specifically recognize and bind to the antigen.

- Antibody reagents may be polyclonal or monoclonal.

1975, by Georges Köhler and Cesar Milstein
- Be awarded a Nobel Prize in1984

Punya kemampuan replikasi unlimited

Setelah digabung: banyak Ab dengan replikasi baik



Nanti dipisah2 spesifik jd Ab monoclonal

Polyclonal Antibodies

Polyclonal Antibodies

- Polyclonal antibody reagents are produced as different classes of immunoglobulins by many B-cells clones and react with various epitopes on an antigen. (Figure 2)

- They are more tolerant of small changes in the nature of the antigen since they often recognize multiple epitopes.

- They may be generated in a variety of animals like rabbit, goat, sheep, horse, etc. The rabbit is the most commonly used animal for generating polyclonal antibodies.

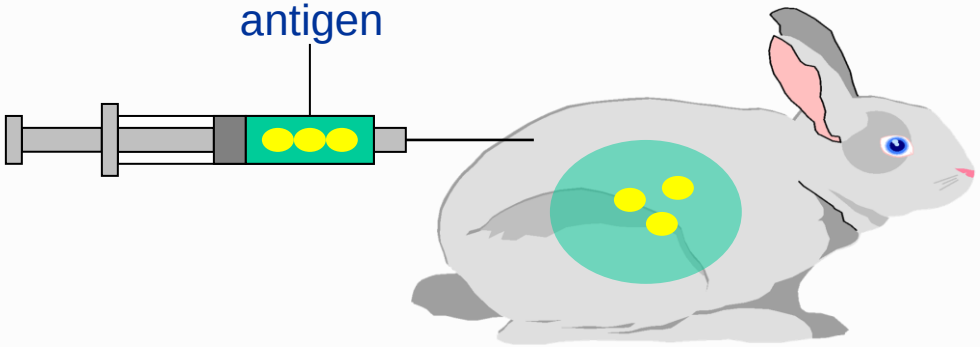
Polyclonal Antibodies

Figure 2

Polyclonal antibodies reacting with various epitopes
Each antibody is made by a different B-cell

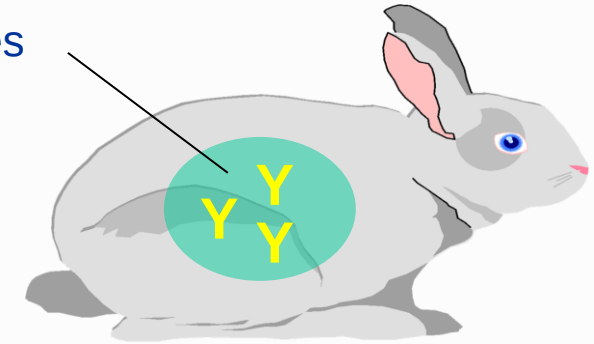
Polyclonal Antibody Production

Polyclonal Antibody Production



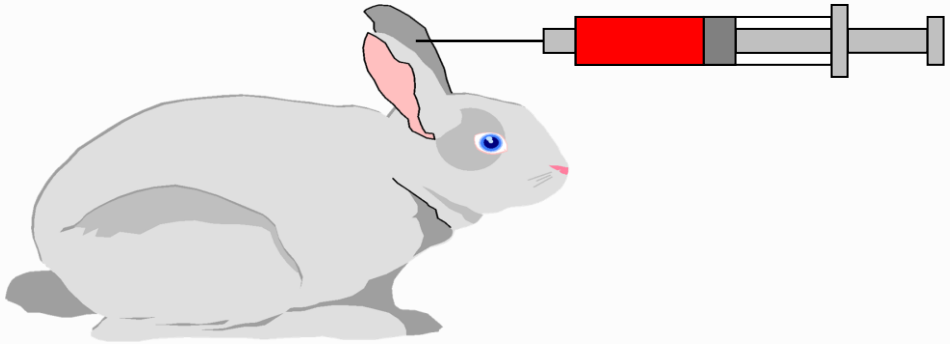
A rabbit is injected (intradermally or subcutaneously) with a purified dose of antigen.

Polyclonal Antibody Production



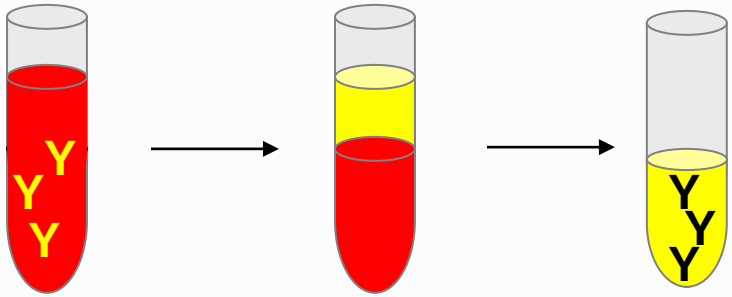
The rabbit's immune system responds by producing antibodies specific to the injected antigen.

Polyclonal Antibody Production



Blood is harvested from the ear at the peak of antibody production.

Polyclonal Antibody Production

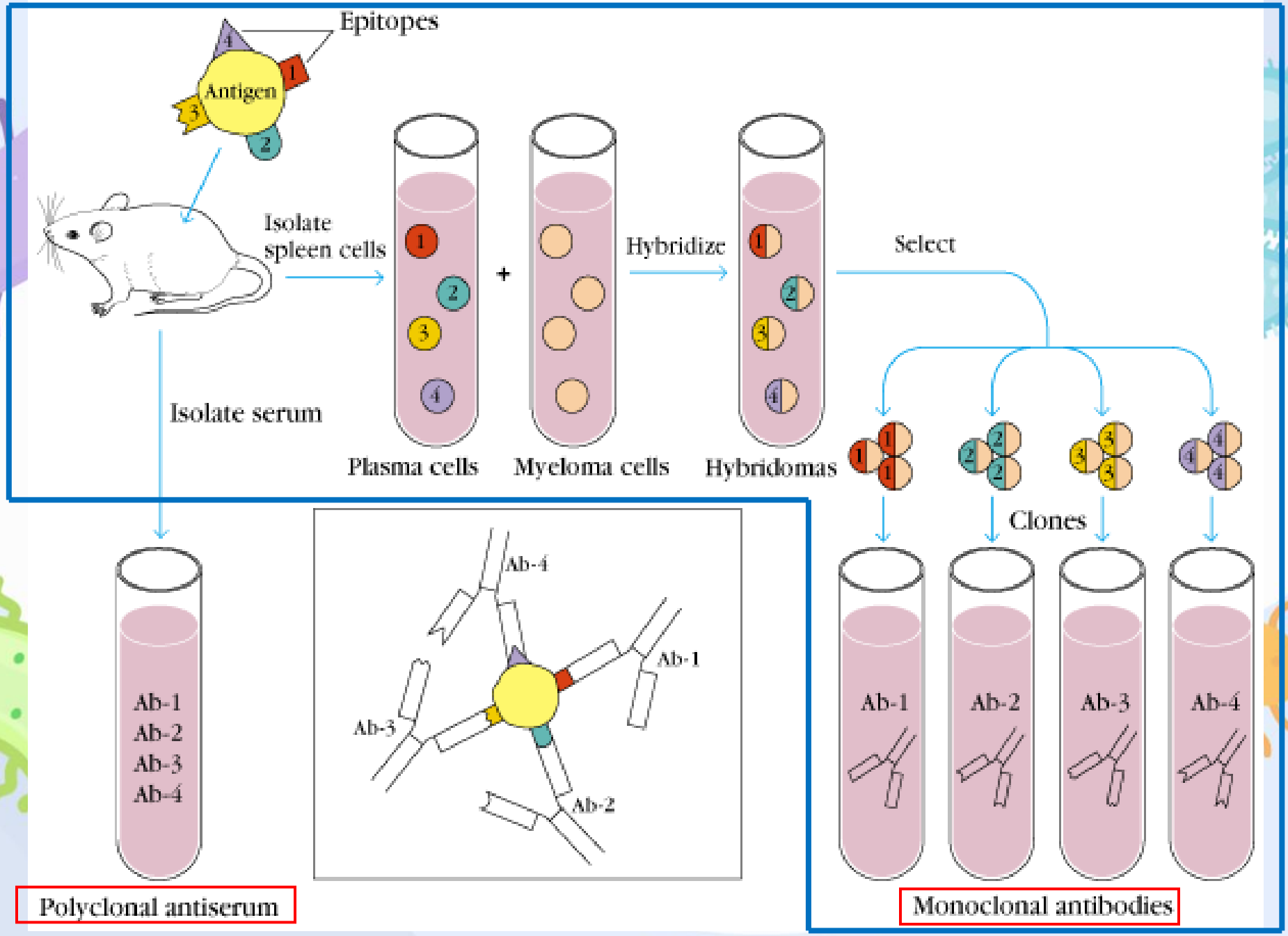


Red blood cells and clotting proteins are removed and the antiserum is purified.

Polyclonal Antibody Production

- Polyclonal antibodies are purified either by Protein Purification or Antigen Affinity Chromatography.
  - *Protein Purification* eliminates the bulk of serum proteins but does not eliminate nonspecific immunoglobulin fraction.
  - *Antigen Affinity Purification* eliminates the bulk of the nonspecific immunoglobulin fraction using antigen to capture the antibody leaving only the immunoglobulin of desired specificity.

Monoclonal Antibodies


Epitopes
Antigen
1
2
3
4
Isolate spleen cells
Isolate serum
Plasma cells
Myeloma cells
Hybridize
Hybridomas
Select
Clones
Ab-1
Ab-2
Ab-3
Ab-4
Polyclonal antiserum
Monoclonal antibodies

Monoclonal Antibodies

- Monoclonal antibodies are derived from a single B-cell and are produced as a single class of immunoglobulin.
- They are raised by fusion of the specific B-cells with immortal myeloma (B-cell) cancer cells to form a hybridoma.
- A hybridoma can multiply indefinitely and continuously produce a specific monoclonal antibody.

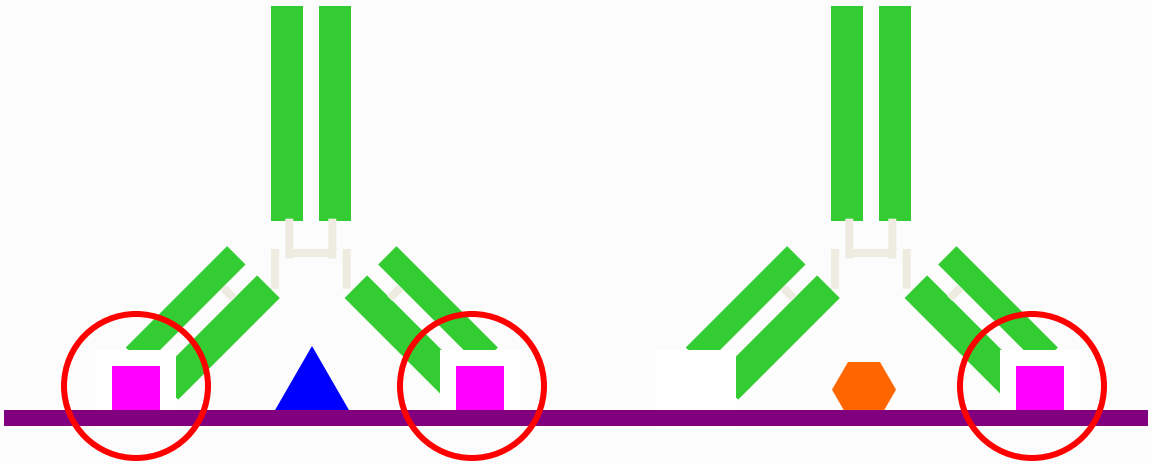
Monoclonal Antibodies

- They react with a specific epitope on a given antigen (Figure 3), giving less background staining.

- Because they react with only one epitope, they are more vulnerable to the loss of epitope through chemical treatment of the antigen (e.g., fixation).

Monoclonal Antibodies

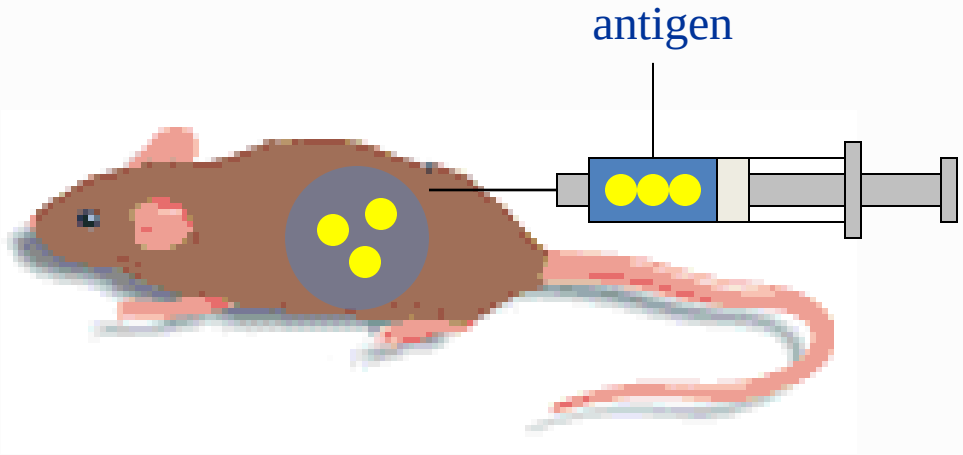
Figure
3



Monoclonal antibodies reacting with similar epitopes

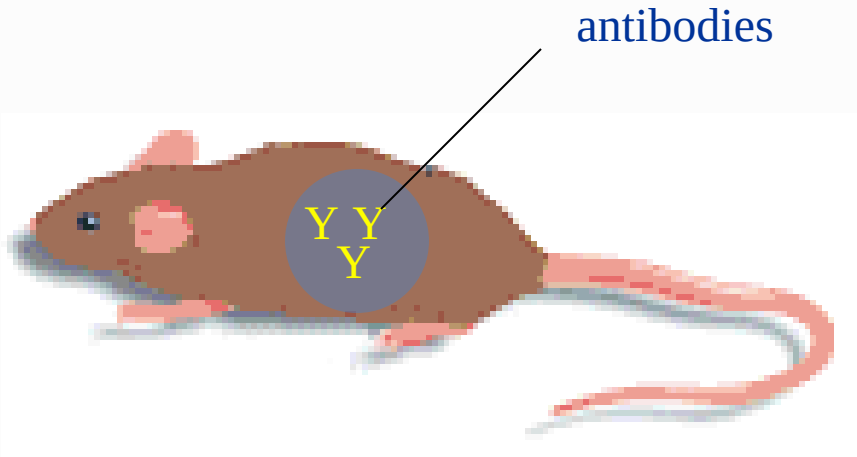
Monoclonal Antibody Production

Monoclonal Antibody Production



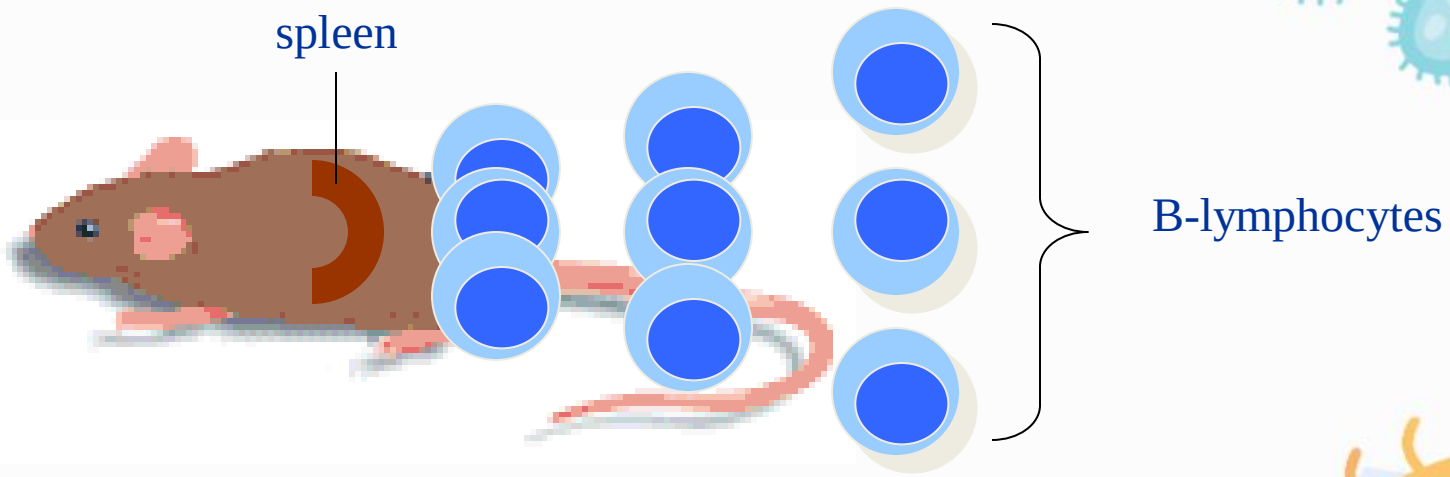
A mouse is injected (intradermally or subcutaneously) with a purified dose of antigen.

Monoclonal Antibody Production



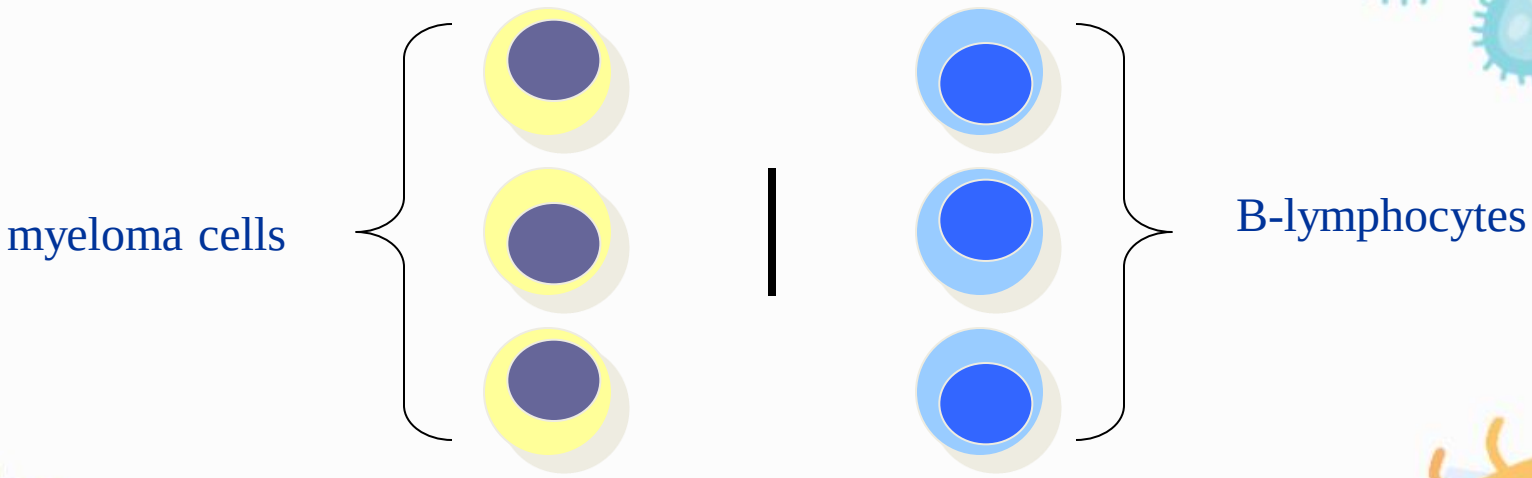
The mouse's immune system responds by producing antibodies specific to the injected antigen.

Monoclonal Antibody Production



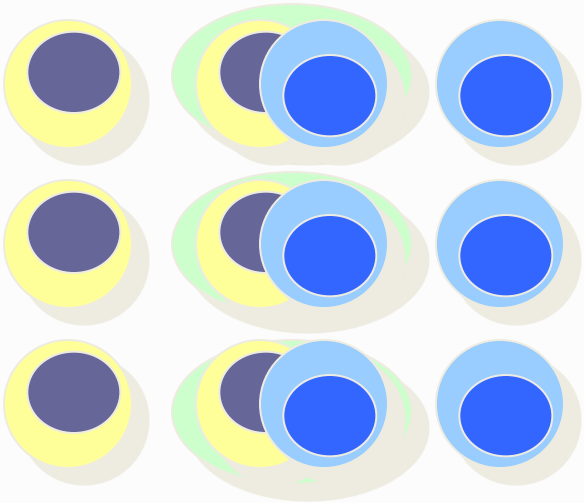
The antibody-producing B-cells are harvested from the spleen or lymph nodes.

Monoclonal Antibody Production



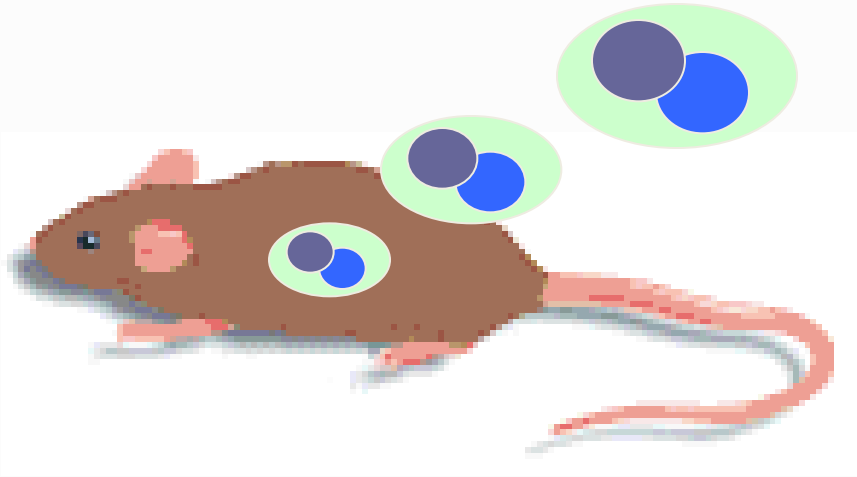
The B-cells are fused with mouse myeloma cells forming immortal hybrid cells or hybridomas.

Monoclonal Antibody Production



The generated hybridomas will produce many copies of the exact same antibody.

Monoclonal Antibody Production

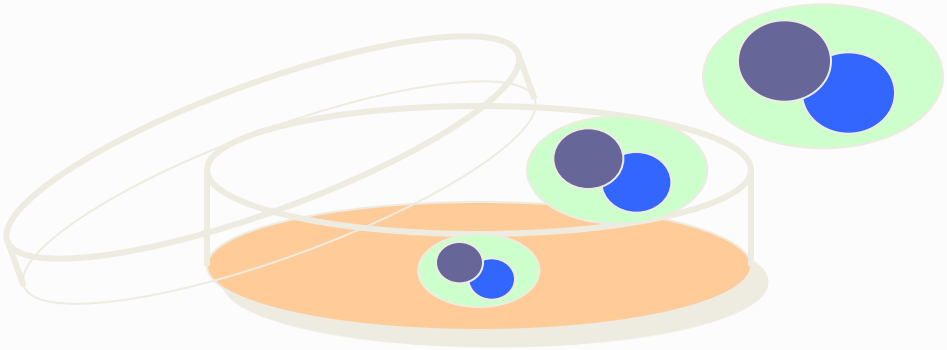


The hybridomas are either transplanted into the peritoneal cavity of a syngeneic mouse and the antibodies produced are harvested as ascitic fluid.

NB:

1) Syngeneic: genetically similar or identical and hence immunologically compatible, especially so closely related that transplantation does not provoke an immune response.

Monoclonal Antibody Production

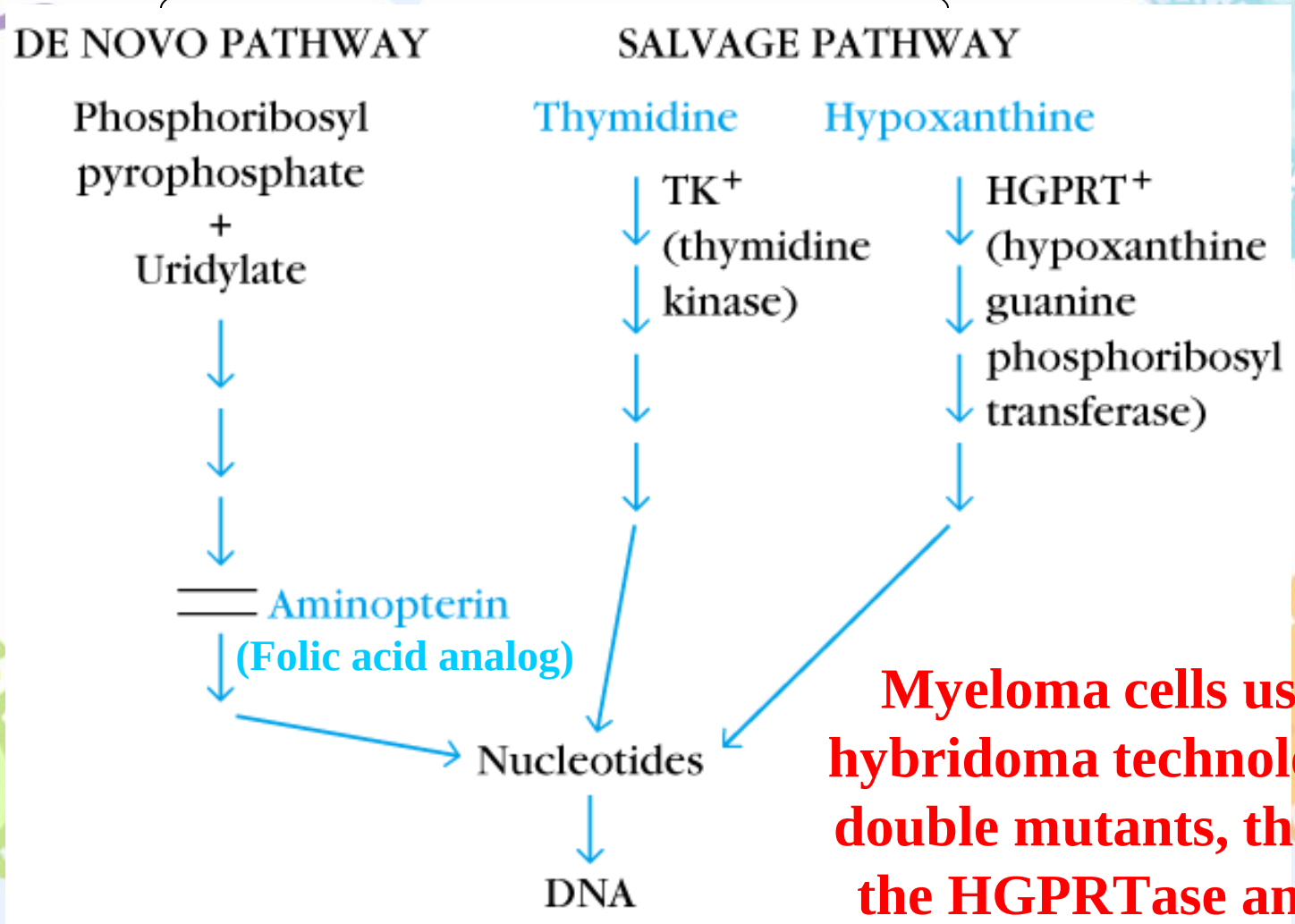


Or are propagated in a tissue culture medium and the antibodies produced are harvested as a hybridoma supernatant

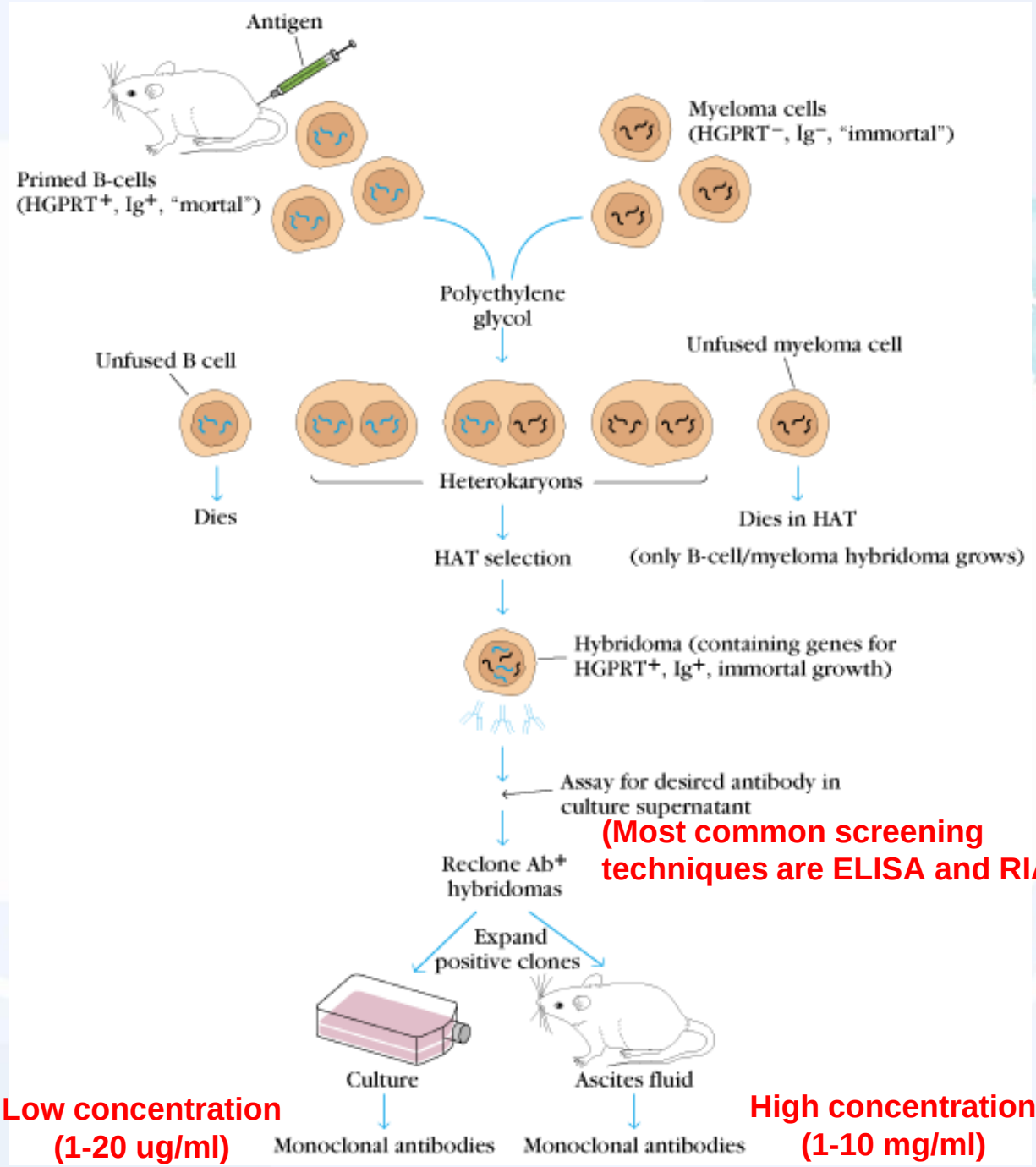
Formation and Selection of Hybrid Cells

- Hybridoma: the B cell X myeloma cell
  - To be produce by using polyethylene glycol (PEG) to fuse cells
  - The myeloma cells: immortal growth properties
  - The B cells: to contribute the genetic information for synthesis of specific antibody
  - Selected by using HAT medium (hypoxanthine, aminopterin, and thymidine)
    - Myeloma cells are unable to grow
    - B cells are able to survive, but can not live for extended periods

Two different pathways to synthesis nucleotide in mammalian cells



Myeloma cells used in hybridoma technology are double mutants, they lack the HGPRTase and lose the ability to produce Ig



(Most common screening techniques are ELISA and RIA)

Low concentration (1-20 ug/ml)

High concentration (1-10 mg/ml)

Clinical Uses for Monoclonal Antibodies

- Very useful as diagnostic, imaging, and therapeutic reagents in clinical medicine
  - Monoclonal antibodies were used primarily as in vitro diagnostic reagents
  - Radiolabeled monoclonal antibodies can also be used in vivo detecting or locating
- Immunotoxins
  - To compose of tumor-specific monoclonal antibodies coupled to lethal toxin
  - Valuable therapeutic reagent

Primary Antibody

Primary Antibodies

- Primary antibody: antibody that is directed against the antigen of interest
  - Eg. CD20 used as the primary antibody to demonstrate B-cells on a tissue section.
- Both polyclonal and monoclonal antibodies can be used as primaries.
- They are typically supplied by manufacturers in two formats: concentrated or ready-to-use (RTU).

usually provided with a recommended dilution range but also depends on the lab setup

Antibody Titer and Dilution

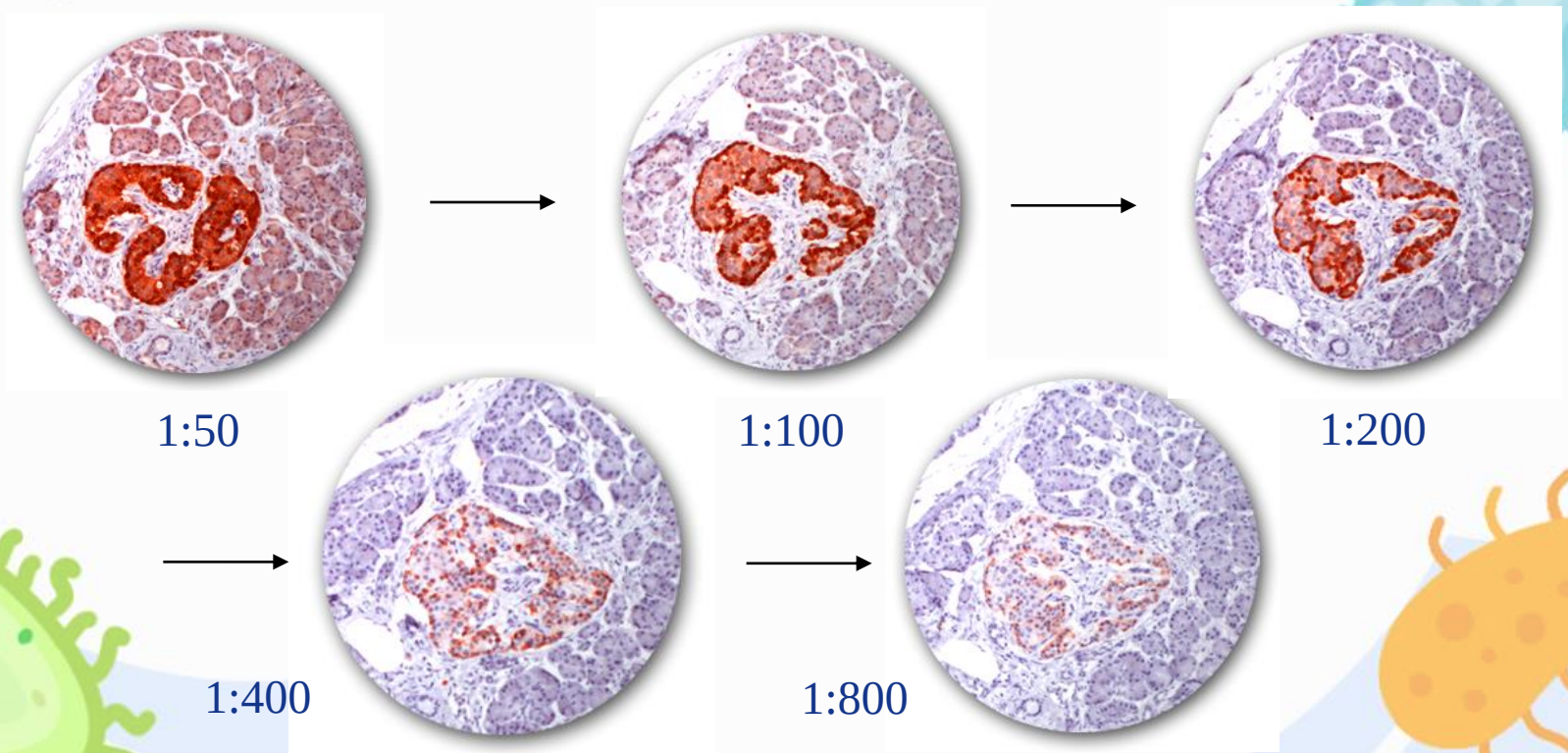
Antibody Titer and Dilution

- Titer: highest dilution of the antibody resulting in strong specific staining with the least amount of background
  - Background includes all nonspecific staining as a result of procedural artifacts.

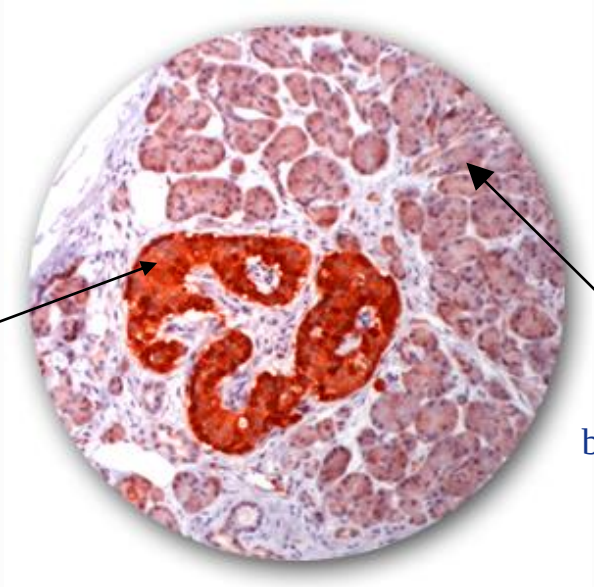
- Dilution: ratio of the concentrated antibody to the total volume of the desired dilution
  - Eg. A 1:5 dilution one part concentrated antibody + four parts diluent.

Optimal working dilution is typically determined by titration or dilution series

IHC staining results of serially diluted *Chromogranin A* antibody on pancreas

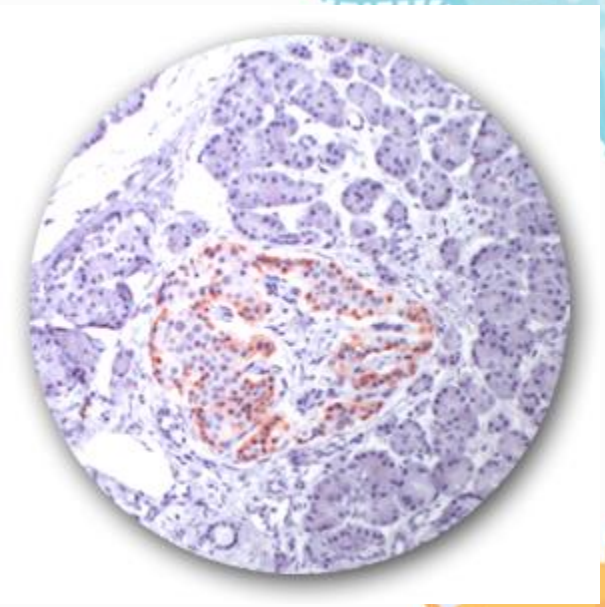


1:50



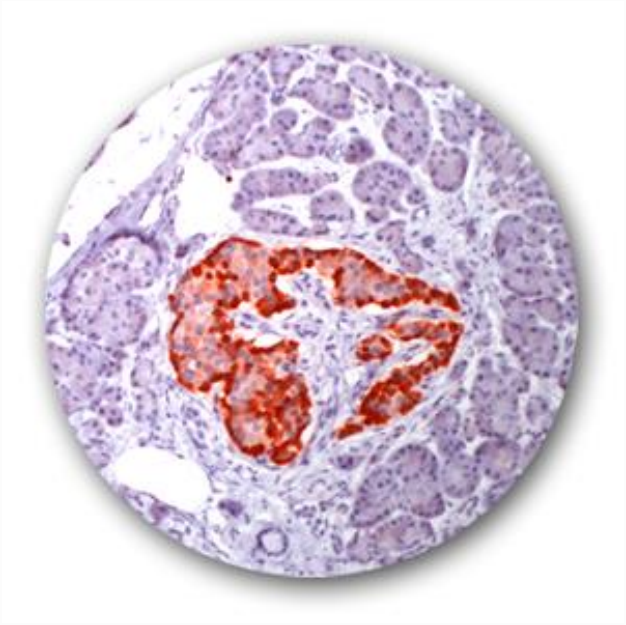
At 1:50, the Islet cells stain strongly but there is also a strong background staining.

1:800



At 1:800, there is no background staining but the Islet cells stain very weak.

1:200



At 1:200, there is good contrast and no background staining, it is therefore the optimal working dilution.

Antibody Incubation

Incubation Time

- Incubation time is inversely proportional to antibody concentration
  - Higher concentration of antibody □ □ shorter incubation time.

↓

- It can be from minutes to hours (most commonly 30-60 minutes)

Incubation Temperature

- Antibody-antigen reaction is hastened at 37°C as compared to room temperature an increase in temperature also allows for a higher dilution of the antibody.
  - Humidity chambers must be used when incubating at higher temperature to prevent drying of tissue sections.

Antigen Retrieval

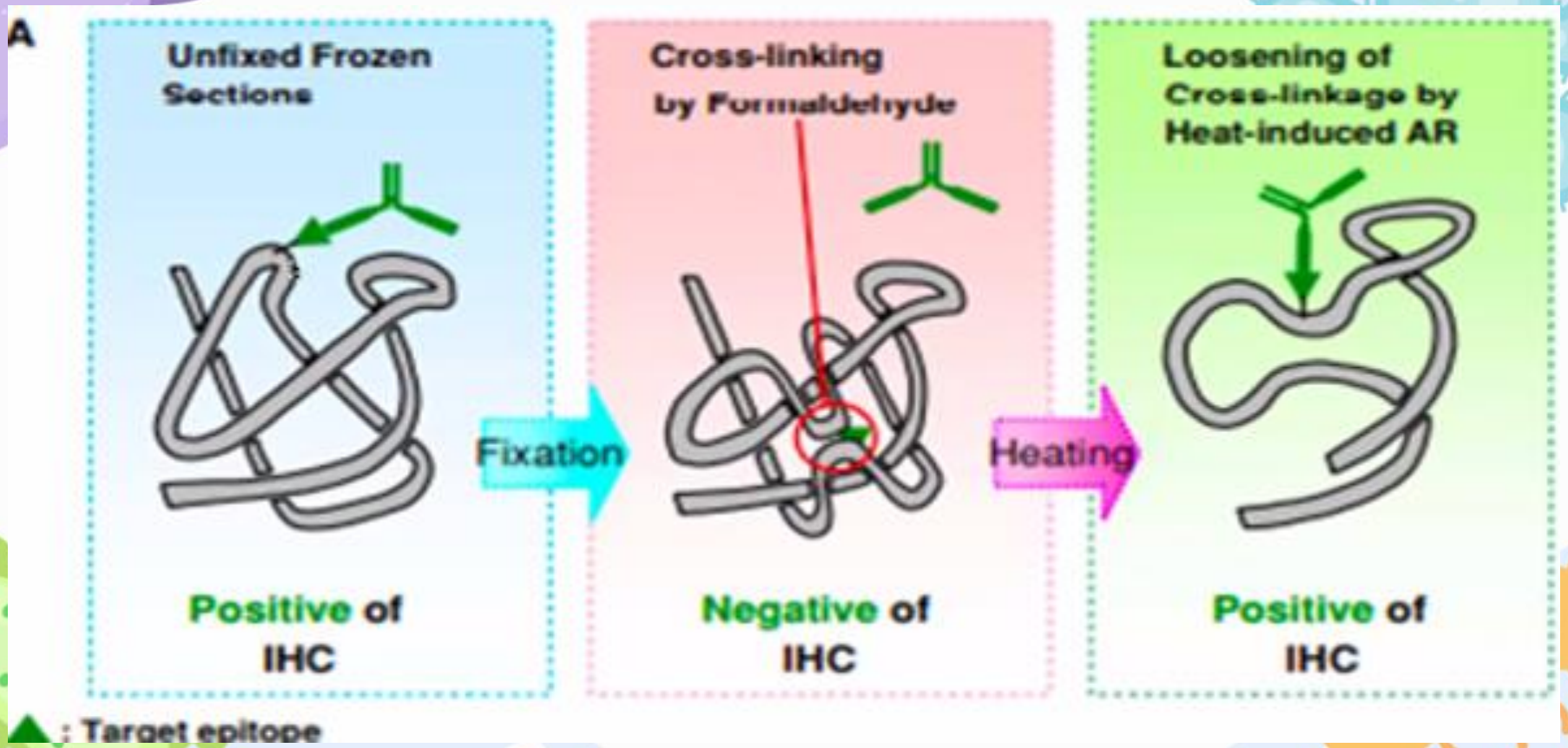
Antigen Retrieval

- During the process of formalin fixation, many antigenic sites are 'masked' → sometimes difficult or impossible to stain

↓

- Antigen retrieval: process of treating formalin fixed-paraffin embedded tissue sections with proteolytic enzymes or heating them in various buffer solutions in order to expose the antigen.
  - Commonly used proteolytic enzymes: trypsin, pepsin and protease.
  - Heat induced epitope retrieval (HIER) includes microwaving, pressure cooking, steaming, autoclaving or using the PreTreatment Module™ → requires buffer of different concentrations and pH

Commonly used buffers include
- citrate at pH 6.0
- EDTA at pH 8.0
- Tris-HCL at pH 10.0


A
Unfixed Frozen Sections
Positive of IHC
Fixation
Cross-linking by Formaldehyde
Negative of IHC
Heating
Loosening of Cross-linkage by Heat-induced AR
Positive of IHC
: Target epitope

These photos show the staining results of CD3 antibody on tonsil, with and without antigen retrieval.

Citrate buffer, pH 6.0

No Antigen Retrieval

Antigen Retrieval

- Formaldehyde fixation usually generates methylene bridges which cross-link proteins and therefore mask the epitope of interest It is essential to unmask the antigen epitopes in order to allow the antibodies to bind
  - By heat (Heat Induced Epitope Retrieval: HIER), or
  - Enzymatic digestion (Proteolytic Induced Epitope Retrieval: PIER).

HIER

- The HIER method can be implemented by microwave, high pressure or water bath. It breaks the methylene bridges and exposes the epitope to allow the antibodies to bind by continuously heating the higher the temperature of the HIER solutions, the more effective the recovery of the epitope.
- The following antigen retrieval reagent is required:
  - 0.01 M citrate buffer solution (pH 6.0)
  - 0.01 M PBS buffer (pH7.0)
  - 0.05 M EDTA (pH 8.0)
  - 0.05 M Tris-EDTA (pH 9.0)
  - 0.05 M Tris-HCl (pH 1~12)

- HIER solutions can be grouped in three categories based on pH and buffer compositions:
  - Low pH (pH ~3-5) solutions frequently buffered by glycine-HCl.
  - Low to neutral pH (pH ~ 6-7) solution buffered with citric acid.
  - High pH (pH~ 8-10) buffered by Tris or EDTA.

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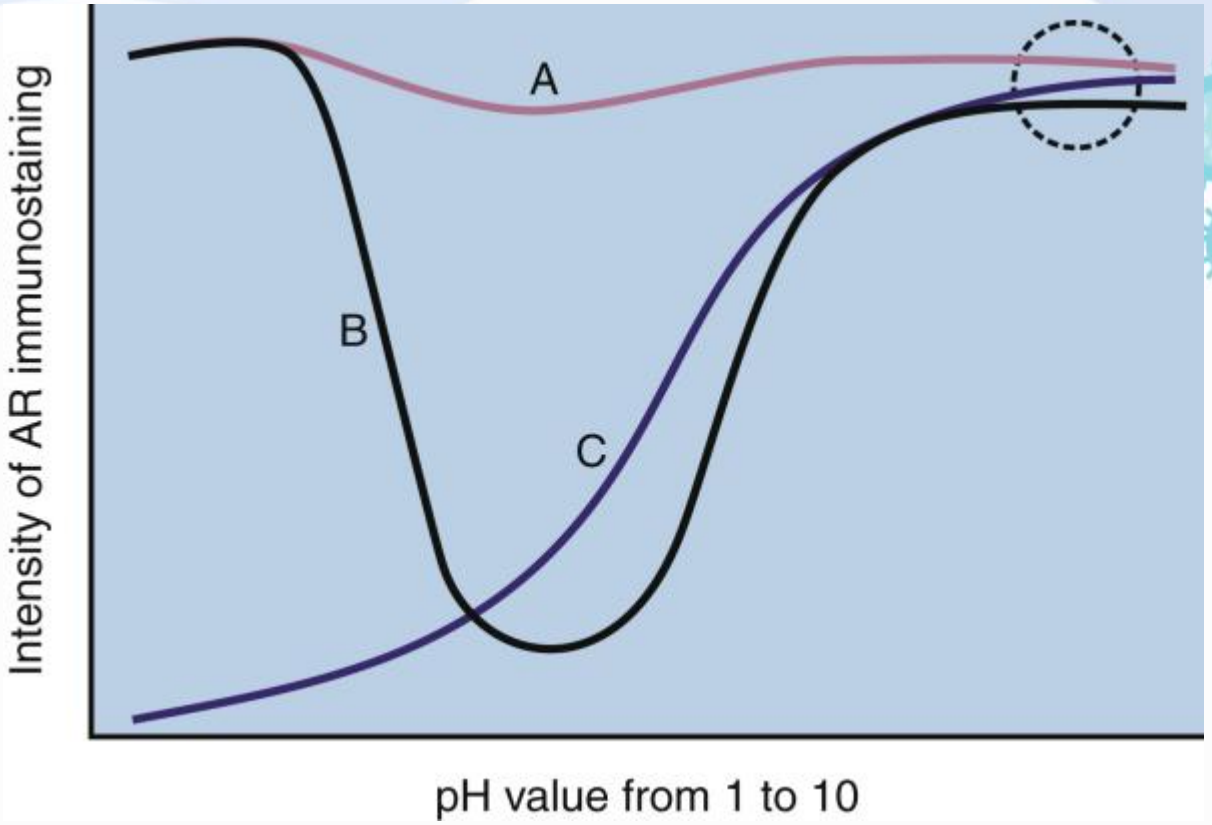
Current evidence suggests that the pH of the HIER solutions is more important than the composition of the buffer. Optimal recovery for most epitopes occurs in alkaline buffers with a pH range of 8-10.



- **FIGURE 1.18 Schematic diagram of the three patterns of pH-influenced AR immunostaining. Line A (pattern of type A) shows a stable pattern of staining with only a slight decrease in staining intensity between pH 3 and pH 6. Line B (pattern of type B) shows a dramatic decrease in staining intensity between pH 3 and pH 6. Line C (pattern of type C) exhibits an ascending intensity of AR immunostaining that correlated with increasing pH values of the AR solution. Circle *(right)* indicates the advantage of using an AR solution of higher pH value.**

Reproduced, with permission, from Shi S-R, Imam SA, Young L, et al. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem. 1995;43:193-201.

1. Microwave Method
   1. Place the sample section into a microwaveable vessel where antigen retrieval reagent is present
   2. Place the vessel inside a microwave oven
   3. Apply microwave radiation to the sample for 5-20 min

Uneven heat distribution → violent boiling → tissue detachment

2. High Pressure Method
   1. Place the sample section into an appropriate vessel where antigen retrieval reagent is present
   2. Place the vessel inside a pressure cooker
   3. Turn on the cooker and heat the sample until it boils
   4. Once boiling starts, turn off the cooker after the sample is allowed to reach full pressure for 1-4 min

higher temperature may damage or distort morphology: connective tissue may appear shredded or burnt

Requires more heating time than microwave or pressure cooker

3. Water Bath Method
   1. Place the sample section into an appropriate vessel where antigen retrieval reagent is present
   2. Place the vessel and thermometer inside a water bath chamber
   3. Heat the sample to 92℃ in the chamber
   4. Remove the sample from the chamber after it is heated at 92℃ for 20-40 min

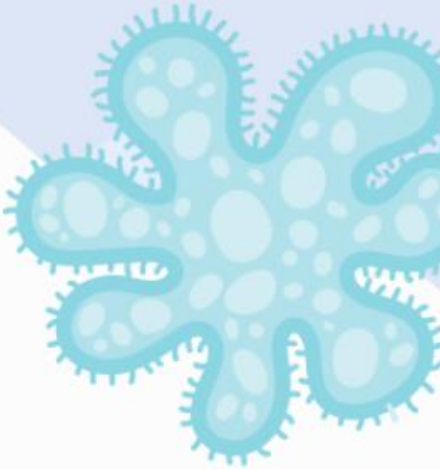
PIER

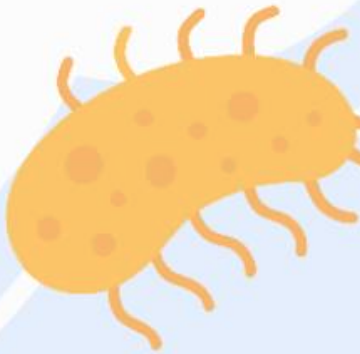
- Epitope can be exposed by incubation with proteases which can break the methylene bridges the choice for digestion enzymes depends on the antigenic components
  - Pepsin and bromelin are used for retrieving antigens in intercellular substance
  - Other enzymes can be used for intracellular antigen exposure.

PIER

Enzyme	Working Concentration	Digestion Condition
Trypsin	0.05% to 0.1%	37℃ (10 to 40 min)*
Proteinase K	20 μg/mL	37℃ (20 min)
Pepsin	0.40%	37℃ (30 to 180 min)

- The reaction time can be increased for certain worn-out tissues. Fresh trypsin solution should be prepared with pH adjusted to 7.6 and used at 37℃.



Enzymes and Chromogens

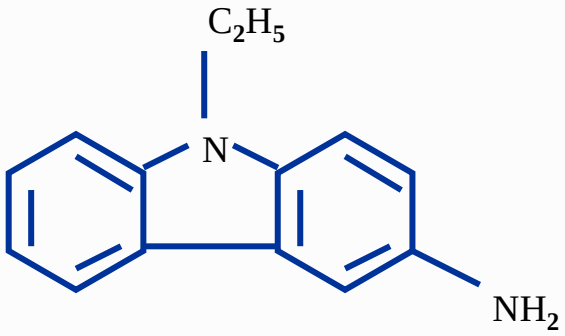
Enzymes and Chromogens

- Detection systems attach enzyme labels to primary or secondary antibodies to visualize the localized antibody-antigen binding in tissue section.
  - Enzymes: proteins that act as catalysts to increase the rate of chemical reaction
    - Used in IHC to convert a colorless reagent into a stable colored product (chromogen) that marks the site of antibody-antigen complex.
  - A chromogen is a substance that absorbs light, producing color.

- Commonly used enzyme labels for IHC procedures include
  - Alkaline phosphatase (AP)
  - horseradish peroxidase (HRP) catalyze the reduction of hydrogen peroxide (H_2O_2) to water and oxygen
    - Commonly used chromogens for HRP include
      - 3-amino-9-ethylcarbazole (AEC)
      - 3,3’-diaminobenzidine (DAB)

AEC (3-amino-9-ethylcarbazole)

- AEC is oxidized by HRP producing a bright red reaction product not stable and may fade over time.



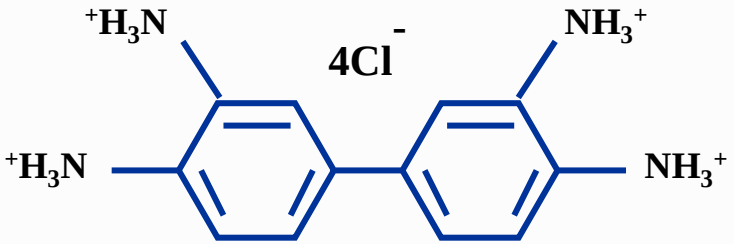
Structure of AEC

- AEC is soluble in alcohol stained slides should therefore be counterstained with non-alcoholic hematoxylins (Mayer’s or Gills).

- AEC-stained slides should be coverslipped with an aqueous mounting media.

DAB (3,3’-diaminobenzidine)

- DAB is oxidized by HRP producing a dark brown reaction product stable and does not fade over time.



Structure of DAB

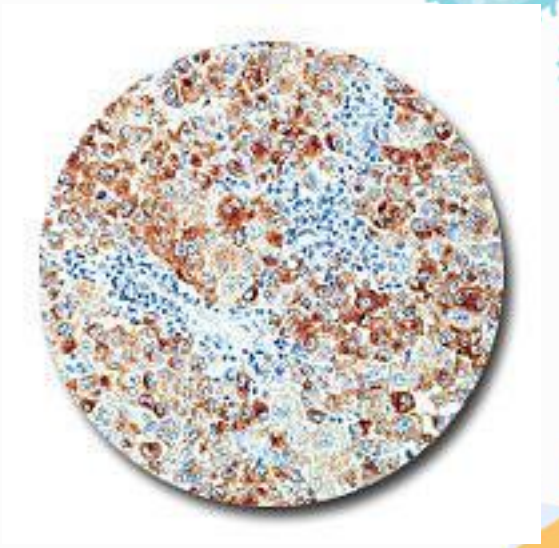
- DAB stained slides can be coverslipped with permanent organic based mounting media.

- In some IHC procedures, the dark brown reaction product can be modified and intensified by adding metals (copper or cobalt) to DAB solution.

- DAB is not suited for staining melanoma cases because the endogenous dark brown pigments in tumor cells can be confused with DAB reaction product.

Examples of staining results using AEC and DAB chromogens

AEC chromogen
Mart-1
Melanoma



DAB chromogen
Mart-1
Melanoma

IHC Staining Methods

- **Direct Method**
- **Two-Step Indirect Method**
- **Three-Step Indirect Method**
- **Peroxidase-Antiperoxidase (PAP) Method**
- **Alkaline-Antialkaline Phosphatase (APAAP) Method**
- Avidin-Biotin Complex (ABC) Method
- Labeled Streptavidin-Biotin (LSAB) Method

Direct Method

Direct Method

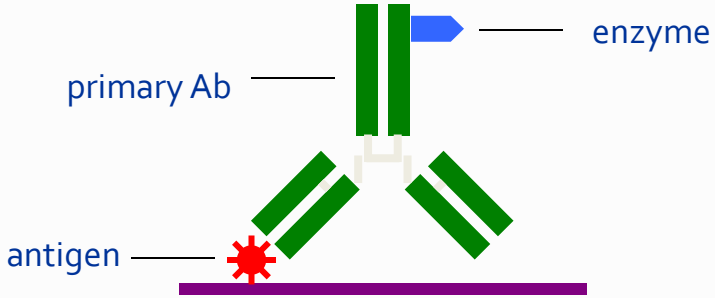
- This method uses an enzyme-labeled primary antibody with known antigenic specificity.
- Enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (AP) are either conjugated to the primary antibody.
- Less sensitive compared to other methods because it produces little signal since only one enzyme-labeled antibody is involved.

Direct Method

- Although rarely used nowadays, it has the advantages of rapidity, ease of performance and limited nonspecific reaction.

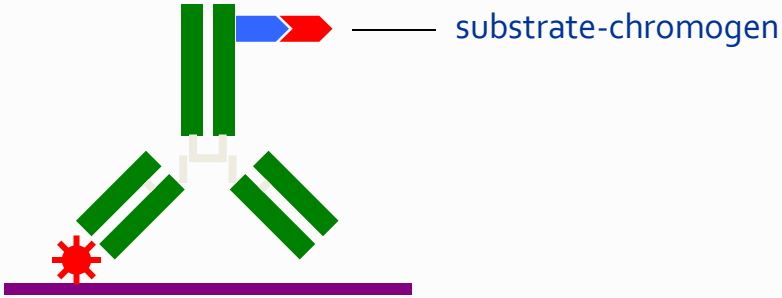
- Mostly used in fluorescent techniques where the primary antibody is labeled with fluorescent compound (e.g., fluorescein isothiocyanate or FITC).

Direct Method - Procedure



An enzyme-labeled primary antibody binds to the tissue antigen.

Direct Method - Procedure



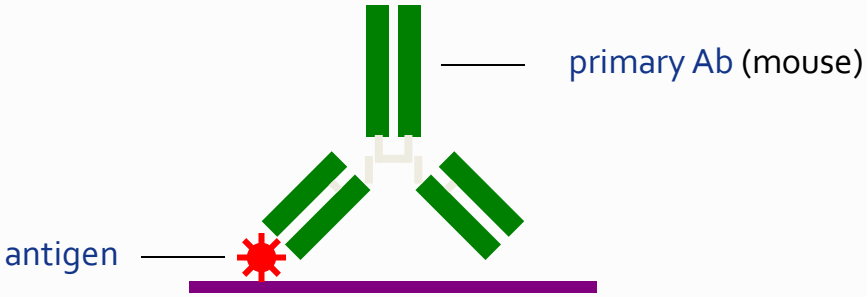
A substrate-chromogen solution is added producing a colored end-product.

Two-Step Indirect Method

Two-Step Indirect Method

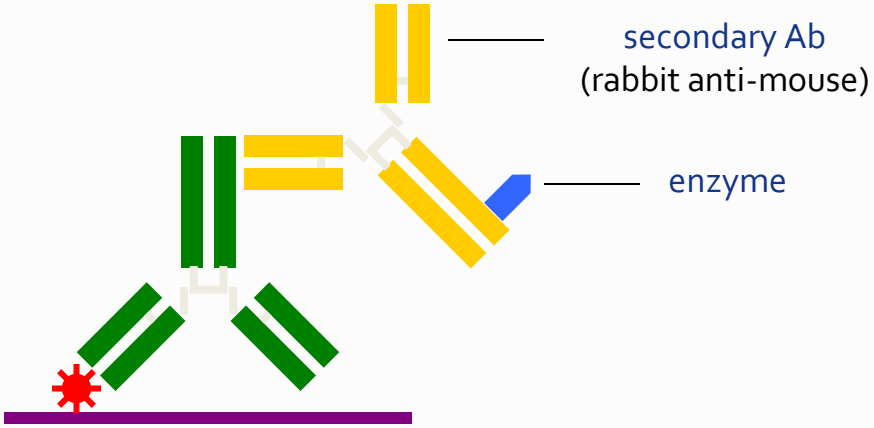
- Uses an enzyme-labeled secondary antibody that is directed against the unlabeled primary antibody.
- If the primary antibody (which is now the antigen) is made in mouse, the secondary antibody must be against mouse immunoglobulin.
- More sensitive than the *Direct Method* because several secondary antibodies are likely to bind with a number of various epitopes on the primary antibody increasing the enzyme labels involved.

Two-Step Indirect Method - Procedure



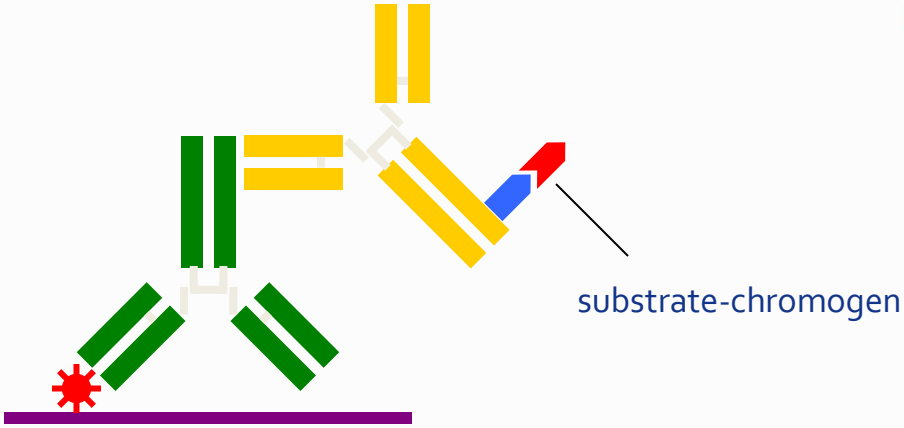
An unlabeled primary antibody binds to the tissue antigen.

Two-Step Indirect Method - Procedure



An enzyme-labeled secondary antibody binds to the primary antibody.

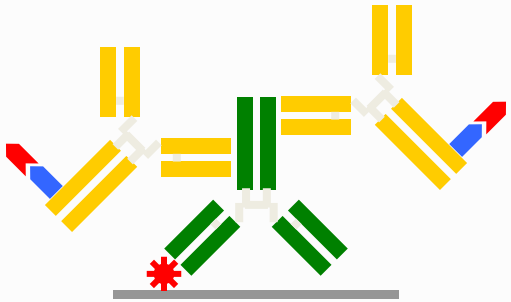
Two-Step Indirect Method - Procedure



A substrate-chromogen solution is added producing a colored end-product.

Two-Step Indirect Method - Procedure

- Only one secondary antibody is shown bound to the primary antibody in the previous illustration. However, several secondary antibodies are likely to bind with various epitopes on the primary antibody, thus increasing the signal.



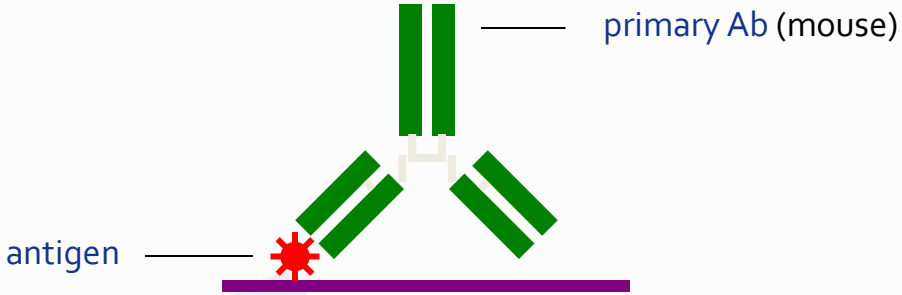
Three-Step Indirect Method

Three-Step Indirect Method

- Uses another layer of enzyme-labeled (tertiary) antibody that is added to the previously described *Two-Step Indirect Method*.
- The added antibody layer increases signal and staining intensity that is helpful when staining antigens with few or limited available epitopes.
- Both secondary and tertiary antibodies must be conjugated to the same enzyme.

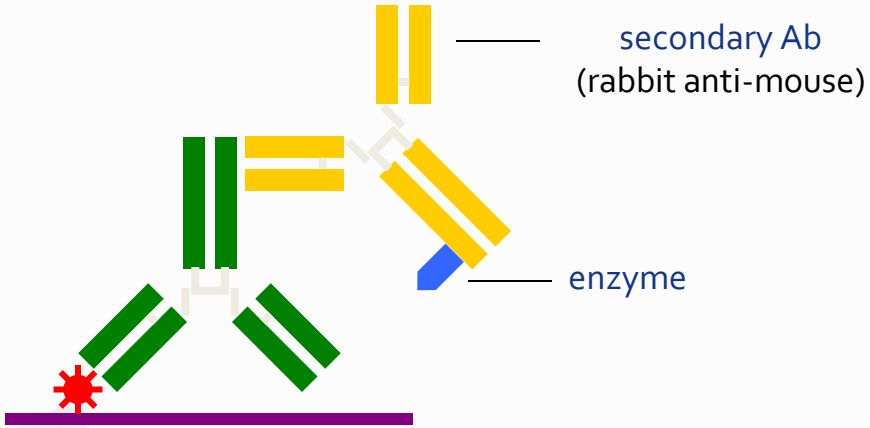
Three-Step Indirect Method - Procedure

An unlabeled primary antibody binds to the tissue antigen.



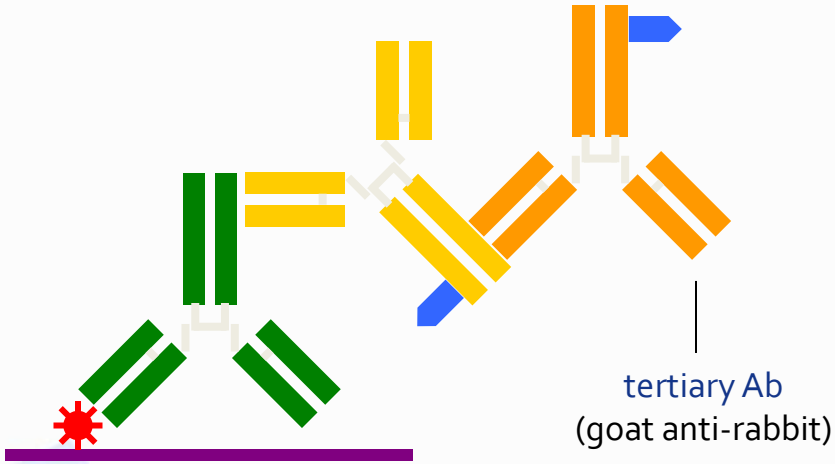
Three-Step Indirect Method - Procedure

An enzyme-labeled secondary antibody binds to the primary antibody.



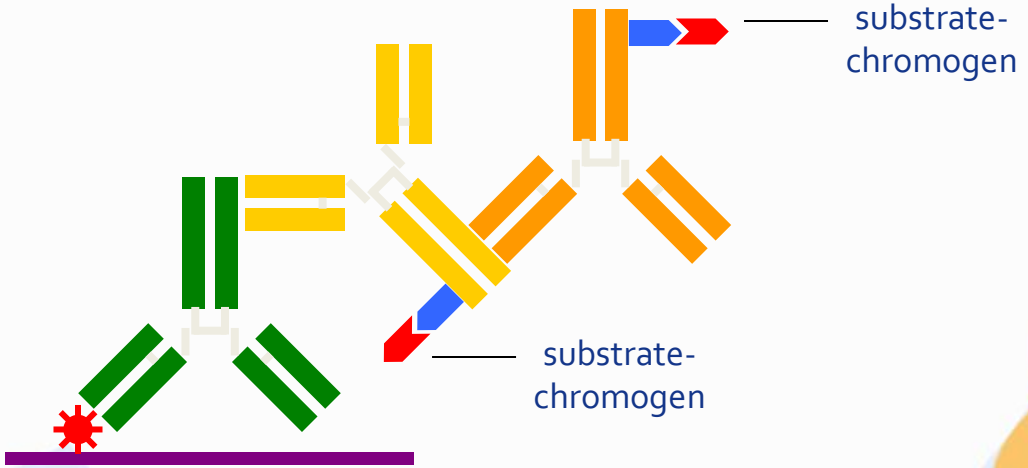
Three-Step Indirect Method - Procedure

An enzyme-labeled tertiary antibody is added and binds to the secondary antibody.



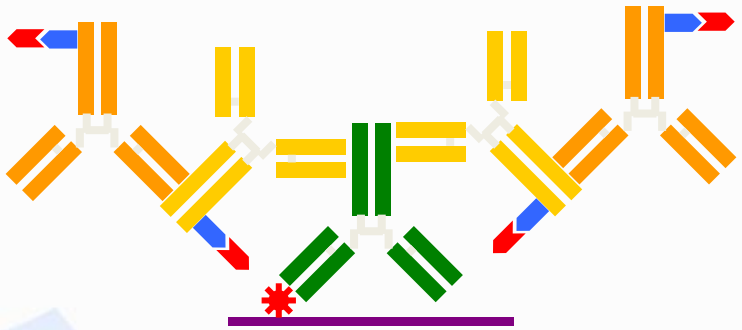
Three-Step Indirect Method

A substrate-chromogen solution is added producing a colored end-product.



Three-Step Indirect Method - Procedure

- Only one secondary antibody is shown bound to the primary antibody in the previous illustration. However, several secondary antibodies are likely to bind with different epitopes on the primary antibody, thus increasing the signal.



Soluble Enzyme Immune Complex Techniques

Soluble Enzyme Immune Complex Techniques

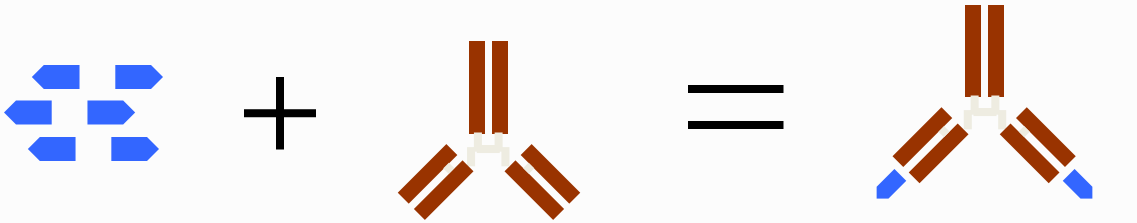
- These methods are among the sensitive IHC techniques, attributed to more enzyme molecules being localized per antigenic site.

- Methods include
  - peroxidase-antiperoxidase (PAP)
  - alkaline phosphatase-antialkaline phosphatase (APAAP)

- The secondary antibody must be directed against both the primary and the antibody of the enzyme-antienzyme immune complex.

Soluble Enzyme Immune Complex Techniques

- Preformed soluble enzyme-antienzyme immune complex is prepared by mixing excess enzyme to its antibody.



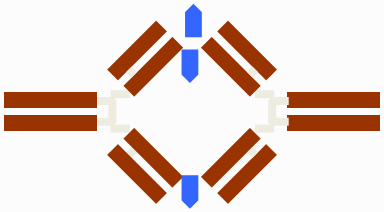
enzymes

antienzyme

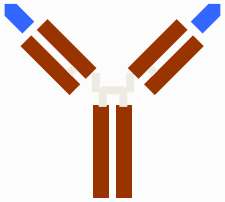
enzyme-antienzyme immune complex

Soluble Enzyme Immune Complex Techniques

- The PAP immune complex consists of three molecules of peroxidase and two antibodies.
- The APAAP immune complex consists of two molecules of alkaline phosphatase and one antibody.



peroxidase-antiperoxidase complex



alkaline phosphatase-antialkaline phosphatase complex

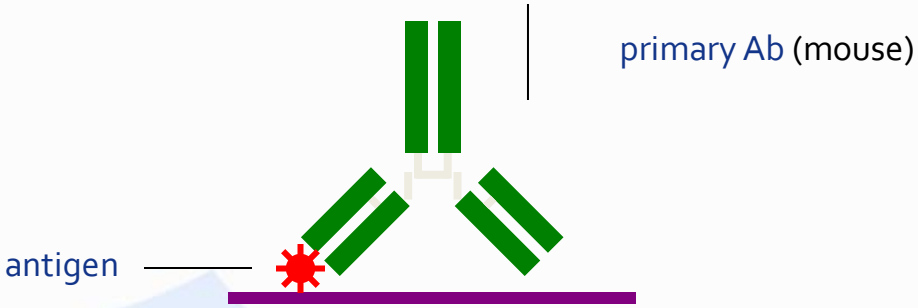
Soluble Enzyme Immune Complex Techniques

PAP and *APAAP* procedures are identical except for the enzyme-antienzyme immune complex used. Only *PAP* will be illustrated.

Peroxidase-Antiperoxidase (PAP) Method

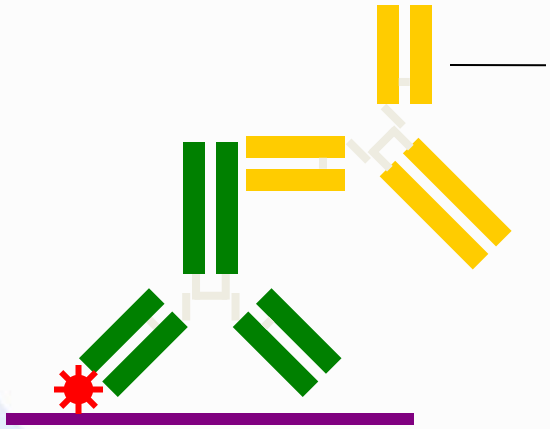
PAP - Procedure

An unlabeled primary antibody binds to the tissue antigen.



PAP - Procedure

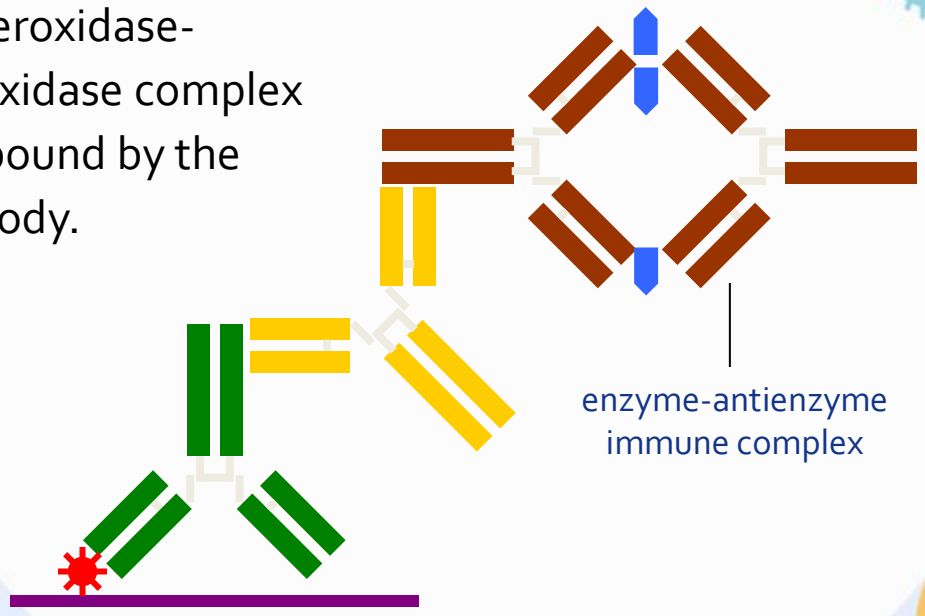
A secondary antibody binds to the primary antibody.



secondary Ab
(rabbit anti-mouse)

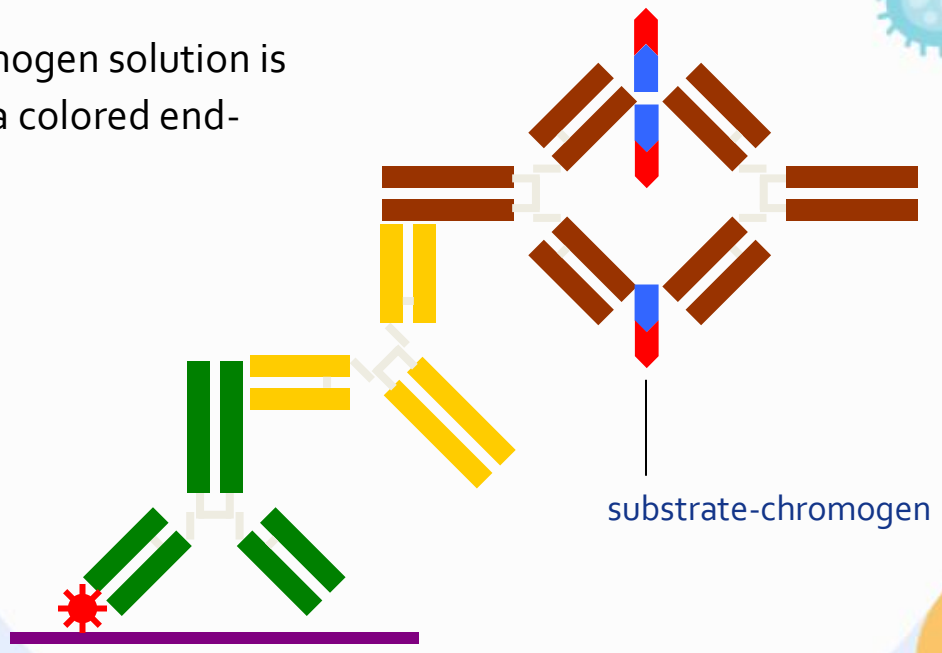
PAP - Procedure

A horseradish peroxidase-mouse-antiperoxidase complex is added and is bound by the secondary antibody.



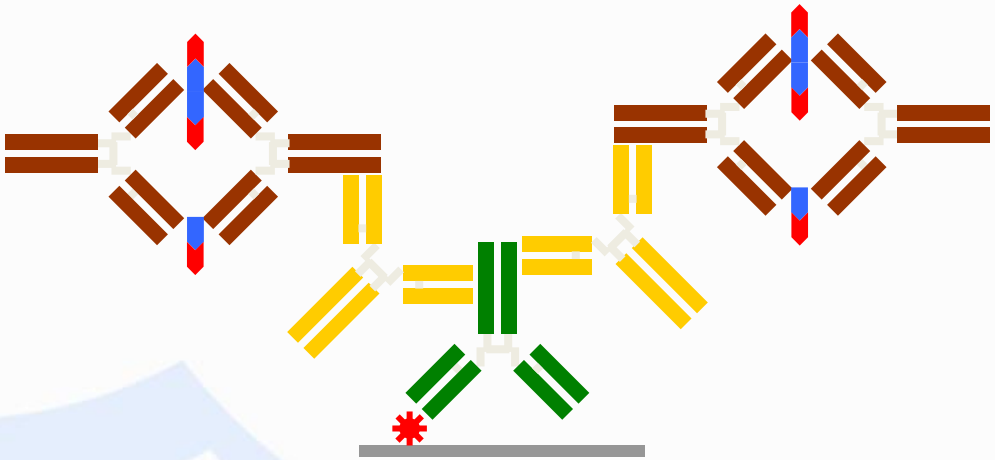
PAP - Procedure

A substrate-chromogen solution is added producing a colored end-product.



PAP - Procedure

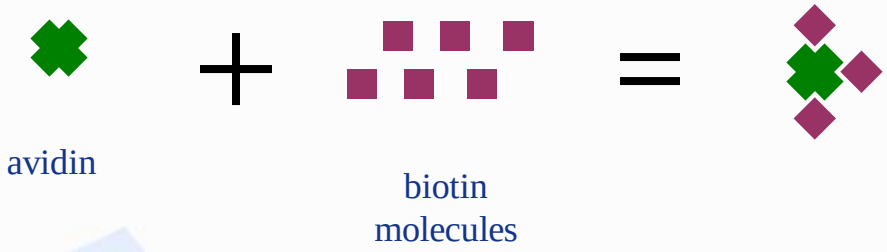
- Only one secondary antibody is shown bound to the primary antibody in the previous illustration. However, several secondary antibodies are likely to bind with different epitopes on the primary antibody, thus increasing the signal.



Avidin-Biotin Methods

Avidin-Biotin Methods

- Uses the strong and high affinity of avidin (egg white glycoprotein) for biotin (water-soluble vitamin).
- Avidin has four binding sites for biotin but fewer than four molecules of biotin will actually bind to avidin.



Avidin-Biotin Methods

- Two of the most common methods include
  - Avidin-Biotin enzyme Complex (ABC)
  - Labeled StreptAvidin-Biotin (LSAB)

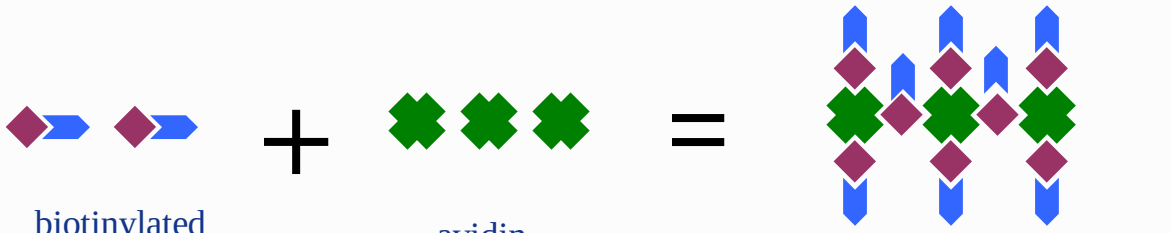
- The inherent amplification of sensitivity offered by avidin and biotin makes these methods more favorable than *PAP* or *APAAP*.

- Streptavidin, a bacterial protein has recently replaced avidin because it produces less background staining than avidin.

Avidin-Biotin Enzyme Complex (ABC)

ABC Method

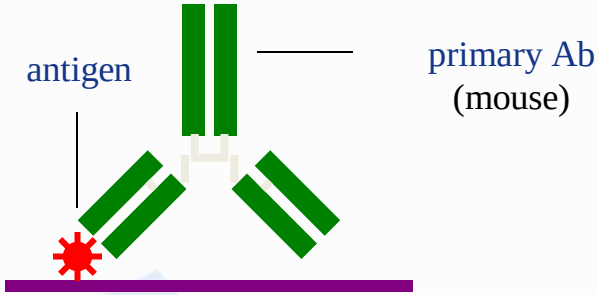
- The enzyme complex is prepared by mixing biotinylated enzyme (HRP or AP) and avidin.



- This preformed avidin-biotin-enzyme complex then reacts with the biotinylated secondary antibody.

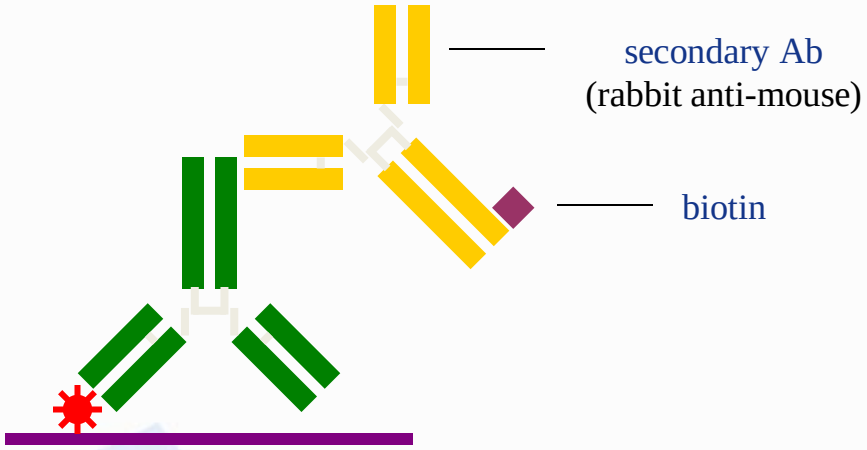
ABC - Procedure

An unlabeled primary antibody binds to the antigen.



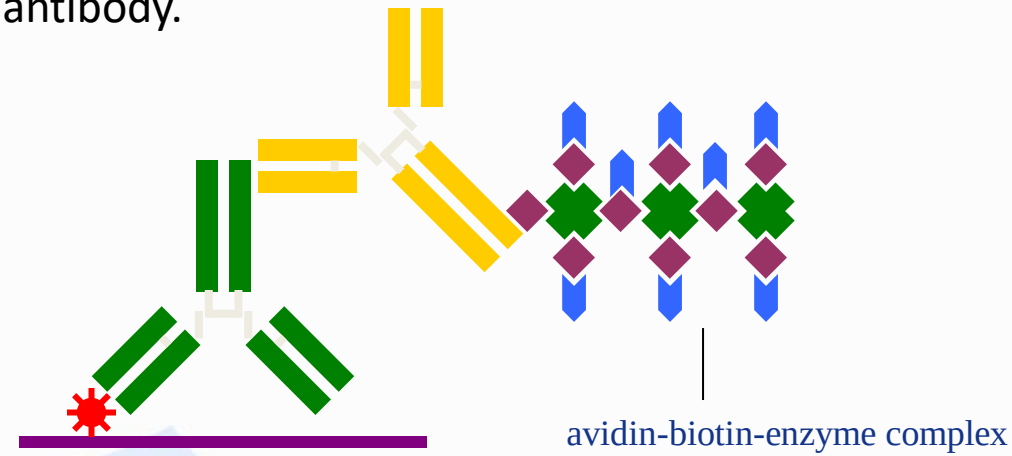
ABC - Procedure

A biotinylated secondary antibody binds to the primary antibody.



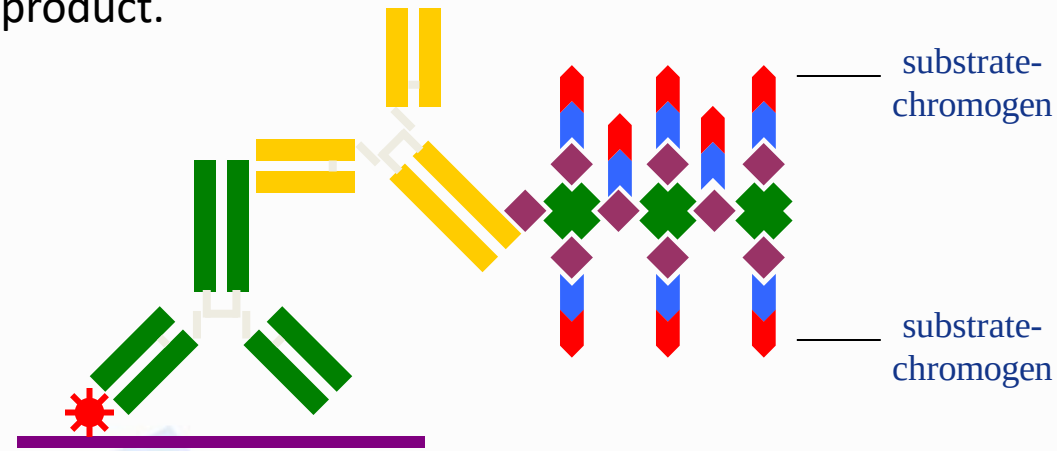
ABC - Procedure

A preformed avidin-biotin-enzyme complex solution is added and binds to the biotinylated secondary antibody.



ABC - Procedure

A substrate-chromogen solution is added ending the reaction and producing a colored end-product.



Labeled StreptAvidin-Biotin (LSAB)

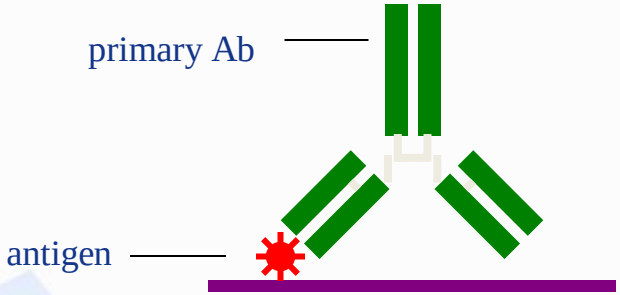
LSAB Method

- A Uses enzyme-conjugated streptavidin. Streptavidin is conjugated to several molecules of enzyme horseradish peroxidase (HRP) or alkaline phosphatase (AP).

- The secondary antibody is conjugated to numerous biotin molecules, each of which can potentially bind to an enzyme-conjugated streptavidin.

LSAB – Procedure

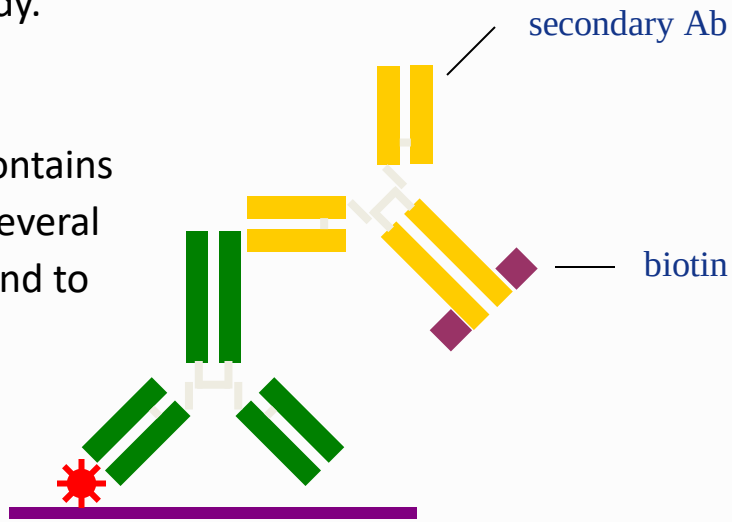
An unlabeled primary antibody binds to tissue antigen.



LSAB – Procedure

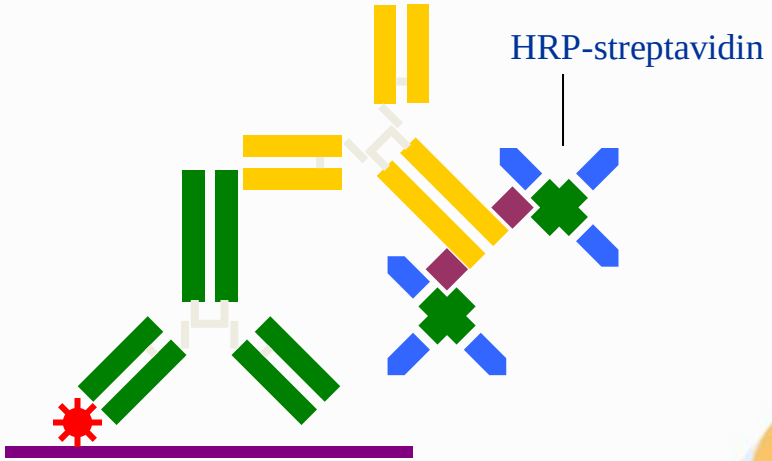
A biotinylated secondary antibody binds to the primary antibody.

Each secondary antibody contains multiple biotin molecules; several secondary antibodies can bind to the primary antibody.



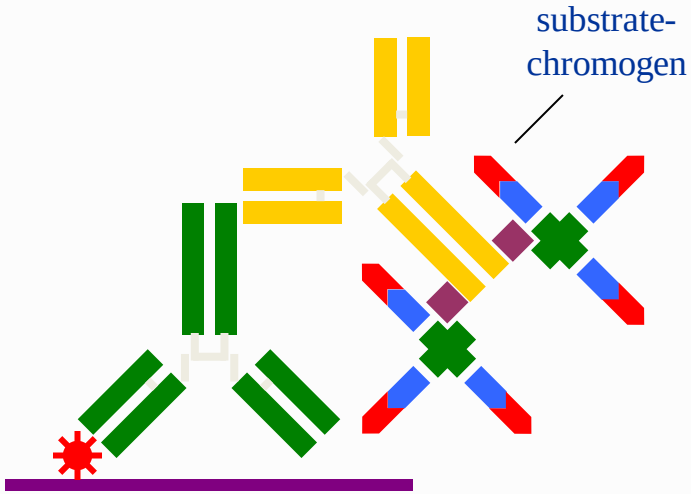
LSAB – Procedure

An enzyme-labeled streptavidin is added and binds to the secondary antibody.



LSAB – Procedure

A substrate-chromogen solution is added producing a colored end-product.



Step by Step LSAB Staining Procedure

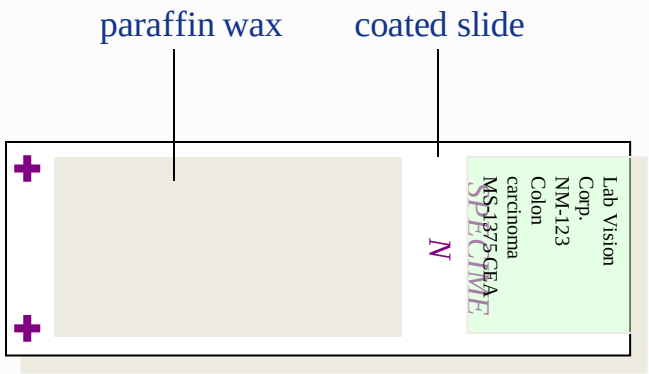
Introduction

This section shows the step by step *LSAB* staining procedure using carcinoembryonic antigen (CEA) antibody

on colon carcinoma.

Step by Step LSAB Staining Method

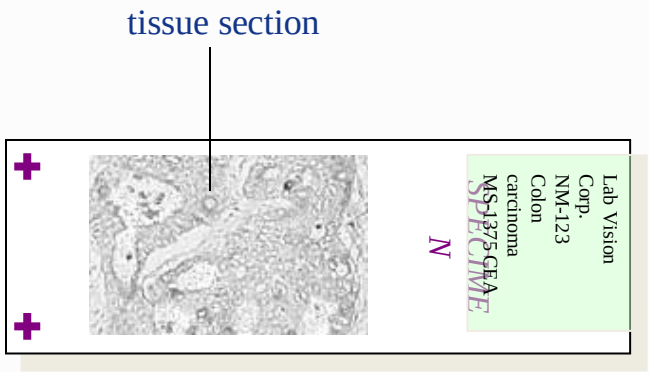
Deparaffinization and Rehydration



Remove paraffin wax and hydrate tissue section.

Step by Step LSAB Staining Method

Deparaffinization and Rehydration

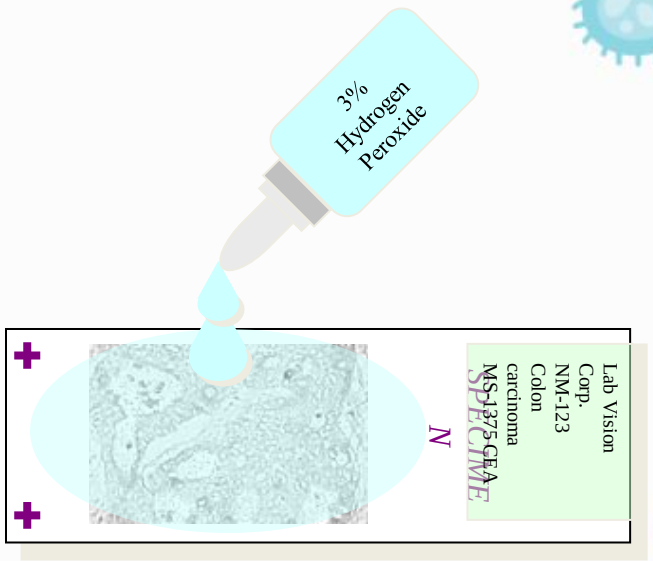


Remove paraffin wax and hydrate tissue section.

Step by Step LSAB Staining Method

Deparaffinization and Rehydration

Block Endogenous Peroxidase



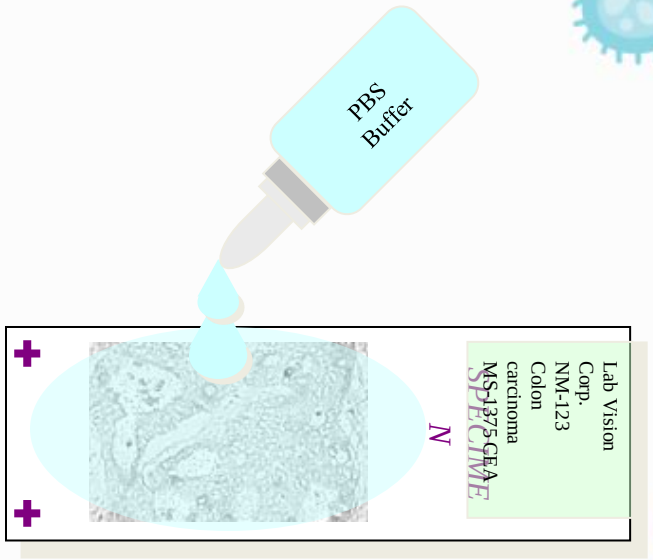
3% hydrogen peroxide solution for 10 minutes to inactivate endogenous peroxidase activity.

Step by Step LSAB Staining Method

Deparaffinization and Rehydration

Block Endogenous Peroxidase

Rinse



Rinse in distilled water and wash 2 times in PBS buffer.

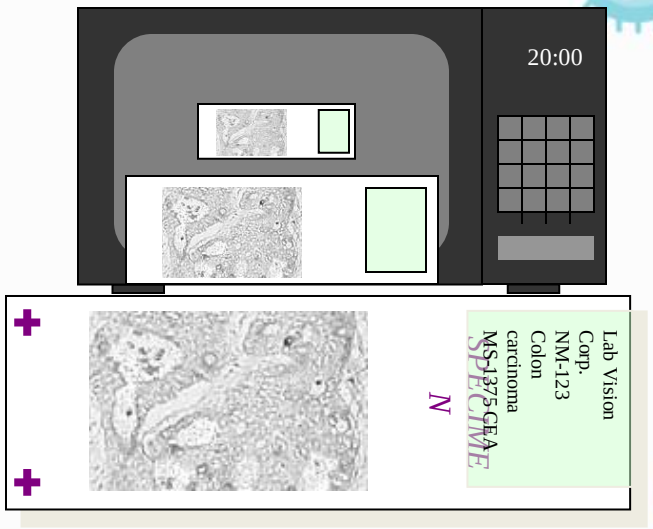
Step by Step LSAB Staining Method

Deparaffinization and Rehydration

Rinse

Block Endogenous Peroxidase

Antigen Retrieval



Microwave for 20 minutes using citrate buffer solution pH 6.0.

Step by Step LSAB Staining Method

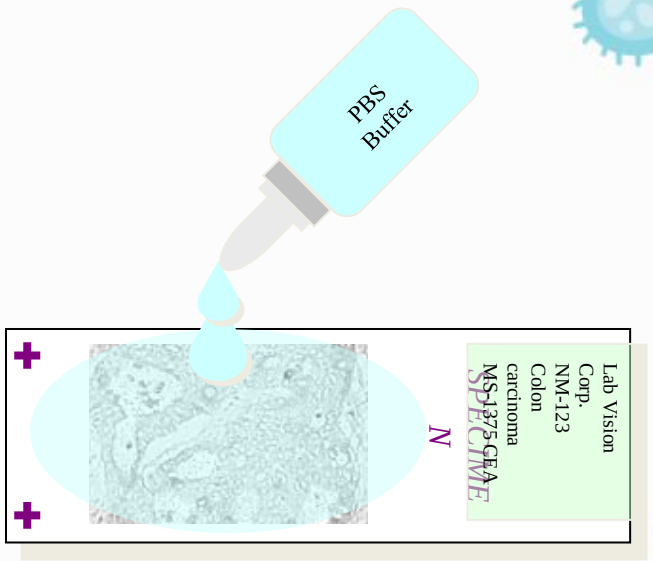
Deparaffinization and Rehydration

Block Endogenous Peroxidase

Rinse

Antigen Retrieval

Rinse



Thorough rinse in distilled water and wash 2 times in PBS buffer.

Step by Step LSAB Staining Method

Deparaffinization and Rehydration

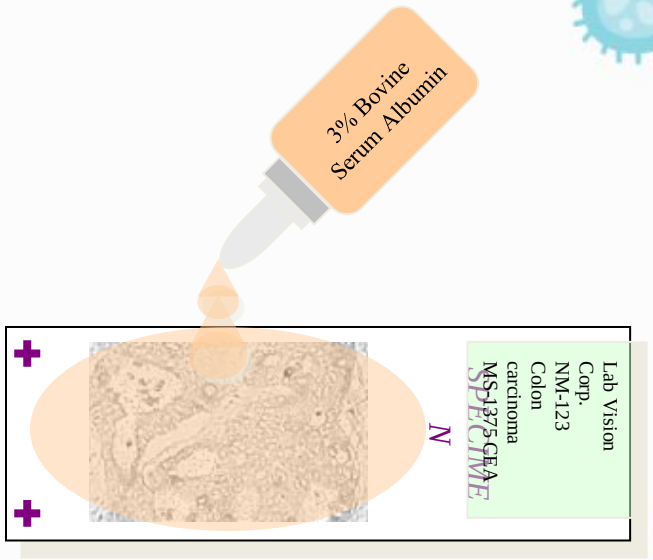
Block Endogenous Peroxidase

Rinse

Antigen Retrieval

Rinse

Protein Block



3% bovine serum albumin (BSA) to block nonspecific staining. Drain excess BSA after incubation.

Step by Step LSAB Staining Method

Deparaffinization and Rehydration

Block Endogenous Peroxidase

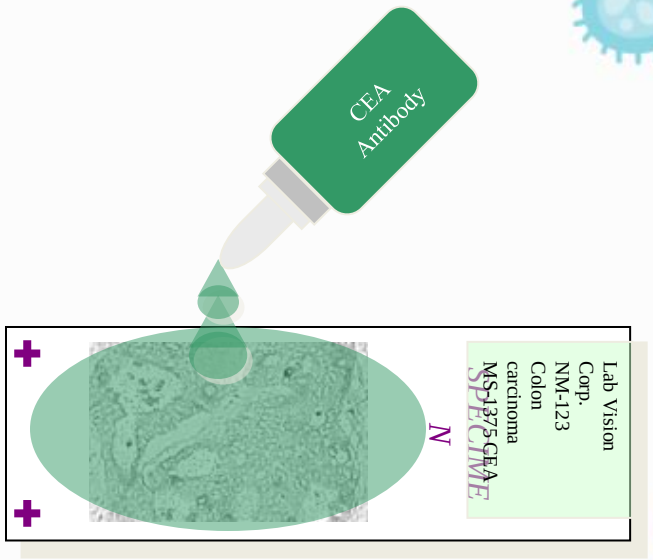
Rinse

Antigen Retrieval

Rinse

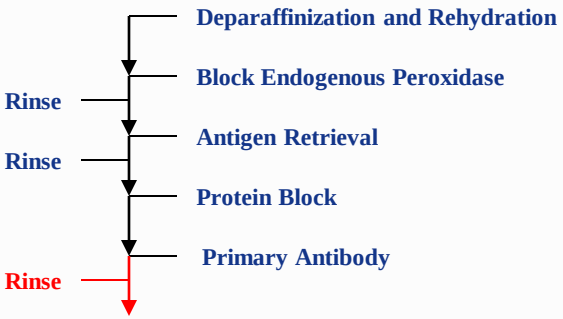
Protein Block

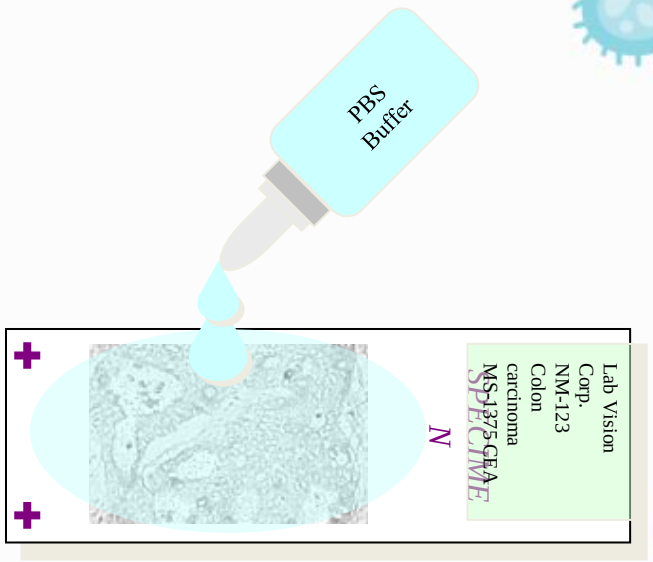
Primary Antibody



Optimally diluted CEA antibody for 30 minutes.

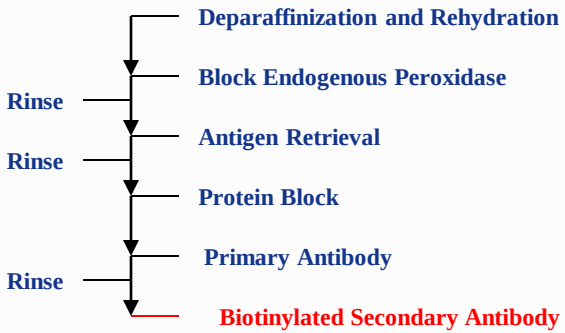
Step by Step LSAB Staining Method

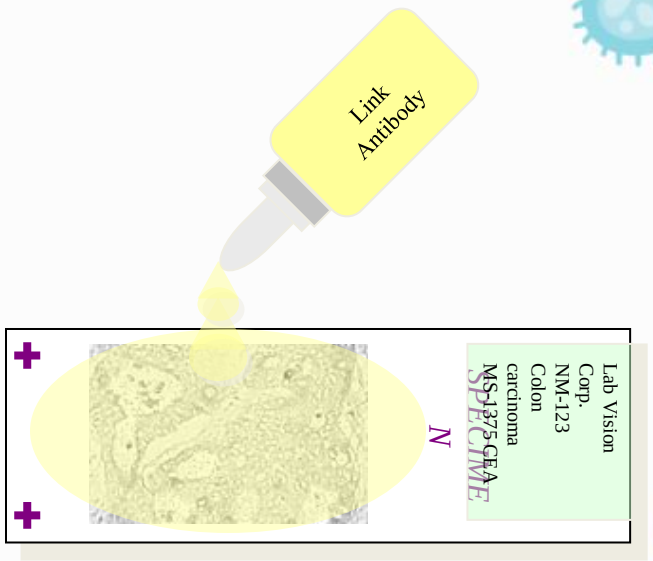




Wash 2 times in PBS buffer.

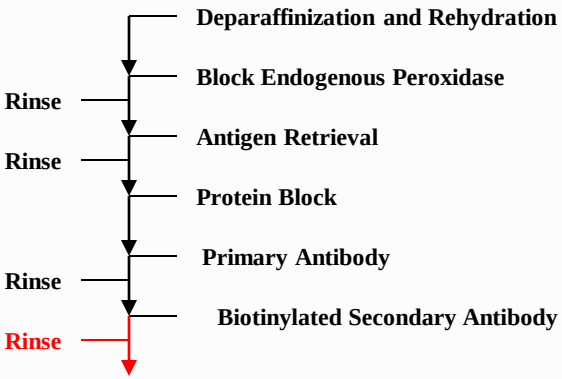
Step by Step LSAB Staining Method

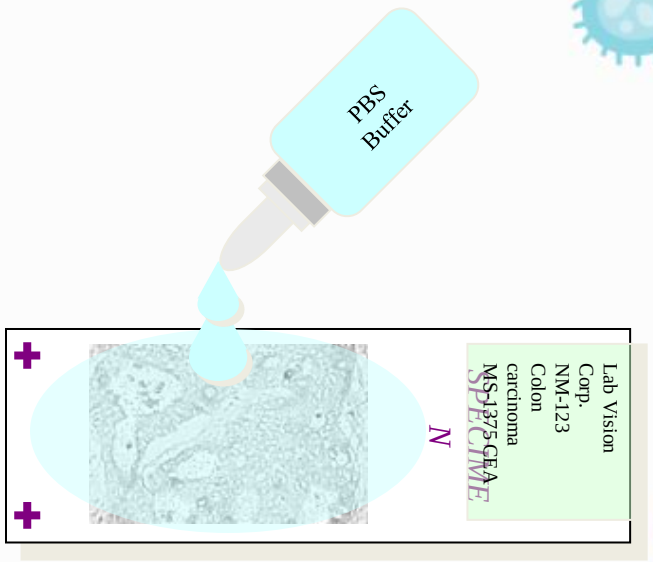




Biotinylated link antibody for 10 minutes.

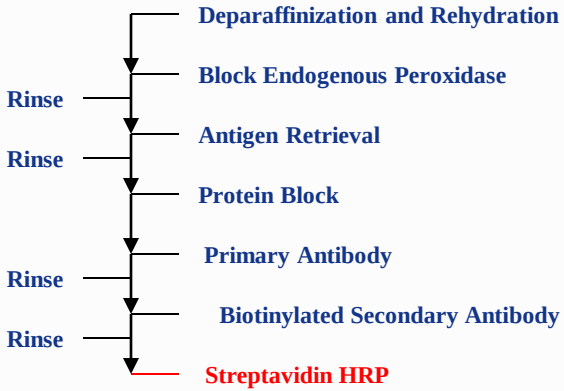
Step by Step LSAB Staining Method





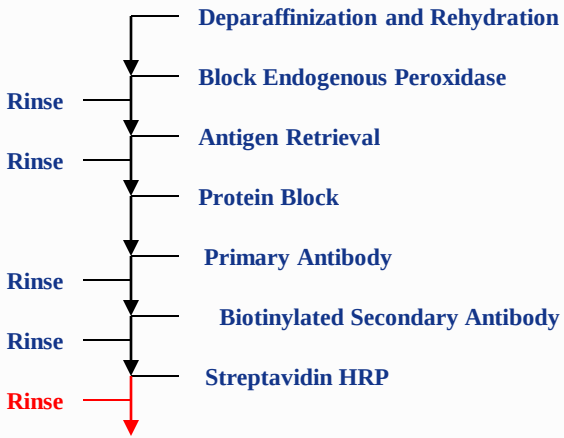
Wash 2 times in PBS buffer.

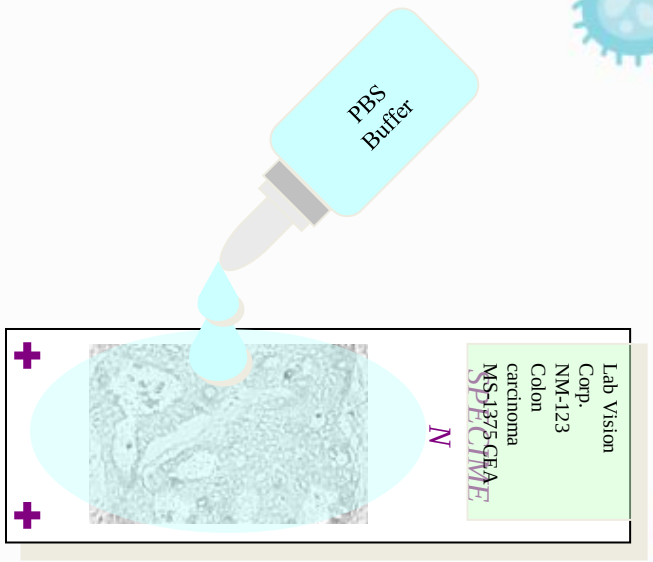
Step by Step LSAB Staining Method



HRP conjugated streptavidin for 10 minutes.

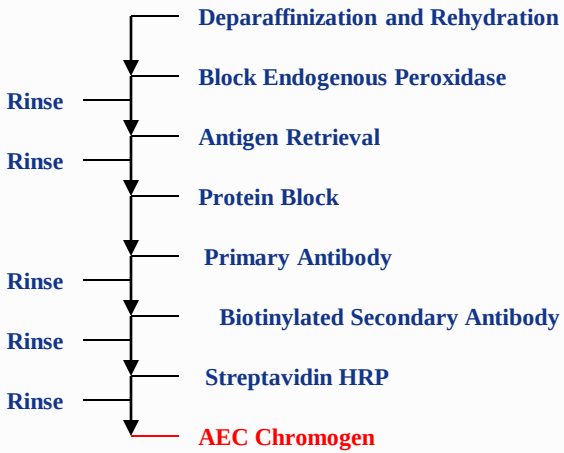
Step by Step LSAB Staining Method

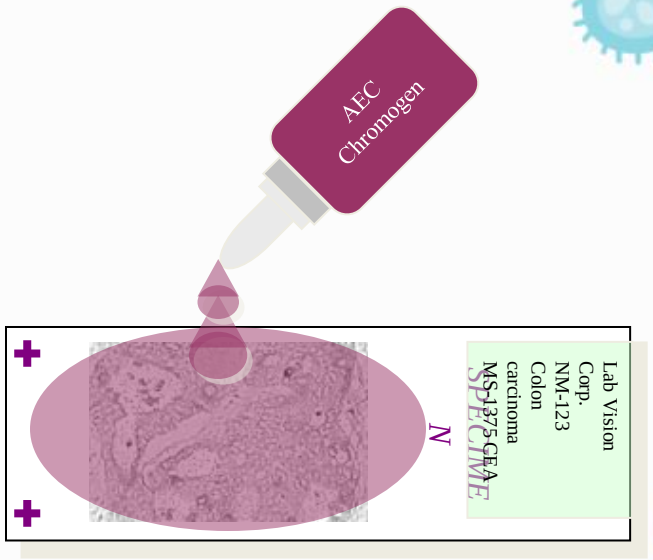




Wash 2 times in PBS buffer.

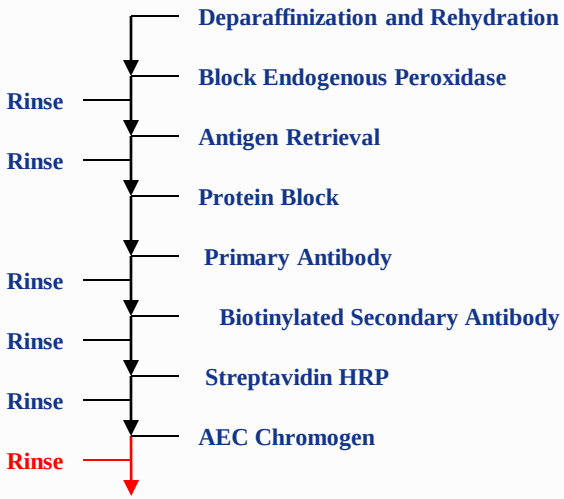
Step by Step LSAB Staining Method

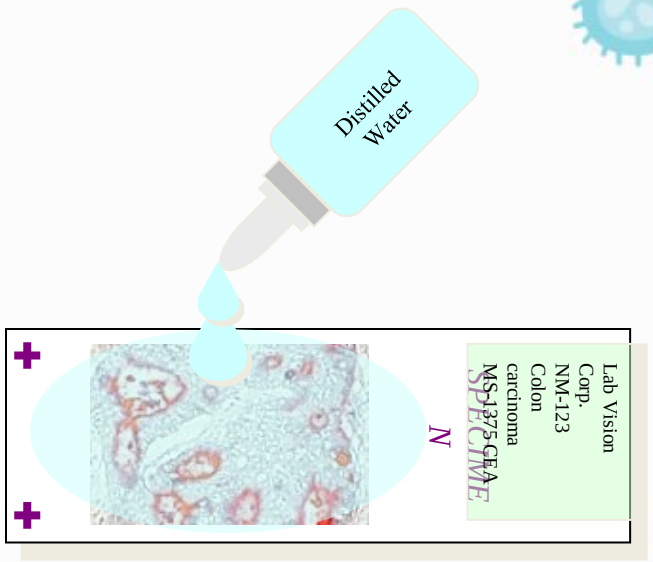




AEC chromogen for 10 minutes.

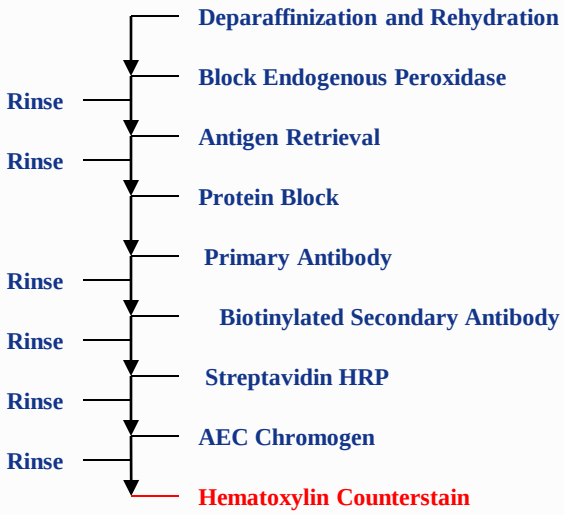
Step by Step LSAB Staining Method

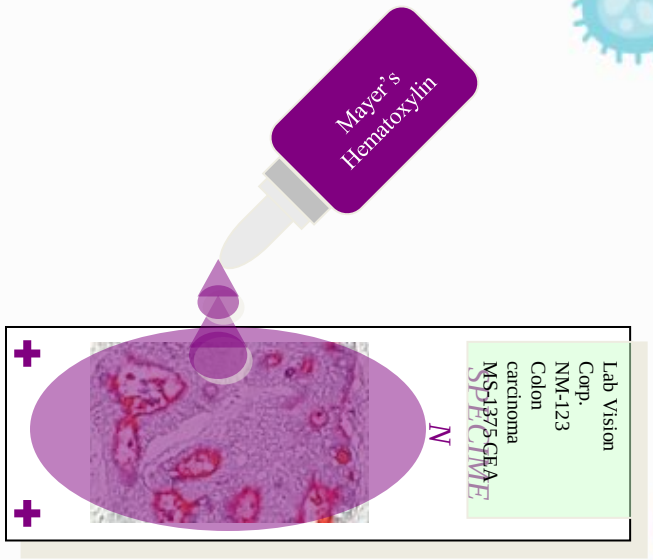




Wash 2 times in PBS buffer and rinse in distilled water.

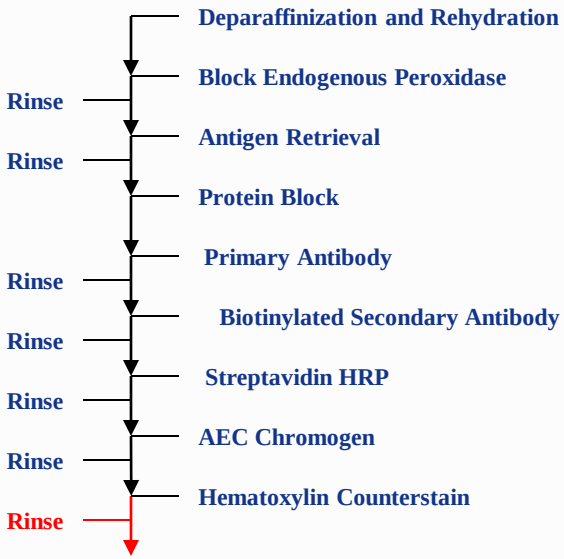
Step by Step LSAB Staining Method

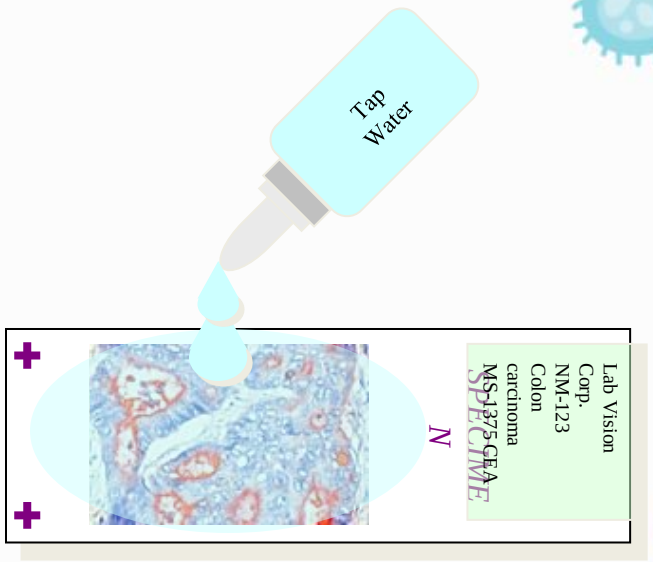




Counterstain in Mayer's Hematoxylin for 1 minute.

Step by Step LSAB Staining Method

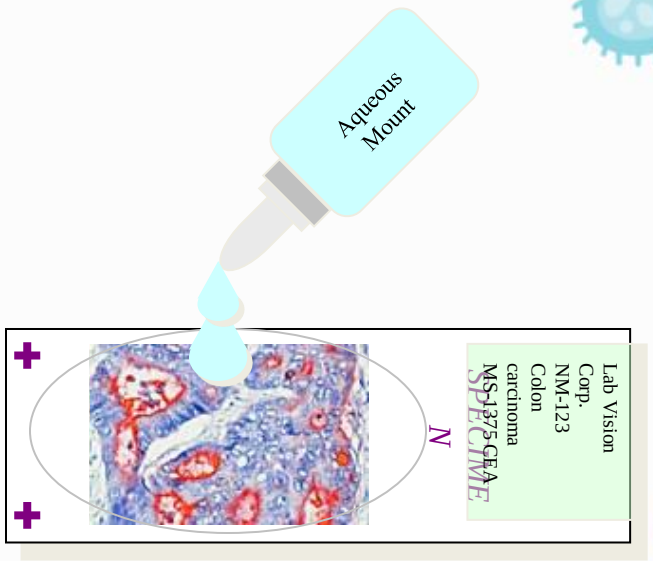




Thorough wash in tap water to “blue” the nuclei.

Step by Step LSAB Staining Method

- Deparaffinization and Rehydration
- Block Endogenous Peroxidase
- Rinse
- Antigen Retrieval
- Rinse
- Protein Block
- Primary Antibody
- Rinse
- Biotinylated Secondary Antibody
- Rinse
- Streptavidin HRP
- Rinse
- AEC Chromogen
- Rinse
- Hematoxylin Counterstain
- Rinse
- Aqueous Mount



Coverslip using an aqueous mount.

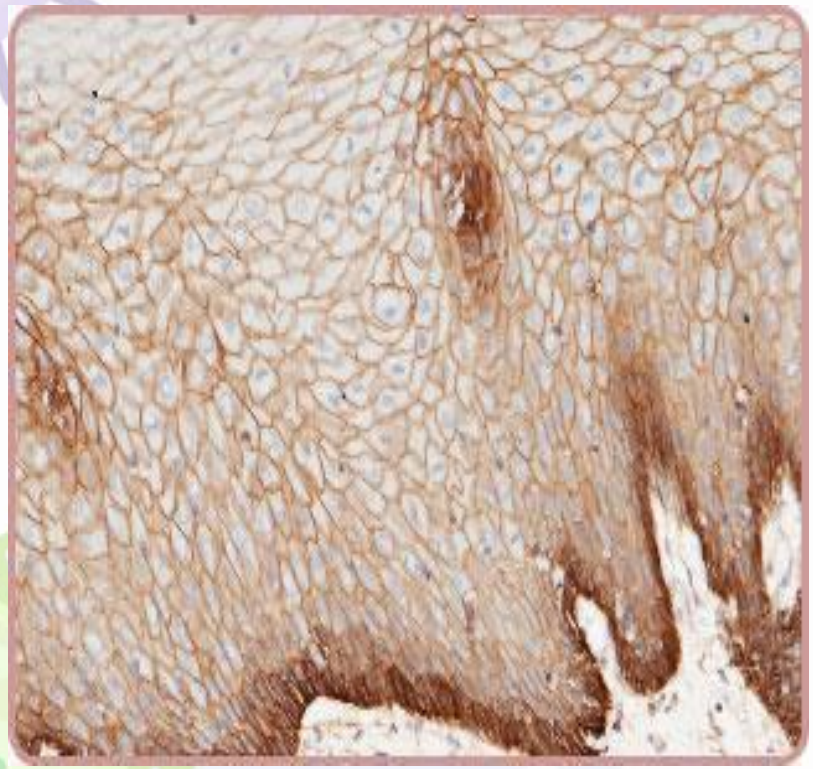
IHC Staining Patterns

Introduction

The following slides show photos of various staining patterns that can be achieved after performing an IHC stain.

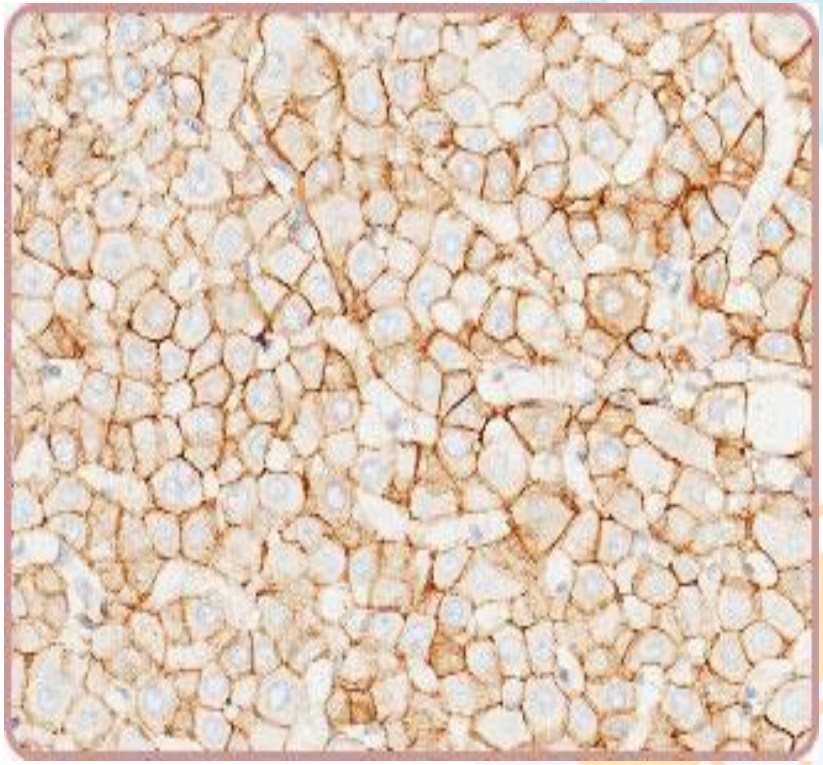
Pola Pulasan – Membran Sel

CD147



Immunochemical staining of human CD147 in human esophagus with mouse monoclonal antibody (10 µg/mL, formalin-fixed paraffin embedded sections). The image showing membrane staining of squamous epithelium cell.

E-cadherin

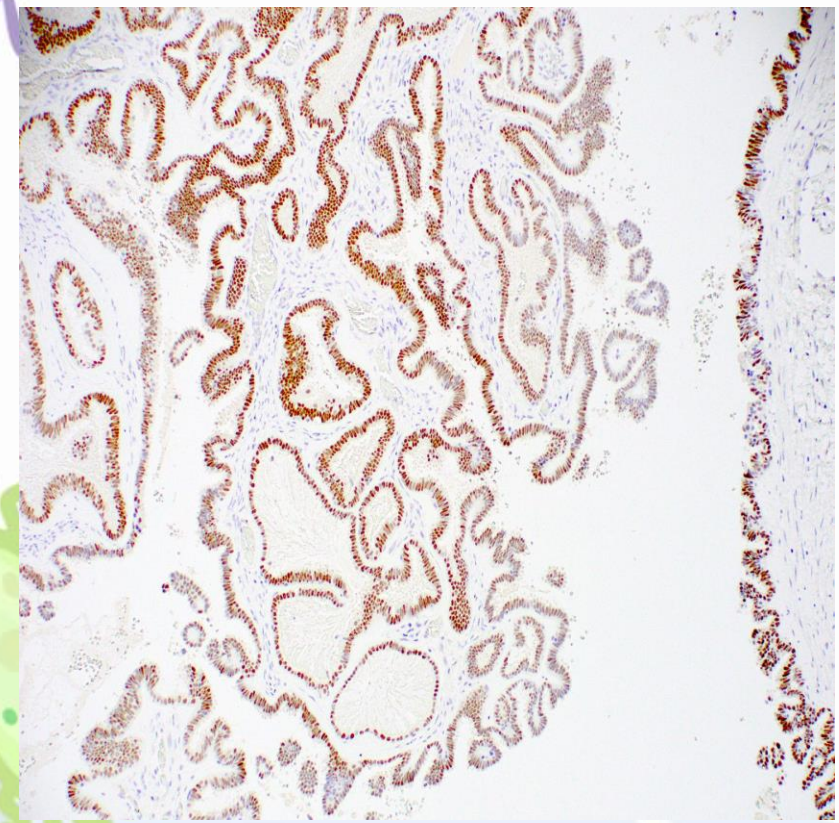


Immunochemical staining of human E-cad in human liver with rabbit polyclonal antibody (1 µg/mL, formalin-fixed paraffin embedded sections).The image showing cytomembrane staining.

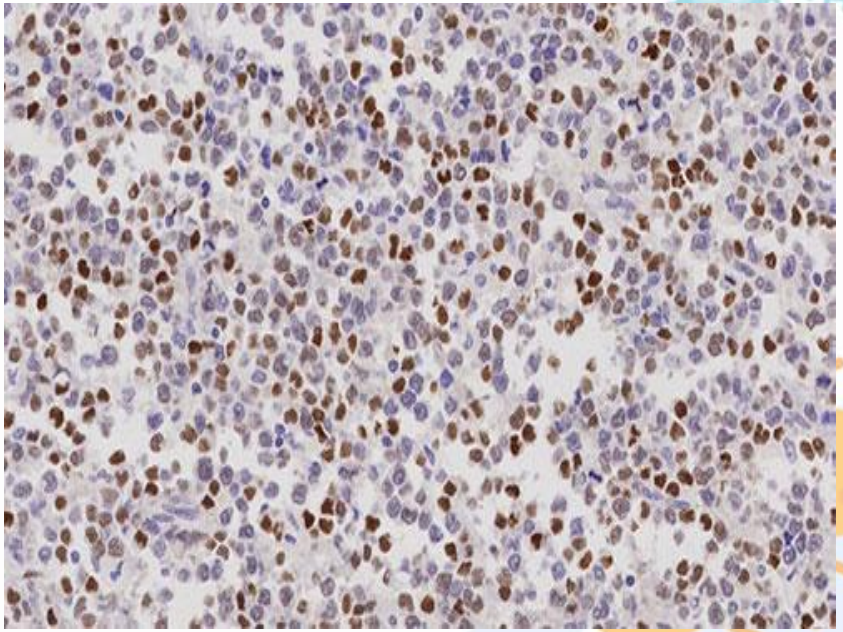
https://www.sinobiological.com/category/ihc-antibody-cytomembrane-staining

Pola Pulasan – Inti Sel

ER

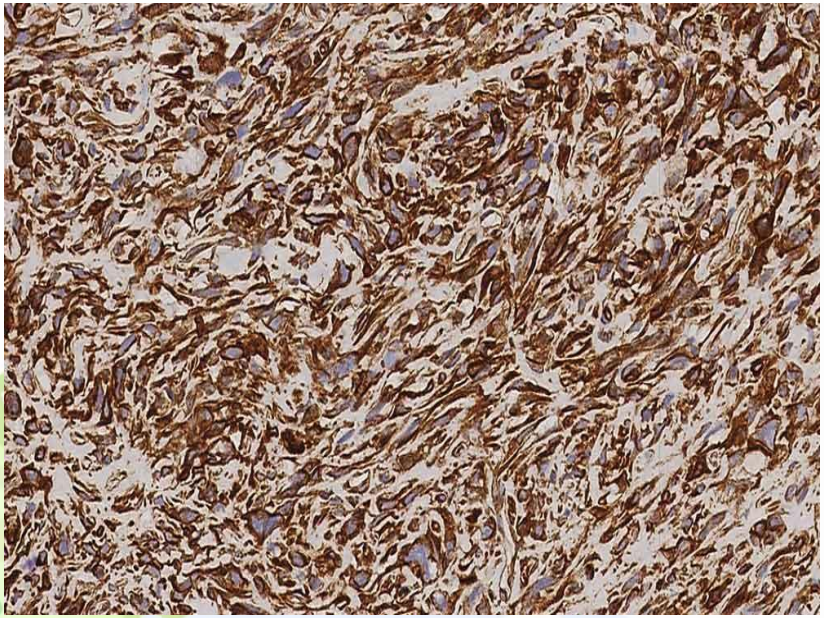


Cyclin D1

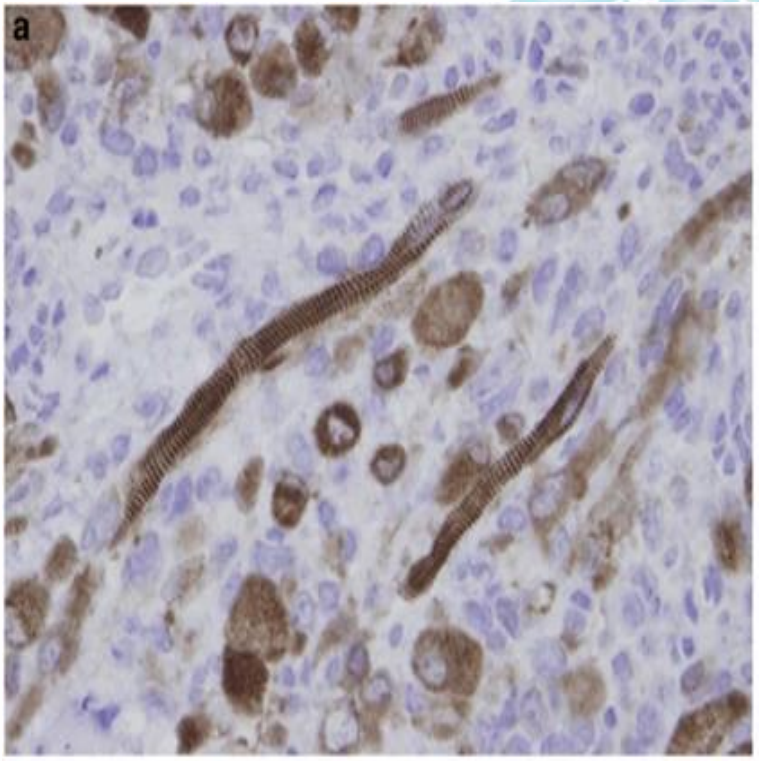


Pola Pulasan - Sitoplasma

Vimentin

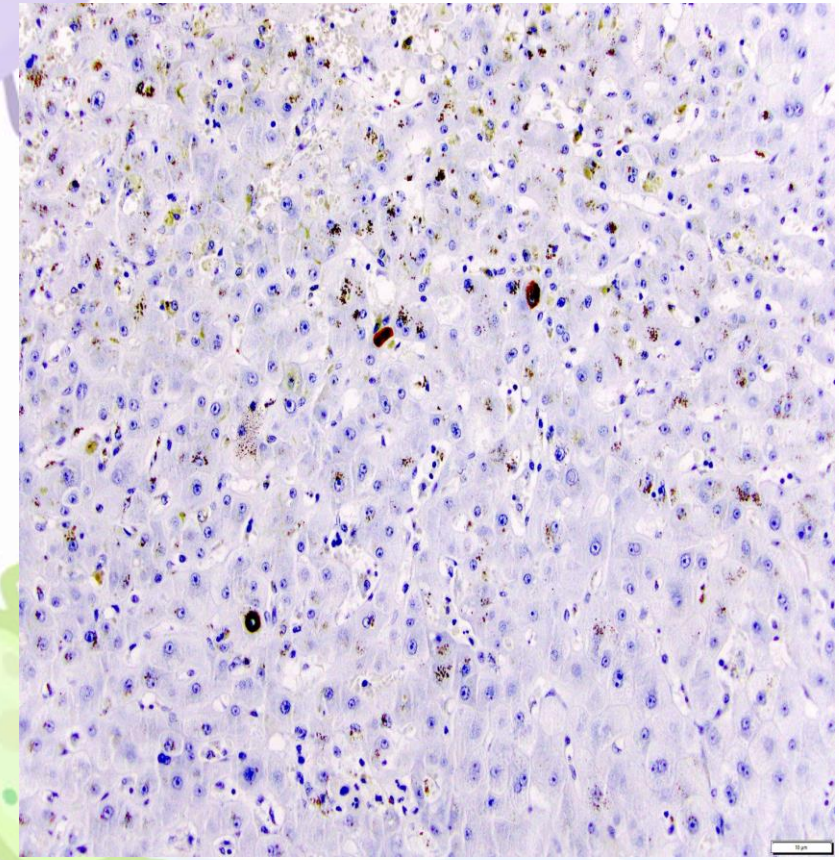


p63

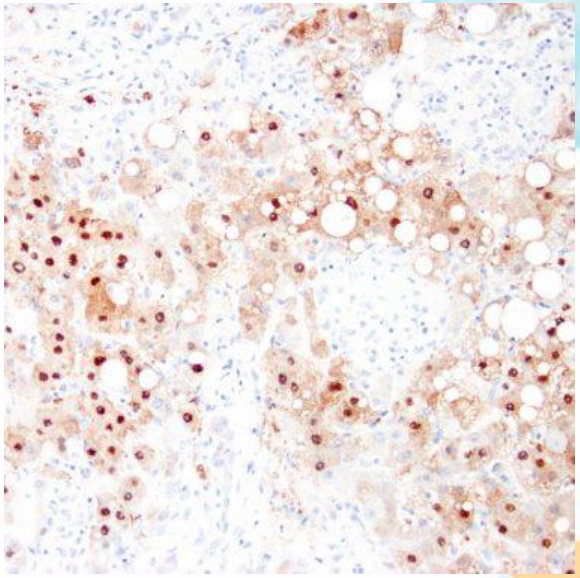


https://shop.leicabiosystems.com/ihc-ish/ihc-primary-antibodies/pid-vimentin
https://www.nature.com/articles/modpathol201189/figures/1

Pola Pulasan – Agen Infeksius



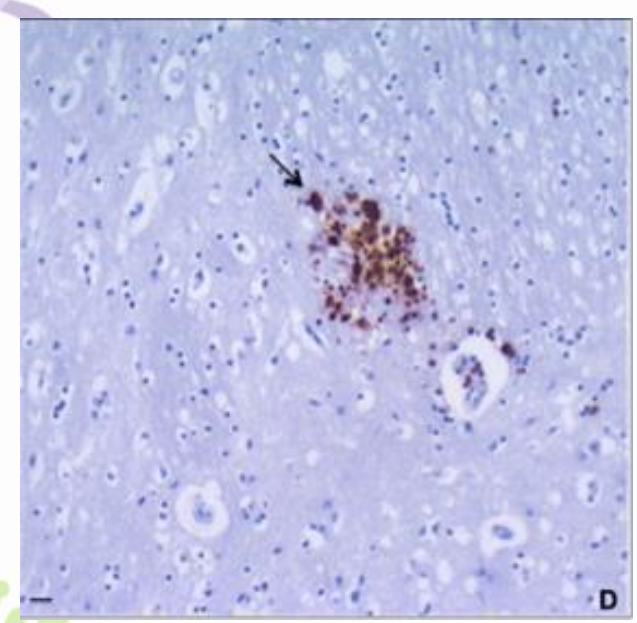
HBsAg inclusions in the hepatocyte cytoplasm highlighted by HBsAg immunohistochemical stain.



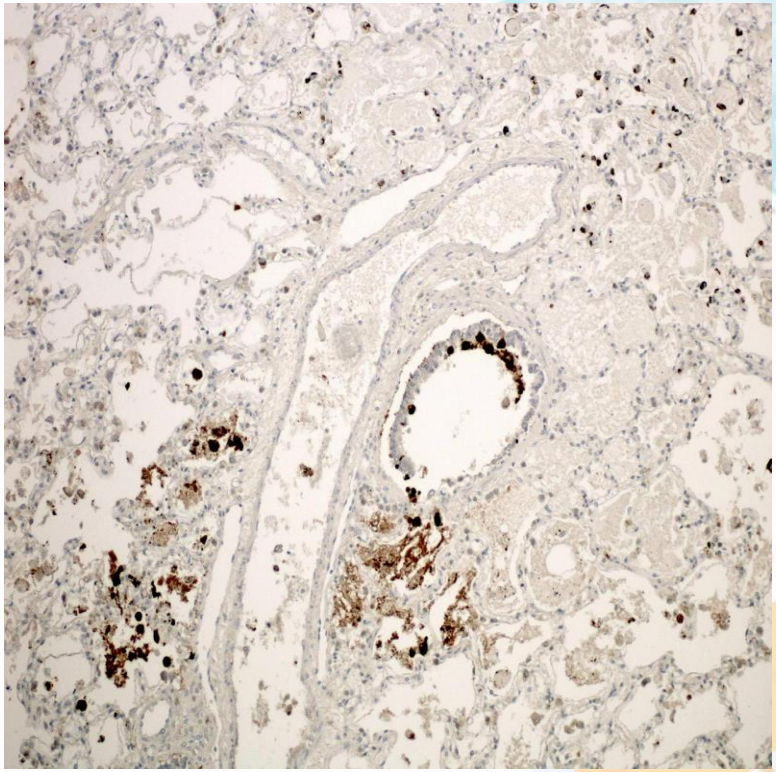
Human liver infected with hepatitis B virus stained with anti-HBcAg antibody using peroxidase-conjugate and DAB chromogen. Note the nuclear staining of infected hepatocytes.

https://zeta-corp.com/product/hbcag-antibody/
https://www.pathologyoutlines.com/topic/liverhepb.html

Pola Pulasan – Agen Infeksius

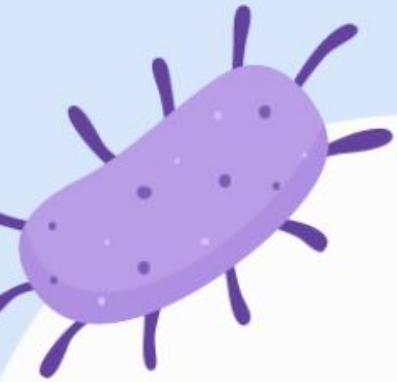


Immunostained section of *T. gondii*-infected human brain (positive control)-numerous and strongly marked cysts with bradyzoites (arrow).



Immunohistochemical stain for adenovirus: nuclear and cytoplasmic expression (IHC, 20x).

https://www.sciencedirect.com/science/article/pii/S0304401712004797
https://www.pathologyoutlines.com/topic/stainsadenovirus.html



THANK YOU