Preparation for the Qualification in Immunohistochemistry Examination

DEB VAN EYCK CT(ASCP)QIHC

BRANDON WARD HTL(ASCP)QIHC

Objectives

- Why take the QIHC examination?
- ASCP eligibility requirements, taking the exam & requalification
- Topic outline – the nitty gritty
- Governmental Regulations/Accrediting Agencies
- Resources

Why should I take the QIHC examination?

- Show your knowledge
- Some employers require qualification
- Some employers may have a higher pay scale for qualified individuals
- Be a better troubleshooter
- Being qualified will help you to teach others about a complex and technical aspect of the lab

Eligibility requirements for the QIHC Examination

- Route 1:
  - ASCP or ASCP^{i} certified as a histotechnicial (HT) or histotechnologist (HTL)
  - 6 months full-time (minimum 35 hrs/week) acceptable experience in IHC (clinical, veterinary, industry or research) in the U.S., Canada or an accredited lab within the last 5 years
- Route 2:
  - ASCP or ASCP^{i} certified as a technologist/scientist (eg. CT, MLS) or specialist (eg. SCT)
  - 12 months full-time acceptable experience in a combination of routine histology and IHC, with a minimum of 6 months in histology (clinical, veterinary, industry or research) and a minimum of 6 months in IHC in the U.S., Canada or an accredited lab within the last 5 years

Eligibility requirements for the QIHC Examination

- Route 3:
  - MLT(ASCP) or MLT(ASCP^{i}) certification
  - 18 months of full-time acceptable experience in a combination of routine histology and IHC, with a minimum of 6 months in histology (clinical, veterinary, industry or research) and a minimum of 12 months in IHC in the U.S., Canada or an accredited lab within the last 5 years
- Route 4:
  - Baccalaureate degree or higher from a regionally accredited college/university
  - 24 months of full-time acceptable experience in a combination of routine histology and IHC, with a minimum of 9 months in histology (clinical, veterinary, industry or research) and a minimum of 15 months in IHC in the U.S., Canada or an accredited lab within the last 5 years

Eligibility requirements for the QIHC Examination

- *Note that for work in an accredited laboratory, the lab must have achieved accreditation via CMS, CLIA, JCI or accreditation under ISO 15189*

Apply online

- After reviewing the eligibility requirements and determining eligibility, apply online at https://www.ascp.org/content/board-of-certification/get-credentialed
  - Application fee of $240 is non-refundable
    - Pay online with a credit card OR
    - If you are unable to pay with a credit card, instructions for pay-by-mail will be available upon completion of the application process
- Following online application, submit necessary documents within 45 days (next slide)

Documents needed for application

- Work experience documentation forms (All routes)
  - ASCP Experience Documentation Form completed by immediate supervisor
  - Letter of Authenticity signed by this individual verifying the authenticity of the form
- Verification of academic education (Route 4)
  - Official transcript from the regionally accredited college/university, bearing the embossed seal of the college/university, signature of the registrar and the date the degree was conferred.
- Mail to:

  ASCP Board of Certification

  33 W. Monroe St., Suite 1600

  Chicago, IL 60603

Application status update

- When the status of the application changes (determined deficient, ineligible, eligible, etc.) you will receive an email notification informing you to login to your ASCP account for specific information/details regarding the status of your application

Scheduling and taking your exam

- Once your eligibility has been approved, you will receive an Admission Notification email containing an authorization number to the examination
- Test is self-administered on your own computer at the date/time of your choice within the 60 day time period indicated on your Admission Notification

QIHC Examination format

- The examination consists of 50 multiple choice questions
- Timed test – 90 minutes

Examination Results

- You will receive your exam score within four business days of your exam date
- Wall certificate will arrive within 4-8 weeks

Use of Qualification

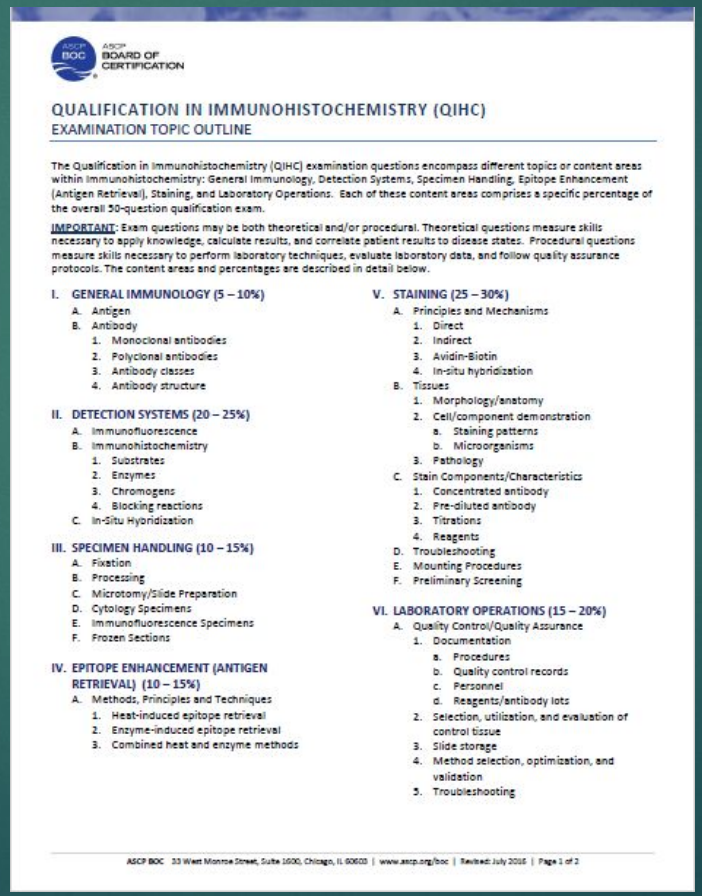
- Elizabeth Jones, QIHC^{CM}
- Elizabeth Jones, HT(ASCP)QIHC^{CM}

QIHC Requalification

- To maintain qualification, you **must requalify every three years** by:
  - Completing 6 contact hours of acceptable continuing education in the area of qualification OR
  - Completing 3 contact hours of acceptable continuing education in the area of qualification and 3 contact hours of other activities related to the qualification
- Requalification fee is $50
- If you hold ASCP certification, you are able to synchronize your requalification with your recertification. Initially will cost the full fee for each. Every time thereafter, full fee for recertification, $15 for requalification.
- See procedure booklet for the steps for requalification
  - https://www.ascp.org/content/docs/default-source/boc-pdfs/exam-content-outlines/ascp-qualification-brochure-web.pdf?sfvrsn=10

Examination Topic Outline

- https://www.ascp.org/content/docs/default-source/boc-pdfs/boc-us-guidelines/qihc_topic_outline.pdf?sfvrsn=4



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QUALIFICATION IN IMMUNOHISTOCHEMISTRY (QIHC)
EXAMINATION TOPIC OUTLINE

The Qualification in Immunohistochemistry (QIHC) examination questions encompass different topics or content areas within Immunohistochemistry: General Immunology, Detection Systems, Specimen Handling, Epitope Enhancement (Antigen Retrieval), Staining, and Laboratory Operations. Each of these content areas comprises a specific percentage of the overall 50-question qualification exam.

IMPORTANT: Exam questions may be both theoretical and/or procedural. Theoretical questions measure skills necessary to apply knowledge, calculate results, and correlate patient results to disease states. Procedural questions measure skills necessary to perform laboratory techniques, evaluate laboratory data, and follow quality assurance protocols. The content areas and percentages are described in detail below.

I. GENERAL IMMUNOLOGY (5 – 10%)
- A. Antigen
- B. Antibody
  1. Monoclonal antibodies
  2. Polyclonal antibodies
  3. Antibody classes
  4. Antibody structure

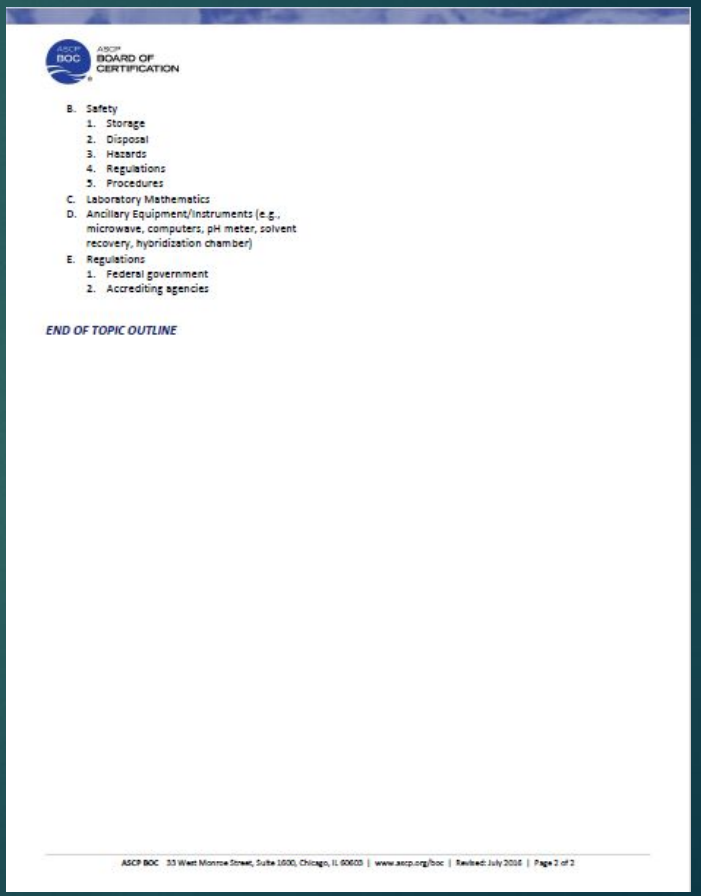
II. DETECTION SYSTEMS (20 – 25%)
- A. Immunofluorescence
- B. Immunohistochemistry
  1. Substrates
  2. Enzymes
  3. Chromogens
  4. Blocking reactions
- C. In-Situ Hybridization

III. SPECIMEN HANDLING (10 – 15%)
- A. Fixation
- B. Processing
- C. Microtomy/Slide Preparation
- D. Cytology Specimens
- E. Immunofluorescence Specimens
- F. Frozen Sections

IV. EPITOPE ENHANCEMENT (ANTIGEN RETRIEVAL) (10 – 15%)
- A. Methods, Principles and Techniques
  1. Heat-induced epitope retrieval
  2. Enzyme-induced epitope retrieval
  3. Combined heat and enzyme methods

V. STAINING (25 – 30%)
- A. Principles and Mechanisms
  1. Direct
  2. Indirect
  3. Avidin-Biotin
  4. In-situ hybridization
- B. Tissues
  1. Morphology/anatomy
  2. Cell/component demonstration
     a. Staining patterns
     b. Microorganisms
  3. Pathology
- C. Stain Components/Characteristics
  1. Concentrated antibody
  2. Pre-diluted antibody
  3. Titrations
  4. Reagents
- D. Troubleshooting
- E. Mounting Procedures
- F. Preliminary Screening

VI. LABORATORY OPERATIONS (15 – 20%)
- A. Quality Control/Quality Assurance
  1. Documentation
     a. Procedures
     b. Quality control records
     c. Personnel
     d. Reagents/antibody lots
  2. Selection, utilization, and evaluation of control tissue
  3. Slide storage
  4. Method selection, optimization, and validation
  5. Troubleshooting



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- B. Safety
  1. Storage
  2. Disposal
  3. Hazards
  4. Regulations
  5. Procedures
- C. Laboratory Mathematics
- D. Ancillary Equipment/Instruments (e.g., microwave, computers, pH meter, solvent recovery, hybridization chamber)
- E. Regulations
  1. Federal government
  2. Accrediting agencies

END OF TOPIC OUTLINE

General Immunology

5-10%

General Immunology

- Immunology is the branch of science that deals with the immune system and the cell-mediated and humoral aspects of immunity and immune responses https://www.merriam-webster.com/dictionary/immunology
  - Cell-mediated immune response
    - Macrophages engulf foreign antigen and present the antigen to T-Helper cells.
    - Certain T-Helper cells secrete cytokines which activate Cytotoxic T-Cells.
    - Cytotoxic T-Cells will kill the infected cell through initiation of apoptosis
  - **Humoral (aka "Antibody-mediated") immune response**
    - **Macrophages engulf foreign antigen and present the antigen to T-Helper cells**
    - **Certain activated T-Helper cells interact with B cells, causing the B cells to proliferate, differentiate and produce antibodies**

General Immunology

- A. Antigen
  - Biomarker that can activate an antibody-generation (immune) response
  - In IHC, biomarkers of choice are typically proteins
    - May exist on the surface of a cell, in the cytoplasm, in the nucleus or be transmembrane
  - Part of the antigen that is specifically recognized by the immune system is the epitope
    - Epitope - the antigenic determinant that is bound by the antibody
      - Linear epitope – defined by primary amino acid sequence; typically 5-20 amino acids in length. Chain of amino acids is called a peptide.
      - Conformational epitope – defined by spatial structure of the protein, brought together via folding
  - Antigens used in IHC for generating antibodies from animals are often full length or protein fragments due to their potency (ie. they contain many epitopes)

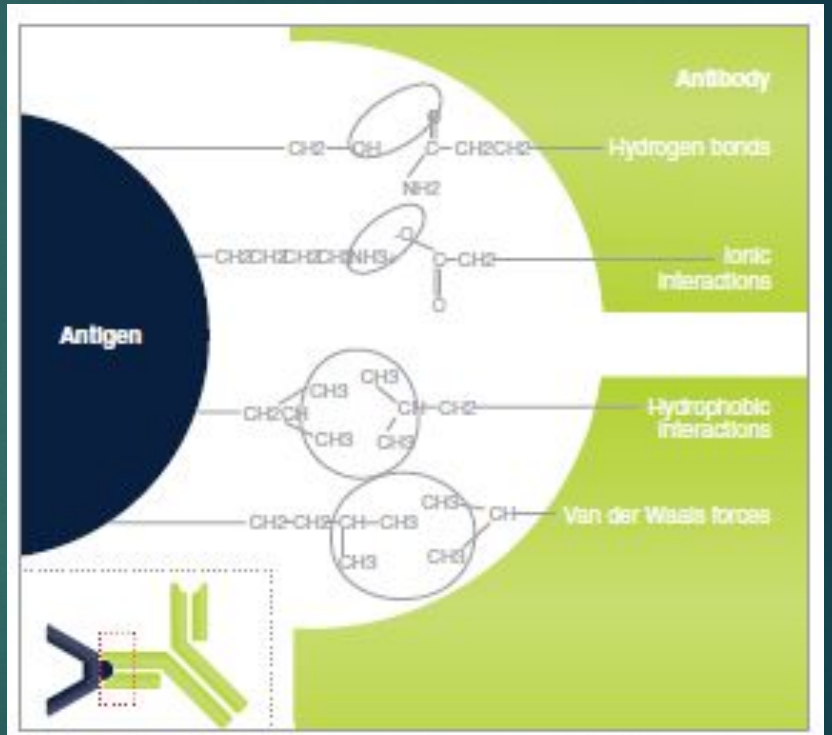


Figure 8.5 Antibodies are attracted to antigens initially through electrostatic interactions, and subsequently through Van der Waals and hydrophobic interactions.

General Immunology

- B. Antibody

An antibody is a protein that belongs to a group called immunoglobulins (Ig) that are present in the blood of immunized individuals

General Immunology

- 1. Monoclonal antibodies
  - Antiserum of homogenous antibodies that recognize a specific epitope on an antigen
  - Often prepared in mice; seeing more and more produced in rabbits
    - Produced by injecting the animal with an antigen (immunized) and boosting the animal every two weeks over a period of two months
    - After acceptable immune response is reached, B lymphocytes from the animal's spleen are isolated and fused with an immortal cell line (myeloma cell line fusion partner)
    - Fused immortal cell line is called a hybridoma and it will produce a stable clone of the specific antibody

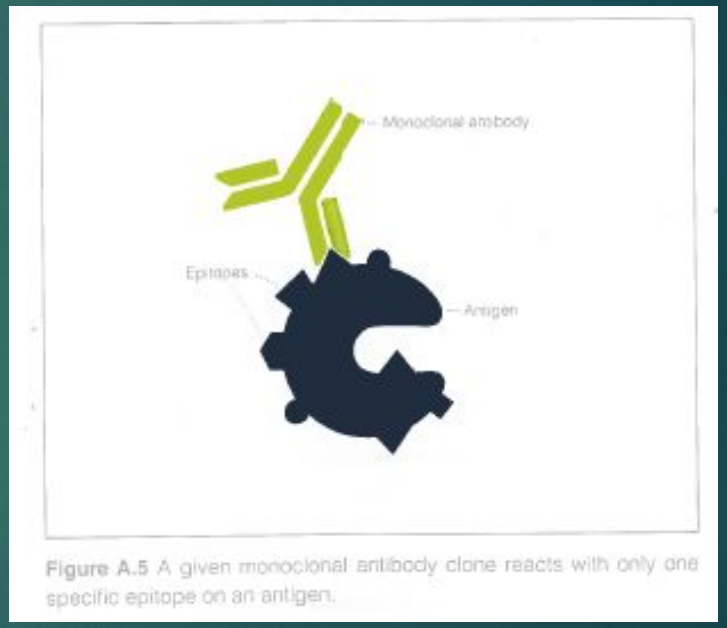


Figure A.5 A given monoclonal antibody clone reacts with only one specific epitope on an antigen.

General Immunology

Mouse Monoclonal Hybridoma	Rabbit Monoclonal Hybridoma
Generate higher yield of Ig	More diverse epitope recognition
Cell lines are more stable in culture	Improved immune response to small-sized epitopes
	Generate antibodies with higher affinity* and overall avidity**

- *Affinity – the strength with which an antibody binds to its epitope
- **Avidity – overall strength of an antibody-antigen complex

General Immunology

- 2. Polyclonal antibodies
  - Antiserum of heterogenous antibodies that recognize many epitopes of a specific antigen
  - Produced by injecting an animal (usually mouse or rabbit)
  - Antibodies are generated by different B lymphocyte clones of the animal and because they are from different B-cell lines, they are immunochemically different

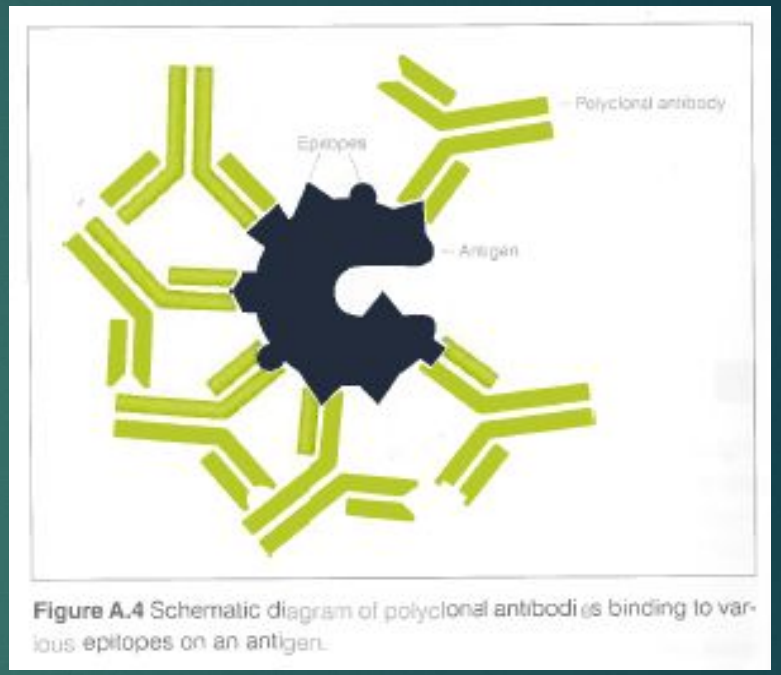


Figure A.4 Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

General Immunology

Polyclonal Antibodies	Monoclonal Antibodies
More robust when used on routinely-processed tissue specimens	Lot-to-lot consistency
Recognize multiple epitopes on a single antigen	Use of a hybridoma enables a sustained production of antibody
Not as subject to negative effects of pre-analytical steps on tissue	Less cross-reactive due to the fact that the antibody only recognizes one specific epitope

General Immunology

- 3. Antibody classes
  - There are five major classes of antibodies: IgG, IgA, IgM, IgD and IgE
  - Of the five major classes, **IgG** and **IgM** are by far the most frequently utilized antibodies in immunohistochemistry

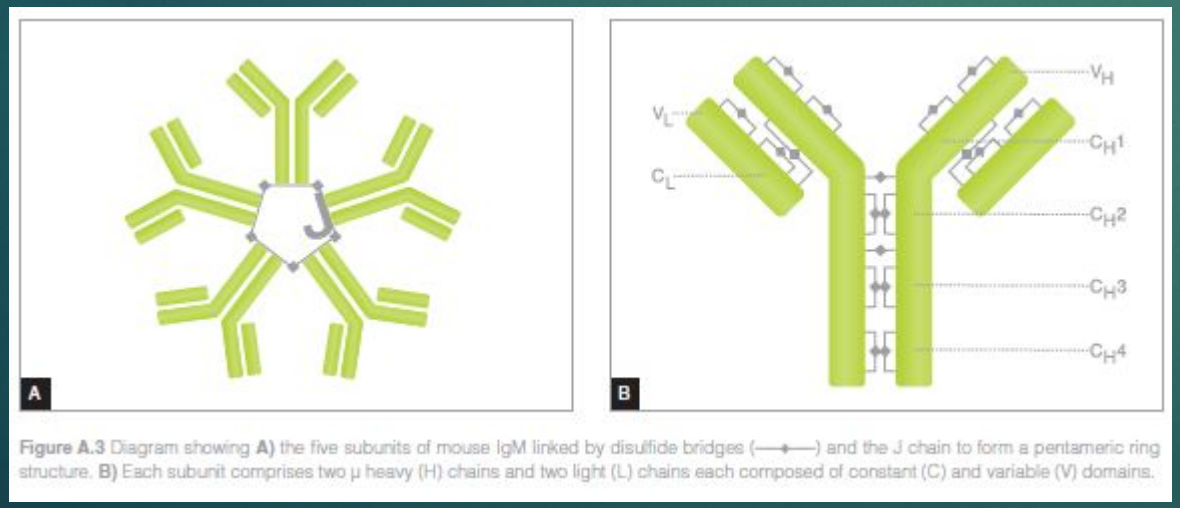
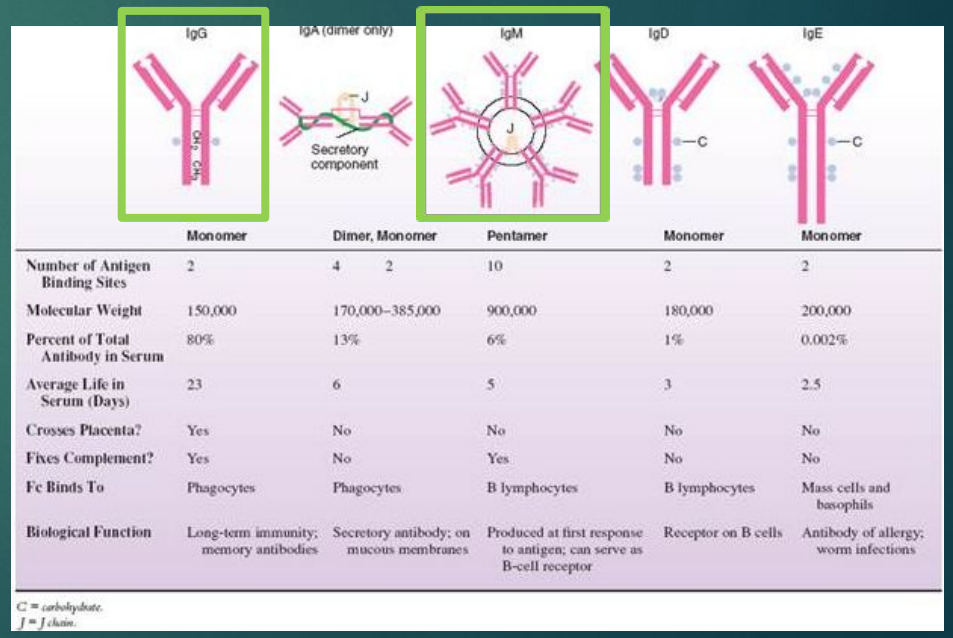


Figure A.3 Diagram showing **A)** the five subunits of mouse IgM linked by disulfide bridges (—•—) and the J chain to form a pentameric ring structure. **B)** Each subunit comprises two μ heavy (H) chains and two light (L) chains each composed of constant (C) and variable (V) domains.



	Monomer	Dimer, Monomer	Pentamer	Monomer	Monomer
Number of Antigen Binding Sites	2	4 2	10	2	2
Molecular Weight	150,000	170,000–385,000	900,000	180,000	200,000
Percent of Total Antibody in Serum	80%	13%	6%	1%	0.002%
Average Life in Serum (Days)	23	6	5	3	2.5
Crosses Placenta?	Yes	No	No	No	No
Fixes Complement?	Yes	No	Yes	No	No
Fc Binds To	Phagocytes	Phagocytes	B lymphocytes	B lymphocytes	Mass cells and basophils
Biological Function	Long-term immunity; memory antibodies	Secretory antibody; on mucous membranes	Produced at first response to antigen; can serve as B-cell receptor	Receptor on B cells	Antibody of allergy; worm infections

C = carbohydrate.
J = J chain.

https://studopedia.su/10_45431_Characteristics-of-the-Immunoglobulin-Ig-Classes.html

General Immunology

- 4. Antibody Structure
  - All classes of antibodies are composed of identical heavy (H) and light (L) chains.
  - The heavy (H) chains determine the subclass of the immunoglobulin. Heavy chain Greek designation corresponds with the antibody class name (thus **IgG** has **G**amma γ long chains, **IgM** has **M**u μ long chains)
  - The light (L) chains are either kappa (κ) or lambda (λ)
  - Covalent bonds join the light chains to heavy chains, and the heavy chains to each other

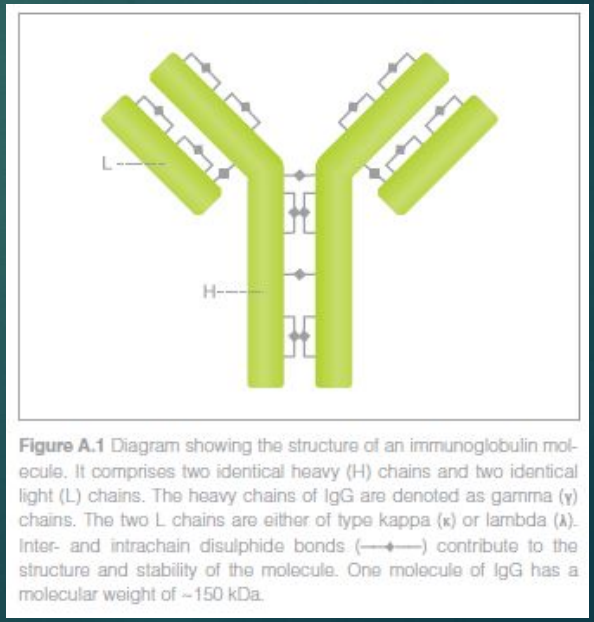


Figure A.1 Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. The heavy chains of IgG are denoted as gamma (γ) chains. The two L chains are either of type kappa (κ) or lambda (λ). Inter- and intrachain disulphide bonds (—◆—) contribute to the structure and stability of the molecule. One molecule of IgG has a molecular weight of ~150 kDa.

General Immunology

- 4. Antibody structure (continued)
  - The IgG antibody can be further divided into variable (V) and constant (C) domains of both the heavy and light chains
  - Immunoglobulins also have antibody binding (Fab) regions and crystalline (Fc) fragments, based on their location in respect to the hinge region
    - The Fab fragment is comprised of the entire light chain, as well as the heavy chain above the hinge region
    - The Fc fragment is comprise solely of the heavy chain beneath the hinge region
    - Note that the fragments can be separated through proteolytic enzyme digestion and that papain digestion yields two Fab fragments while pepsin digestion yields one F(ab')2 fragment. This is due to the location along the heavy chain where the enzyme digests

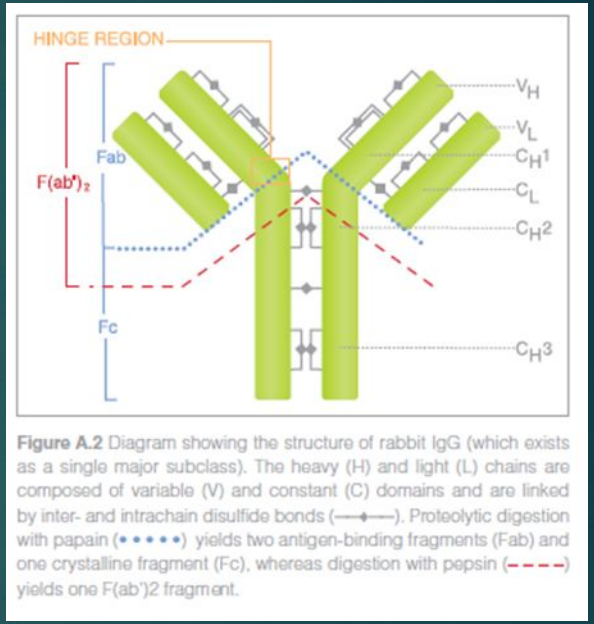


Figure A.2 Diagram showing the structure of rabbit IgG (which exists as a single major subclass). The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter- and intrachain disulfide bonds (——•——). Proteolytic digestion with papain (• • • • •) yields two antigen-binding fragments (Fab) and one crystalline fragment (Fc), whereas digestion with pepsin (– – – –) yields one F(ab')2 fragment.

General Immunology

- 4. Antibody structure (continued)
  - IgM is a pentamer (has five subunits)
  - J (joining) chain holds the five subunits together
  - While IgG is the most abundant antibody in an immunized animal, **IgM is the first humoral antibody detectable**
  - IgM have a relatively short half-life of about 4-6 days
  - Half-life of IgG is approximately 3 weeks

General Immunology

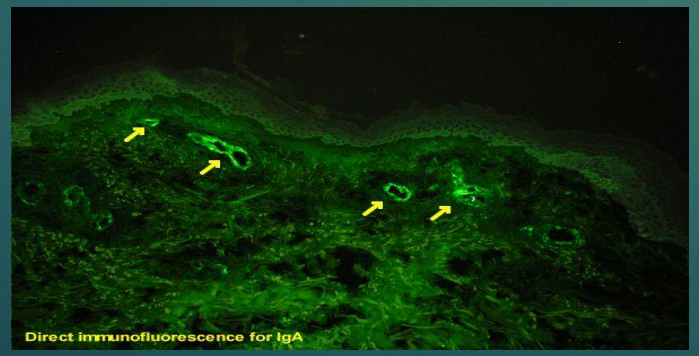
- Other types of antibodies
  - Lectins
  - Chimera

Detection Systems

20-25%

Detection Systems

- Detection Systems are reagent products most often sold and used in a kit format that are designed to target a bound antibody directly or indirectly.
- A. Immunofluorescence (IF)
  - Original method for performing IHC
  - Direct or indirect methods can be used to produce a fluorescent signal for protein detection. In direct detection, the primary antibody specific for the target molecule is directly labeled. Indirect detection uses an unconjugated primary antibody. This can be done by hand or on an instrument.
  - In most labs today this is used in the diagnosis of skin lesions, autoimmune diseases and kidney biopsies.
  - Skill and experience are required for reviewing stained tissue sections. Nuclear details and the architecture of tissue are not as easily visualized as bright field .



Direct Immunofluorescence for IgA picture in Wikipedia.

Detection Systems

- B. Immunohistochemistry (IHC)
- An antibody-antigen reaction done at the molecular level.
- Most clinical labs today use indirect methods.
- Detection systems most widely used:

  Fluorescence labels

  Enzyme labels

  Polymer based detection

Immunohistochemical tests using polymer-based detection systems (biotin-free) are sufficiently free of background reactivity to obviate the need for a negative reagent control and such controls may be omitted at the discretion of the laboratory director following appropriate validation. The Centers for Medicare and Medicaid Services (CMS) recognizes the use of polymer-based detection systems (biotin free) may preclude the use of a negative reagent control. However, there have been no changes to the histopathology regulations. The CMS will be looking into an alternate QC method for these types of stains.

Source: CAP checklist 2018

Detection Systems

- B. Immunohistochemistry (IHC)
  - 1.Substrates
    - The choice of chromogen used is directed by the enzyme used in your detection system or kit.
    - The substance acted on by the enzyme in your tissue is the **substrate**.
    - In clinical labs the detection systems most widely used are **HRP – Horseradish Peroxidase and/or AP - Alkaline Phosphatase** based.
    - The HRP detection technology is very robust and the most commonly used chromogen is **DAB** (brown). The HRP enzyme can also be paired with a brick red chromogen **AEC**.
    - **Fast or Permanent Red** is the most popular chromogen used with the alkaline phosphatase enzyme Add'l chromogens: Fuchsin (fuschin colored), BCIP/NBT (dark blue-black).

Detection Systems

- B. Immunohistochemistry (IHC)
  - 2. Enzymes
    - Enzymes are a protein molecules that speed up a chemical reaction in an organism. For IHC we are talking about the main enzyme reaction in the detection system that you are using in your lab.
    - Different detection systems employ different enzymes and these will limit which chromogens you can use in your reaction and what the colored end product is.
    - Your two main detection enzymes are **Horseradish Peroxidase** and **Alkaline Phosphatase**.
  - 3. Chromogens
    - Chromogens are chemical compounds used in IHC staining that produce a colored end product which can then be visualized under bright-field microscopy.
    - DAB (3,3'-Diaminobenzidine)is the most widely used chromogen for immuno-histochemical staining and immunoblotting. When in the presence of peroxidase enzyme, DAB produces a brown precipitate that is insoluble in alcohol and xylene.
    - The brown colored end product can be darkened by use of an enhancing metal solution usually nickel or copper . (Also used are silver, gold and cobalt).
    - The other commonly used chromogen is a Permanent Red that is used with an Alkaline Phosphatase Detection System. This is often used in the diagnosis of Melanotic lesions so that the brown melanin pigment can be visualized next to the positive red staining end product.

Detection Systems

B. Immunohistochemistry (IHC)

- 4. Blocking reactions
- Blocking reactions are steps taken with different blocking solutions to help eliminate or reduce background staining
- Most technology today, is based on a polymer detection kit, which contains blocking reagents, usually peroxide based.
- The polymer kits do not stain endogenous biotin, which was an issue in certain tissues with older methodologies. You had to know what chemistry you were using as well as understand what tissues contained endogenous pigments/products in order to employ the correct blocker. (Peroxide, Casein, Avidin – Biotin blockers, Levamisole).

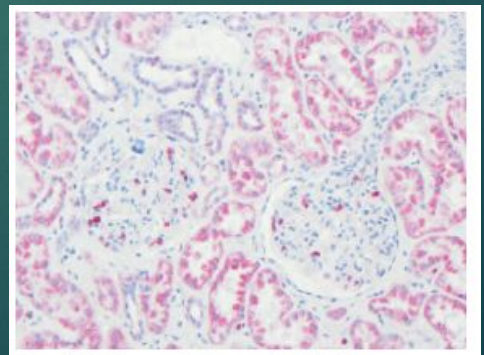


Figure 15.3 Endogenous biotin expression in the kidney.

Detection Systems

- C. In-Situ Hybridization (ISH)
  - This is a sensitive and robust staining method. It is a type of hybridization that uses a labeled complimentary DNA, RNA or nucleic acid strand to localize specific DNA or RNA in a tissue section.
  - Comparison of FISH/CISH

Thermo Fisher Scientific:

Technique	Instrument/ visualization method	Primary advantage	Primary application
CISH	Bright-field microscopy	Ability to view the CISH signal and tissue morphology simultaneously	Molecular pathology diagnostics
DNA-FISH	Fluorescence microscopy	Multiplexible: visualize multiple targets in the same sample	Gene presence, copy number, and location; mutation analysis
RNA-FISH	Fluorescence microscopy, HCS, and flow cytometry	Multiplexible: visualize multiple targets in the same sample	Gene expression, RNA temporal and spatial localization

Detection Systems

COMPARE	CISH	FISH	IHC
Signal stability	Archivable	Fades over time	Archivable
Microscope	Bright-field	Fluorescence	Bright-field
Magnification	40x	60–100x	20–40x
CProtocol length	Overnight + 3 hr, 55 min	Overnight + 3 hr, 12 min	3 hr, 2 min
Morphology	Good	Limited	Good
Amount of training required	Medium	High	Low
Internal control	Yes	Yes	No
Interpretation	Objective/quantitative	Objective/quantitative	Subjective/qualitative
Overall cost	Medium	High	Low

Detection Systems

- C. In-Situ Hybridization (ISH)
  - 1. FISH

    Fluorescent in situ hybridization

    (FISH) is a very sensitive cytogenetic technique that uses **fluorescent** probes to investigate the presence of small, submicroscopic chromosomal changes

    - This has long been considered the Gold standard for the detection of chromosomal abnormalities.
    - Commonly used for Her 2 neu detection and in the diagnosis of lymphomas
    - Requires a fluorescent microscope for review.
    - The signal generated is not permanent and is often photographed for permanence.
    - Requires various filters; Bulb life is short and instrumentation requires more maintenance than light microscopy
    - More regulatory guidelines to be followed.

Detection Systems

- C. In-Situ Hybridization (ISH)
  - 1. FISH
    - Requires the construction of a probe which can bind to the target
    - Probe is tagged with fluorophores.
    - Target and probe are denatured.
    - Probe is applied and incubated which is a lengthy process
    - Note: Rinse steps are very critical in this process.
    - Hybridizer instrument needed
    - Visualization under a fluorescence scope.
    - Kits are available for FISH, Hybridizer instrument used. Lengthy process.

Detection Systems

2. CISH Chromogenic In-Situ Hybridization

- Easier method of molecular testing than FISH, due to the ability to visualize under light microscopy.
- More robust and a more complicated and lengthy method than IHC with several reagent and protocol steps that are critical to good results.
- Kits are available from many Vendors.
- Steps similar to the FISH procedure –
- Probes are applied
- Co-denaturation occurs and hybridization is carried out
- A fluorescent signal results - after stringency washes and rinses, the fluorescent signals are converted to chromogenic signals by an IHC staining protocol on your instrument using specific CISH reagents.

  Usually an antibody mix HRP - labeled anti-FITC and AP labeled anti Texas Red

Detection Systems

- **C. In-Situ Hybridization (ISH)**

2. CISH (continued):

- Following antibody incubation on your instrument red and blue signals are generated by using red and blue permanent chromogens.
- A Hematoxylin counterstain is applied, followed by coverslipping.
- Can be viewed with light microscopy while still visualizing tissue morphology and cell structure.
- Permanent results

3. SISH Silver In-situ Hybridization

- Similar process to CISH except silver is used to visualize the probes and the coloration is black due to the silver precipitation.
- Studies have been done comparing FISH to CISH/SISH with the results being almost a 1:1 conversion ratio of FISH to CISH/SISH signals with a high rate of concordance.
- Many Vendors have developed proprietary reagents and probes to do EBER, KAPPA, LAMBDA, CMV and HPV on their automated instruments in around 5 hours TAT.

Detection Systems

- Many Clinical labs like to use FDA approved kits for their prognostic markers. They are called companion Diagnostics or Pharm Dx and are directly linked to an Oncology drug. These kits are very standardized with literature and controls to help you in your practice. The kits must be followed to the letter to maintain their FDA status and they are matched to certain instrumentation.
- The chart below gives examples of this.

Diagnostic Name	PMA/ 510(k)/ HDE	Diagnostic Manufacturer	Trade Name (Generic) - NDA/BLA
BRACAnalysis CDx	P140020/S016	Myriad Genetic Laboratories, Inc.	Breast Cancer •Lynparza (olaparib) - NDA 208558 •Talzenna (talazoparib) - NDA 211651 Ovarian Cancer •Lynparza (olaparib) - NDA 208558 •Rubraca (rucaparib) - NDA 209115
therascreen EGFR RGQ PCR Kit	P120022/S018	Qiagen Manchester, Ltd.	Non-small cell lung cancer •Iressa (gefitinib) - NDA 206995 •Gilotrif (afatinib)- NDA 201292 •Vizimpro (dacomitinib)- NDA 211288
cobas EGFR Mutation Test v2	P120019/S019	Roche Molecular Systems, Inc.	Non-small cell lung cancer (tissue and plasma) •Tarceva (erlotinib) - NDA 021743 •Tagrisso (osimertinib) - NDA 208065 •Iressa (gefitinib) - NDA 206995

Cont: https://www.fda.gov/medical-devices/vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-vitro-and-imaging-tools

PD-L1 IHC 22C3 pharmDx	P150013 P150013/S006 P150013/S009 P150013/S011 P150013/S014 P150013/S016	Dako North America, Inc.	Non-small cell lung cancer (NSCLC), gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), and esophageal squamous cell carcinoma (ESCC) •KEYTRUDA (pembrolizumab) – BLA 125514
Abbott RealTi*me* IDH1	P170041	Abbott Molecular, Inc.	Acute myeloid leukemia •Tibsovo (ivosidenib) - NDA 211192
MRDx BCR-ABL Test	K173492	MolecularMD Corporation	Chronic myeloid leukemia •Tasigna (nilotinib) - NDA 022068/S026

Specimen Handling
10-15%

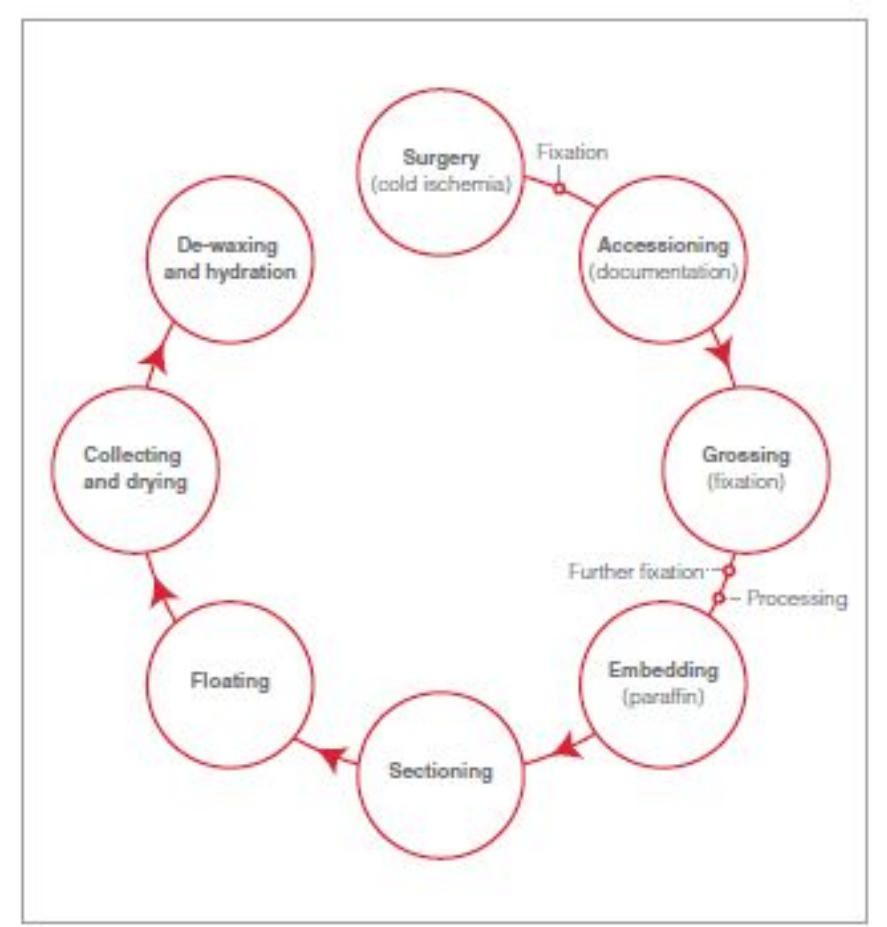


Figure 2.1 An overview of the processing steps included in the pre-analytical phase.

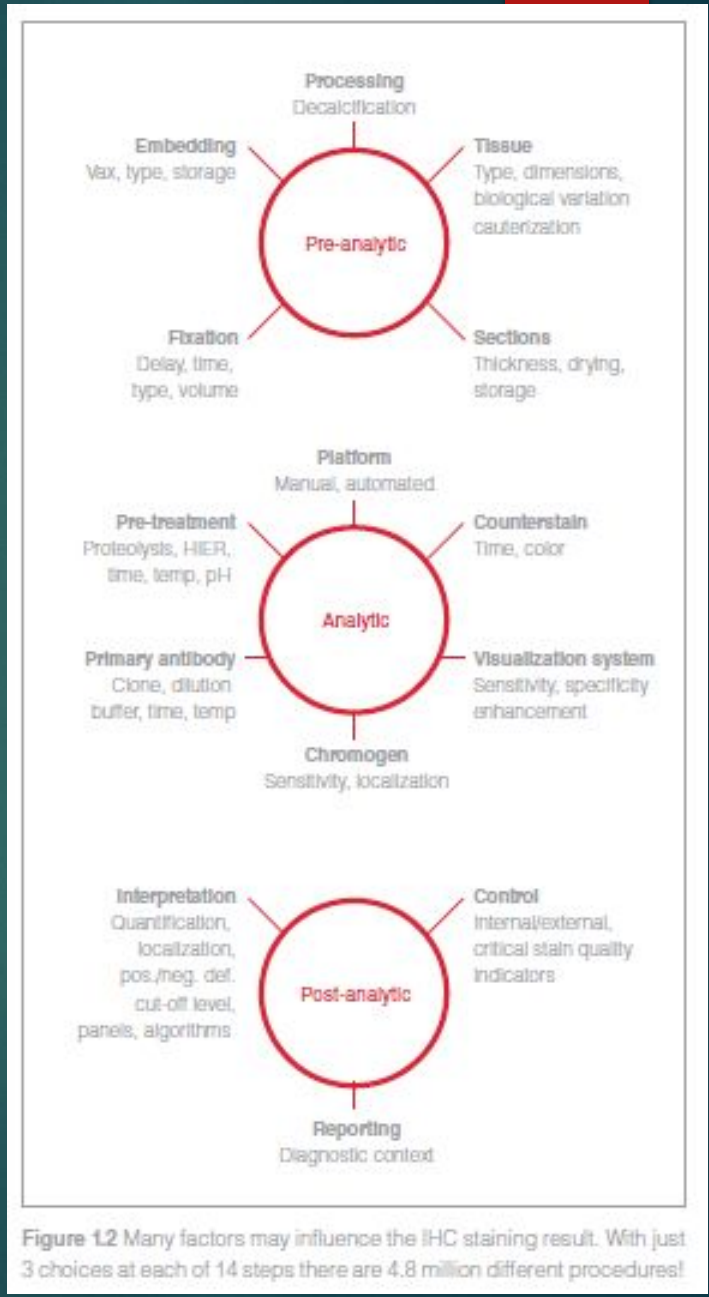


Figure 1.2 Many factors may influence the IHC staining result. With just 3 choices at each of 14 steps there are 4.8 million different procedures!

Specimen Handling

- A. Fixation
  - Most important pre-analytical variable which effects IHC & ISH results
  - Not placing tissue into a fixative as soon as it is removed from the patient can have negative effects
    - Autolysis – autoenzymatic degredation
    - Putrefaction – bacterial degredation
  - Time between the removal of tissue from the patient to placing into fixative is referred to as the "Cold Ischemic Time."
  - Newer terminology also includes the time that blood supply is cut off during surgical procedures. This is known as the "Warm Ischemic Time."
  - Challenge is what changes will be induced by the fixative in relation to antigens or molecular targets
  - Fixatives may destroy or mask the targets
  - Main categories of fixatives:
    - Formalin-based
    - Alcohol-based
    - Combination of formalin & alcohol
  - To date, no single fixative has been shown to be best for all targets and detection methods
  - Underfixed tissue is more problematic for IHC & ISH results than overfixed tissue

Specimen Handling

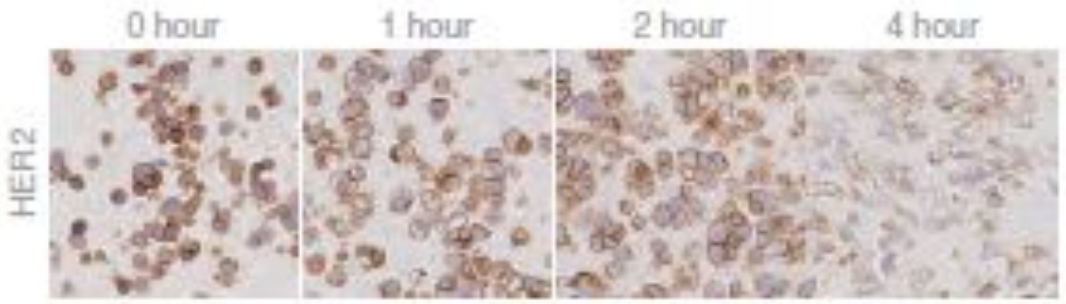


Figure 2.2 Cold ischemia alters the staining intensity of HER2 in MDA-MB-453 cells (2+ cell line). Weak to moderate membrane immunoreactivity on approximately half of the cells is observed in a cell pellet fixed immediately in 10% NBF (0 hour). With as little as one hour cold ischemia (the cell pellet was kept moist under saline-damped gauze), the morphology is already deteriorating and there appears to be increased numbers of cells with membrane staining. After two hours, the staining is even stronger. Following four hours cold ischemia time, much of the membrane staining is lost and the preservation of the cells is poor. This illustrates the need for prompt fixation and that different cold ischemic times can give rise to over staining or under staining of the cell membranes. Cells were stained using IHC and HER2 antibody (Code A0485, Dako) and the Autostainer Link 48, Dako platform.

Specimen Handling

- A. Fixation (continued)
  - 10% Neutral Buffered Formalin
    - Most frequently used fixative. "Gold standard."
    - pH is approximately 7.0-7.4
    - Inexpensive, easy to make, stable when stored
    - Penetrates tissue & forms cross linkages between reactive amino groups in the proteins. Penetrates quickly, but cross-links slowly.
    - Additive & non-coagulant
    - *Ideal to fix tissue grossed at 4mm for 24 hours at room temp
    - ****For ER, PgR & HER2 testing, tissue must fix for minimum of 6 hours** (CAP guidelines). Maximum time for HER2 fixation is 72 hours.

Specimen Handling

- A. Fixation (continued)
  - Due to the cross-linking of NBF, potential IHC and ISH targets are often masked
  - This means the end point of fixation is almost as important as the start time
  - Antigen retrieval has been developed to unmask targets after NBF fixation
    - Heat Induced Epitope Retrieval (HIER)
      - Adjust length of time and pH of retrieval solution
    - Enzyme Induced Epitope Retrieval (EIER)
      - Proteolytic treatment, often using trypsin or pepsi
    - **More will be said about retrieval in the next section (Epitope Enhancement)

Specimen Handling

- A. Fixation (continued)
  - Alcohol fixatives
    - If tissue fixed in 10% NBF fixes for 6 hours or less, the final fixation is likely alcohol fixation during the processing steps
    - This may lead to falsely negative or positive results
    - Alcohol is a non-additive, coagulating fixative
      - Tends to shrink and harden tissues
    - Using alcohol in place of formalin fixatives often eliminates the need for epitope retrieval
    - Alcohol tends to be a faster working fixative than formalin, even though it does not penetrate as quickly
    - *Alcohol is often recommended for work with nucleic acids

Specimen Handling

- A. Fixation (continued)
  - Enhancing of fixation can be done using microwave or ultrasound technology
  - Heat accelerates the rate of reaction
    - Speeds up penetration of fixative
    - May induce uneven fixation depending on the type/size of specimen
    - Microwaving causes protein coagulation & may lead to hard/over-cooked tissues

Specimen Handling

- B. Processing
  - Technique by which fixative reagents that contain water are dehydrated and replaced with wax
    - Waxes may include: polymer-based, non-polymer and microcrystalline formulas
  - Performed by treating the tissue with increasing concentrations of alcohol, ultimately reaching 100% alcohol
  - Tissue is then cleared of the alcohol with an agent such as xylene
  - Tissue is then infiltrated with the molten wax
    - Note that waxes with lower melting points have been shown to produce better IHC staining results than those with higher melting points - especially for T-Lymphocyte markers (CD3, CD4, CD8, etc.)

Specimen Handling

- B. Processing (continued)
  - Use of rapid tissue processors has helped to decrease turnaround time in many laboratories
  - Labs must still ensure adequate fixation prior to use of these processors
  - *When a new fixative or processing method is introduced in a lab, the IHC and ISH methods must be revalidated

Specimen Handling

- C. Microtomy/Slide Preparation
  - Most sections for IHC or ISH are cut at 3-5 micrometers in thickness
  - It is important that sections are flat when picked up on adhesive/charged slides to prevent floating off or bubble formation during staining
  - Tissue tends to be more negatively charged following formalin fixation (formalin blocks positively charged amino acids) thus allowing the tissue to adhere better to positively charged slides
  - Slide choice for immunostaining may be determined by staining platform manufacturer's suggestions
  - Drying steps (air drying, oven drying) must be standardized for IHC and ISH staining
  - Block and slide storage may effect staining as well, so it should be noted for how long slides had been stored prior to staining for IHC or ISH markers

Specimen Handling

- C. Microtomy/Slide Preparation (continued)
  - Infiltrating/embedding medium must be removed prior to staining
    - Due to use of aqueous antibodies, ISH probes and detection reagents
    - Dewaxing & hydrating may be done offline, or some staining platforms may have onboard abilities to perform this function

Specimen Handling

- D. Cytology Specimens
  - Many laboratories prepare cell blocks of cytologic specimens that can be formalin fixed, processed, and embedded similarly to tissue specimens
  - This allows for these specimens to ultimately be stained using the same processes as tissue slides (special stains, IHC, ISH)
  - For cytologic slides prepared with alcohol fixatives, performing IHC and ISH stains must be properly validated, as the pre-analytical steps involved in the preparation of these slides differs from those of tissue slides
    - Note that control material for cytologic slides should also be treated the same as the cytology specimen
  - Microtomy of tissues will destroy some cell membranes, making it easier for markers like cytoplasmic and nuclear markers to access their targets. Conversely, cytologic slides have intact cells with full membranes, which make it more difficult for cytoplasmic and nuclear markers to reach their targets

Specimen Handling

- E. Immunofluorescence (IF) specimens
  - IF may be performed on both fresh and formalin fixed specimens
    - It is best to perform immunofluorescence staining on fresh specimens rather than formalin fixed specimens, because those that are fixed often demonstrate autofluorescence
  - Frozen sectioning of fresh specimens is required for investigation of autoimmune or inflammatory diseases, stained with direct immunofluorescence
  - Holding solutions such as Michel's transport medium or a PBS solution containing 10% sucrose may be used to hold specimens for transport prior to immunofluorescence staining

Specimen Handling

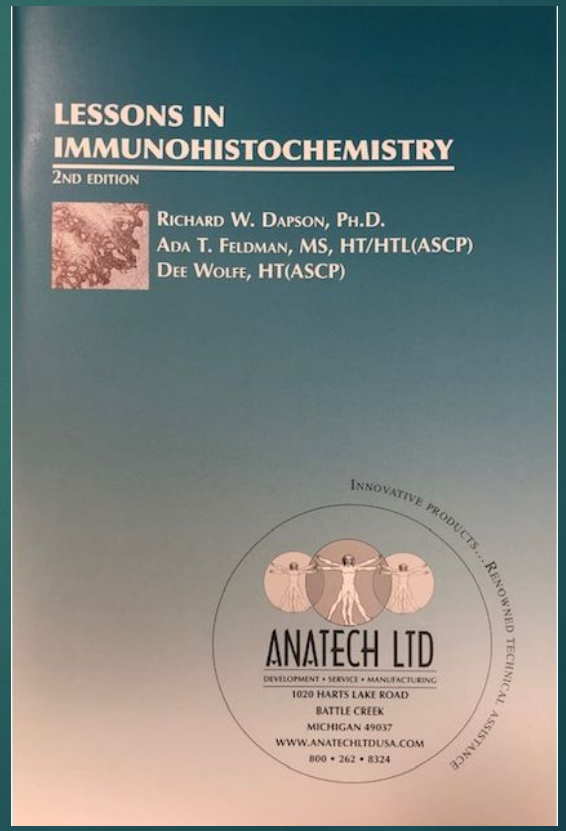
- F. Frozen Section
  - Technique used for demonstrating tissue targets in specimens that cannot be shown following fixation and paraffin processing
  - Tougher to obtain fresh tissue, and must take into consideration logistics and expense concerns
  - Procedures for obtaining fresh specimens must be standardized and validated for the antibodies used
  - Rapid freezing is the greatest challenge when preparing slides of frozen tissue
    - Prevention of freeze artifacts – need to maintain morphology/integrity
  - Tissue thickness should be between 4 and 6 micrometers
  - Blocking of endogenous enzymes must be included in IHC and ISH protocols on slides prepared from fresh tissue
  - *Due to exclusion of the antigen masking effects of formalin when using fresh tissue, little or no epitope retrieval may be needed
    - This shortens the turnaround time for IHC/ISH on fresh frozen sectioned tissues
  - Bloodborne pathogen concerns when frozen sectioning fresh tissue specimens

Specimen Handling

- G. Decalcification
  - Use of decalcifying agents on tissues can destroy antigens
  - The best way to prevent this is to ensure proper fixation prior to placing tissues into a decalcifying agent
  - Stronger acids (Hydrochloric, Nitric) tend to be more damaging than weaker acids (Formic)

Specimen Handling

- Another great resource on Specimen Handling is the Anatech LTD "Lessons in Immunohistochemistry 2nd Edition"



Epitope Enhancement (Antigen Retrieval)

10-15%

Epitope Enhancement

By definition this is a pre-treatment that is needed to make your antigen more accessible to the antibody.

- A. Methods, Principles and Techniques

- Complex topic that many articles and books have been written about.
- Many techniques have evolved over time because NBF is still our fixative of choice.
- NBF yields an excellent H&E section, but the fixation process causes protein modifications or cross links that may result in the masking or loss of reaction between the antigen and the antibody.
- Needs to be reversed or restored by retrieval or enhancement.

Epitope Enhancement

- 1. Enzyme-induced epitope retrieval

  - Enzymes were used in the early days of IHC. (E.g. Pepsin, Trypsin, Ficin, Protease and Pronase)
  - They often had to be made fresh every time they were used
  - Had to know their properties and requirements - concentrations and temperatures needed to work.
  - Enzymes are proteolytic and break up the protein links. They digest tissue and can be quite destructive to morphology.
  - As IHC evolved enzymes have become much more stable and easier to use.

Table 8.1 Proteolytic enzymes and typical incubation conditions.

Enzyme	Approximate activation temperature	Incubation time
Proteinase K	25-37 °C	5 min
Trypsin	37 °C	10 min
Pepsin	37 °C	5-20 min
Protease XXIV	37 °C	5-20 min
Pronase	25-37 °C	30 min

NOTE: Formalin does not preserve tissue proteins by coagulation but it is thought to form cross-links with basic amino acids. Ethanol and mercuric chloride-based fixatives are based on coagulation. With few exceptions retrieval should not be performed on ethanol-fixed tissues. It should only be conducted with limited controlled protocols in mercuric-chloride-based fixatives.

Epitope Enhancement

- 2. Heat-induced epitope retrieval (HIER)
- Since the 1970's many active researchers and Pathologists knew that we needed to do something about the retrieval process.
- Shan-Rong Shi, MD, Pathologist who did a lot of research on this topic in the late 1980's to see if the NBF protein linking could be reversed. He found studies that showed the cross links could be disrupted by heating above 100 degrees or by a strong alkaline treatment. He began by testing different solutions and temperatures.
- **Heat** was found to be most critical element as well as **time** in solution, and time of cool down
- Waterbaths, steamers, ricecookers, pressure cookers, microwave ovens and finally on-line instrumentation has been used
- > 100 degrees may be optimal for breaking links but 90 degrees for a longer period preserves morphology better

Epitope Enhancement

- 2. Heat-induced epitope retrieval (HIER)

- **pH value of the retrieval solution –** Other critical factor for correct retrieval process
- Variable among antibodies
- Most labs use a **low pH retrieval** (Citrate based) and a **High pH** (EDTA based) retrieval solution
- Most effective pretreatment is determined during optimization and validation
- Most vendors have proprietary solutions, often rtu.
- Specification sheets will give you information on pH, preparation and storage
- Most newly developed antibodies require heat retrieval

Epitope Enhancement

3. Combined heat and enzyme methods

- The combination of heat and enzyme digestion can be done for problematic antibodies
- Usually it is a mild form of both and it can be done in either order.

**Keep in mind that too much or too little retrieval or pretreatment can induce false positive results or false negative results.

NordiQC has very nice photos of what inadequate/over retrieval looks like.

Staining
25-30%

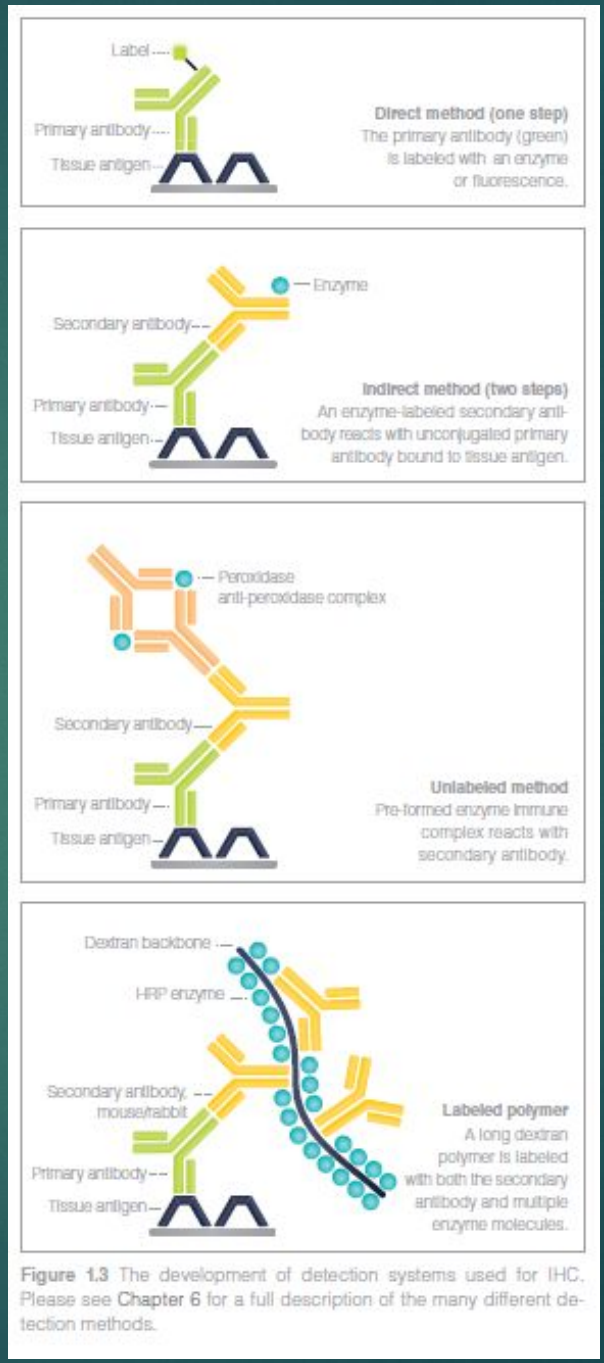


Figure 1.3 The development of detection systems used for IHC. Please see **Chapter 6** for a full description of the many different detection methods.

Staining

- Background
  - IHC is a powerful tool to provide supplemental information to routine (H&E) morphological evaluation of tissue
    - Diagnostic, prognostic, predictive
  - IHC is a molecular-based study which has been adapted and refined, primarily for use in fixed tissue
    - Fixed tissues antigens are much more variable and unpredictable than solution-based immunoassays
  - Evolution of IHC has led to a sensitive and somewhat quantitative assessment of tissue disease

Staining

- A. Principles and Mechanisms
  - 1. Direct
    - First staining of tissues with an antibody were done in 1941 with a fluorescent-labeled antibody.
      - Viewed under fluorescent microscopy
    - When the label is bound to the primary, single antibody which will detect an antigen this is called the **Direct Staining method.**
    - In 1966, enzyme horseradish peroxidase (HRP) was used with 3,3'-diaminobenzidine (DAB) to study mouse kidneys
      - This involved the use of brightfield microscopy

Staining

Fluorescent staining advantages	Fluorescent staining disadvantages
Quick & work well in multiplex staining	Fade over time
Non-masking	Hard to view cell morphology
Sensitive	Requires ability to view w/fluorescent microscope
Fluorescent labels have a small molecular footprint, making them easy to conjugate with an antibody	For each color (fluorophore) used – need a specific filter

Staining

- A. Principles and Mechanisms
  - 2. Indirect
    - In 1967, an antibody linked to horseradish peroxidase was used to visualize antigens in tissue using the first antibody (primary antibody) attached to the antigen
    - A secondary antibody which recognized the Fc (constant/crystalline) part of the primary antibody was added, which makes it possible to recognize all primary antibodies, *as long as they are from the same species*
    - This led the to immunoperoxidase methods for use in routine diagnosis in anatomic pathology, and to the modern day methods of performing IHC
      - Note that while the next methods we discussed have a variety of names and are more complex, they are all founded on the basis of indirect detection methods

Staining

- A. Principles and Mechanisms
  - 2. Indirect (continued)
    - While the most commonly used enzyme in enzymatic labeling of a secondary antibody is horseradish peroxidase, others may be used
      - Second-most common is **alkaline phosphatase** (AP)
    - In order for the enzyme to lead to a visualized product, a **substrate** (the reagent that the enzyme acts on to yield a product) and a **chromogen** must be added
      - Substrate and chromogen are matched, yet an enzyme may act on more than one pairing type
      - Product is viewed as a color with a brightfield microscope

Staining

Chromogenic Dyes

Table 6.2 Examples of enzyme/chromogen pairs suitable for triple staining.

Enzyme	Chromogen	Color
Gal	X-Gal	Turquoise
AP	Fast Blue BB	Blue
HRP	AEC	Red
HRP	DAB	Brown
Gal	X-Gal	Turquoise
AP	Liquid Fast Red	Red
HRP	DAB	Brown
AP	New Fucsin	Red
HRP	TMB	Green

Gal (beta-galactosidase); X-Gal (5-bromo-4-chloro-3-indolyl β-galactoside); AP (alkaline phosphatase); HRP (horseradish peroxidase); AEC (3-amino-9-ethylcarbazole); DAB (3,3'-diaminobenzidine); TMB (3,3',5,5'-tetramethylbenzidine)

Horseradish peroxidase (HRP) chromogens	**Alkaline phosphatase (AP) chromogens**
Diaminobenzidine – **brown**	Fast red – **fuchsia**
Aminoethylcarbazole – **red**	Fuchsin – **fuchsia**
Tetramethybenzidine - **green**	5-Bromo-4-chloro-3-indolyl-phosphate/Nitro-blue tetrazolium – **blue**

Staining

Advantages of enzymatic labels	Disadvantages of enzymatic labels
Permanent	Takes more time than fluorescent techniques
Using light microscopy makes it easier to see morphology	Harder to interpret multiplex stains due to masking
Only need a light microscope	Toxicity of some chromogens (eg. DAB)

Staining

- A. Principles and Mechanisms
  - 3. Avidin-Biotin (ABC and LSAB methods)
    - Started in 1981
    - Still used (limited) in some labs
    - Relies on the strong affinity of avidin or streptavidin for the vitamin biotin
      - Streptavidin – comes from bacteria *Streptomyces avidinii*
      - Avidin – comes from chicken egg
    - Both types of avidin have four binding sites for biotin
      - Biotin is easily conjugated to antibodies and enzymes

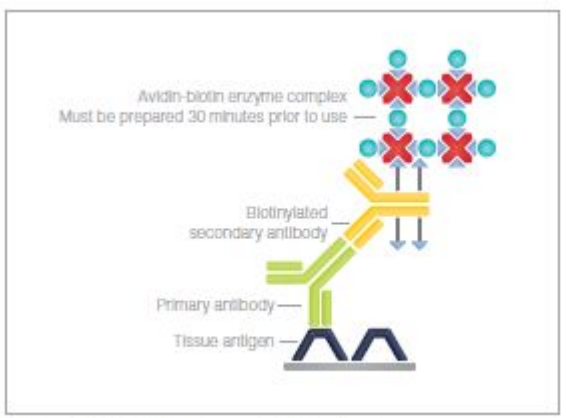


Figure 6.2 Avidin-Biotin Complex (ABC) method.

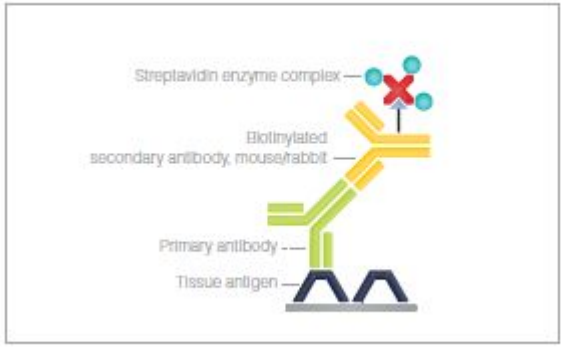


Figure 6.3 Labeled Streptavidin-Biotin (LSAB) Method

Staining

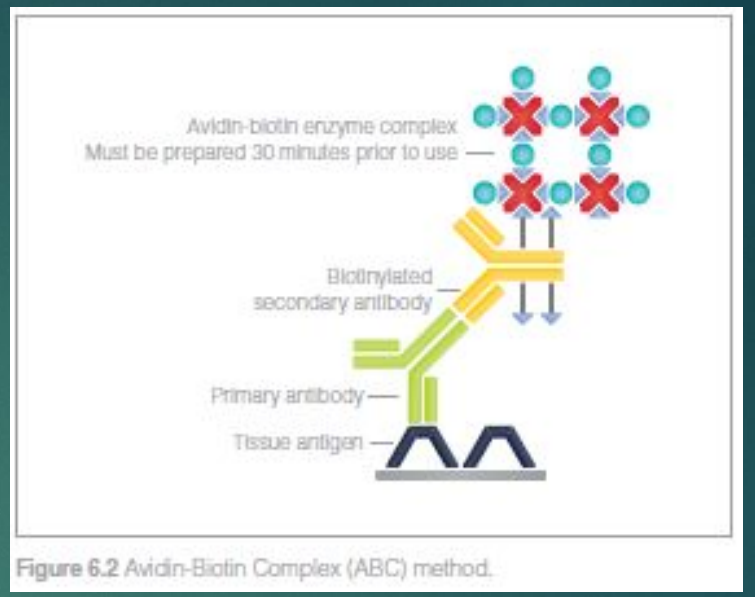


Figure 6.2 Avidin-Biotin Complex (ABC) method.

- A. Principles and Mechanisms
  - 3. Avidin-Biotin – ABC Method
    - Avidin-Biotin Complex
      - Secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and a pre-made avidin-biotin-peroxidase complex
      - Four binding sites for biotin make lattice complexes possible
        - Enzyme must have two biotins attached so it can function as a link between the avidins
        - Colorless substrate in the presence of a chromogen is added and then is converted to a color by the multiple enzymes that are now attached at the site of target antigen
          - Substrate for HRP is hydrogen peroxide

Staining

- A. Principles and Mechanisms
  - 3. Avidin-Biotin – LSAB Method
    - Uses a biotinylated secondary antibody that links primary antibodies to a streptavidin-peroxidase conjugate
    - Advantage over ABC method – preparation of the ABC complex is not needed
    - For both ABC and LSAB, the large enzyme-to-antibody ratio means the end result will be stronger (more sensitive) in comparison to direct peroxidase-conjugate methods

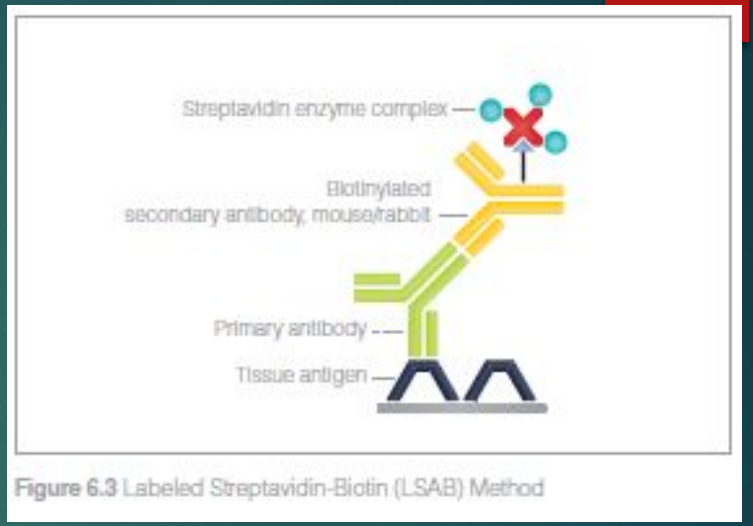


Figure 6.3 Labeled Streptavidin-Biotin (LSAB) Method

Staining

- A. Principles and Mechanisms
  - 3. Avidin-Biotin
    - Note that avidin has a tendency to bind non-specifically to lectin-like and negatively charged tissue components at normal pH
      - Less non-specific binding is noted with streptavidin
    - Also, certain tissues have a high amount of endogenous biotin
      - Formalin fixation & paraffin embedding tends to reduce this, but if performing the method on fresh frozen tissues a biotin block is essential

Staining

- A. Principles and Mechanisms
  - 4. In-situ hybridization (ISH)
    - A sensitive & robust technique, used most frequently to evaluate gene amplification, deletion & translocation
    - Also used to detect chromosomal copy number changes in tissue or cellular material
    - ISH has been used for over forty years in the research setting
    - Used frequently for epidermal growth factor receptor (HER2) status in breast and gastric cancer
    - Allows for specific recognition of DNA target sequences in the nuclei of target cells using fluorescence or hapten-labeled-sequence pairing **probes**.
      - FISH – Fluorescent In-Situ Hybridization
      - CISH – Chromogenic In-Situ Hybridization
      - SISH – Silver In-Situ Hybridization

Staining

- A. Principles and Mechanisms
  - 4. In-Situ Hybridization (continued)
    - FISH uses a fluorescent probe to directly visualize a sequence of DNA
      - Viewed with fluorescent microscopy
    - CISH utilizes a fluorochrome or hapten for traditional IHC visualization of the target DNA sequence
      - Viewed with light microscopy
      - Involves following the FISH staining procedure until the dehydration step
        - Instead, immerse slides in CISH wash buffer, follow an IHC staining protocol with endogenous peroxidase blocking
        - Add primary antibody, which binds to the fluorescent label and visualize the signal with chromogen deposition in the tissue
        - Counterstain with hematoxylin
    - Note that advantages/disadvantages to fluorescent staining versus chromogenic staining have already been discussed and also apply to ISH staining

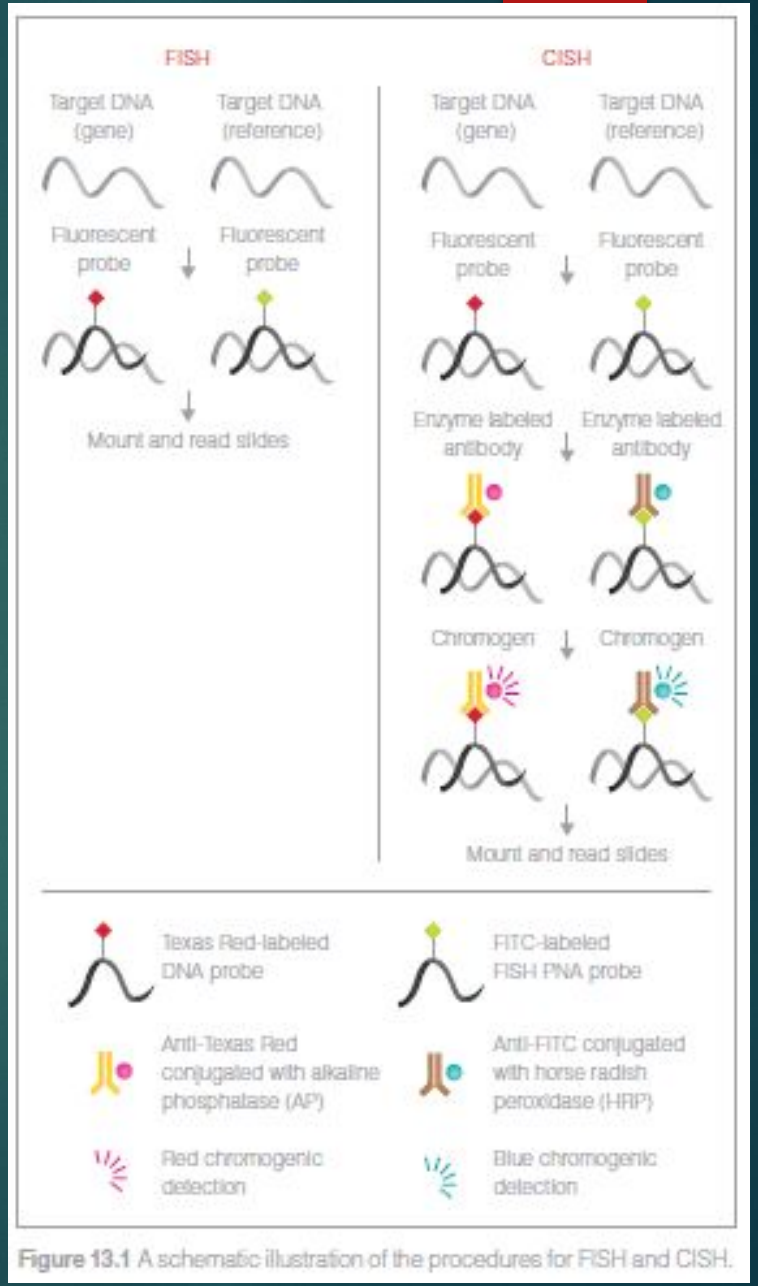


Figure 13.1 A schematic illustration of the procedures for FISH and CISH.

Staining

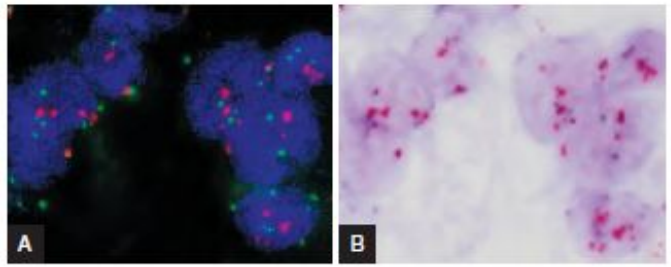


Figure 13.2 The images illustrate a "dot-to-dot" conversion of FISH signals **(A)** to CISH signals **(B)**. The red florescent signals are converted to red chromogenic signals and the green fluorescent signals to blue chromogenic signals (Dako DuoCISH™). In practice the sample was stained in the FISH procedure, evaluated by fluorescence microscopy and subsequently converted to CISH. Due to the nature of the image capture, i.e. by using an already mounted FISH slide to create the CISH stain, the CISH staining quality is compromised (12).

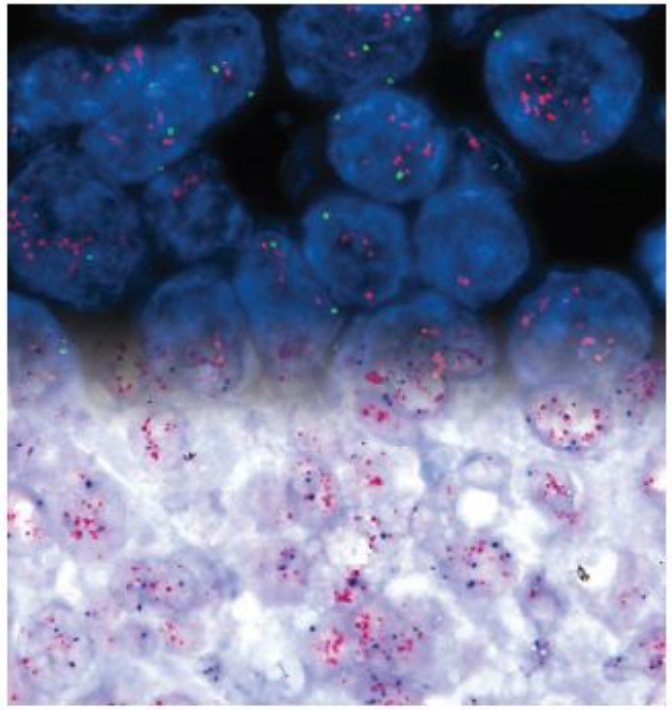


Figure 13.3 A graphical morphing of two sections stained with *HER2* FISH pharmDx™ and *HER2* CISH pharmDx™ kits, respectively.

Staining

- Additional methods
  - Polymer-based
    - Use a polymer backbone to which multiple antibodies and enzyme molecules are conjugated
    - As many as 70 enzyme molecules at about 10 primary antibodies may be conjugated to a single dextran backbone
    - This design has allowed for the entire IHC staining procedure, from primary antibody to enzyme, to be accomplished in a single step
      - This makes it faster than previously discussed avidin-biotin methods
      - Limitation – restricted to a select group of primary antibodies provided by the manufacturer (not useful for user-supplied primary antibodies)

Staining

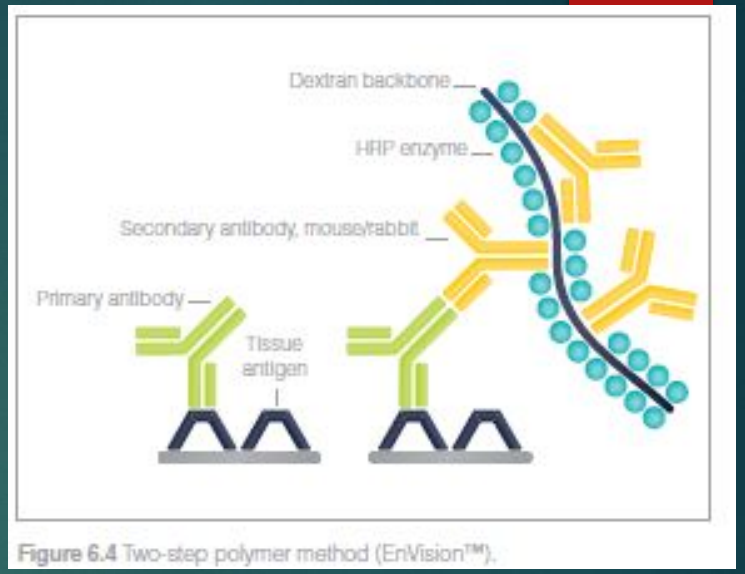


Figure 6.4 Two-step polymer method (EnVision™).

- Additional methods (continued)
  - Polymer-based
    - Latest development of polymer-based detection involves the use of a dextran backbone with **secondary** antibodies conjugated (anti-mouse or rabbit)
    - "Universal" reagent can be used to detect any tissue-bound primary antibody of mouse or rabbit origin
    - This method is comparable or often greater that LSAB or ABC methods
      - Less background staining due to absence of biotin binding/endogenous biotin
    - Note that due to the large molecular size of the polymer complex, it can be hard to detect some epitopes due to inaccessibility/steric hindrance.

Staining

- Other considerations
  - Take note of wash buffers used in the staining process
    - These are used in automated staining platforms for rinsing of reagents between steps
    - Typically kept around a pH of 7.6; monitor with pH strips or pH meter
      - Often contain antimicrobial agents
    - Recommendations on usage come from the manufacturer
    - Wash buffers used are typically Phosphate Buffered Saline (PBS), Tris Buffered Saline (TBS) and Tris hydrochloric acid buffer (TRIS-HCl)
  - There are other, newer detection methods with which one who is preparing for the QIHC exam should be familiar. In respect for time, we will simply list these here:
    - Catalysed Signal-Amplification (CSA)
    - Fluorescyl-tyramide Amplification
    - Improved Catalysed Signal Amplification (iCSA)

Staining

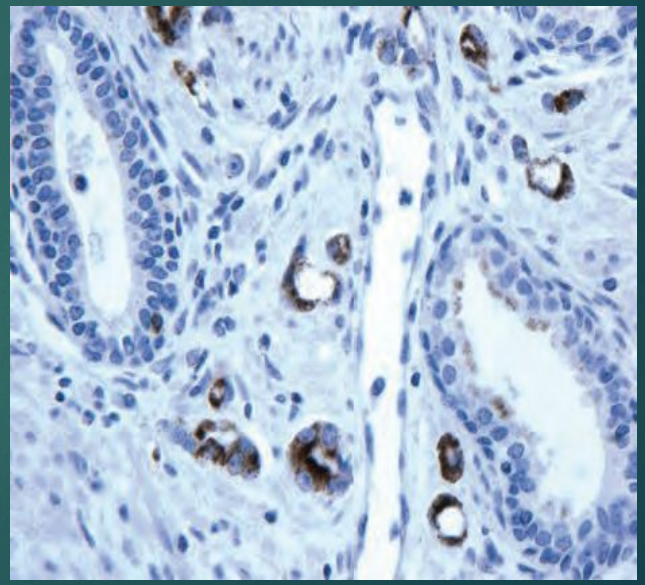
- B. Tissues
  - 1. Morphology/Anatomy – the form and structure of cells
  - Learn about the tissue and cell structures you are staining
  - There are excellent resources available to you.
  - Read **Vendor catalogs** and specification sheets, they describe the antibody you are staining, the staining pattern, controls and disease states. They often have a resource section that gives you different panels.
  - **NordiQC** – this excellent resource will teach you about the antibody, what the best antibody clones are, and the staining patterns in different tissues
  - **Pathology Outlines** is an excellent resource for Special stains IHC, Molecular methods and different diagnoses.
  - Learn your most commonly ordered stains and the panels they are used in,

    E.g. Melanoma – MelA/Mart 1; s100; HMB45; Mitf; Sox 10

Staining

- Tissues
  - 2. Cell/component demonstration – there are specific stain patterns or locations in the cells where the staining reaction takes place.
  - Staining patterns
  - **Cytoplasmic staining** – the reaction occurs in the cytoplasm

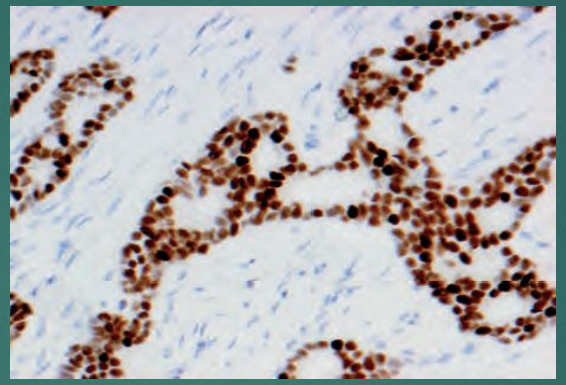
- **Monoclonal Rabbit Anti-Human AMACR (P504S)**
- For identification of prostate adenocarcinoma. The majority of the prostatic adenocarcinoma
- cells should show a granular cytoplasmic staining reaction (Fig. A and Fig. B).
- Cytoplasm.
- Prostate adenocarcinoma: The majority of the carcinoma cells should show a moderate to
- strong granular cytoplasmic staining reaction.
- Benign prostatic hyperplasia: The epithelial cells of the hyperplastic prostate glands should
- be negative or only focally show a weak granular cytoplasmic staining reaction (Fig. C).
- Prostatic adenocarcinoma vs. benign prostatic lesion.
- Figure A. *Prostate adenocarcinoma. The majority of the carcinoma cells*
  - From Dako Flex IHC Stain Atlas



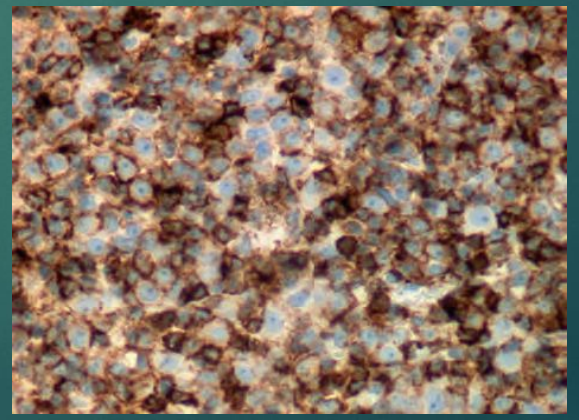
Staining

- Stain Patterns

2. Nuclear – Stains the nucleus of the cell – E.g. Estrogen receptor in image, also PR, TTF, PAX5, PAX 8, Sox 10.



3. Membrane – the nuclear membrane is stained – E.g. many CD markers. CD 43 in B cell Chronic Lymphocytic Leukemia.



Staining

- Stain Patterns
- **4. Combination patterns** - 2 areas may be stained. They can be nuclear + cytoplasmic as in S100 below, or cytoplasmic + membrane.
- Usually one part of the pattern predominates and you must have that staining

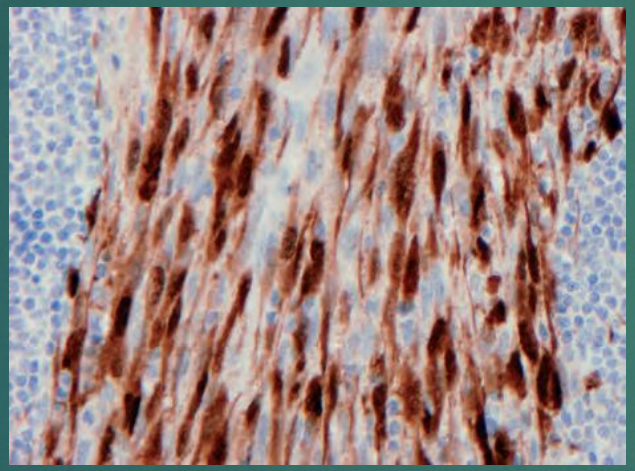
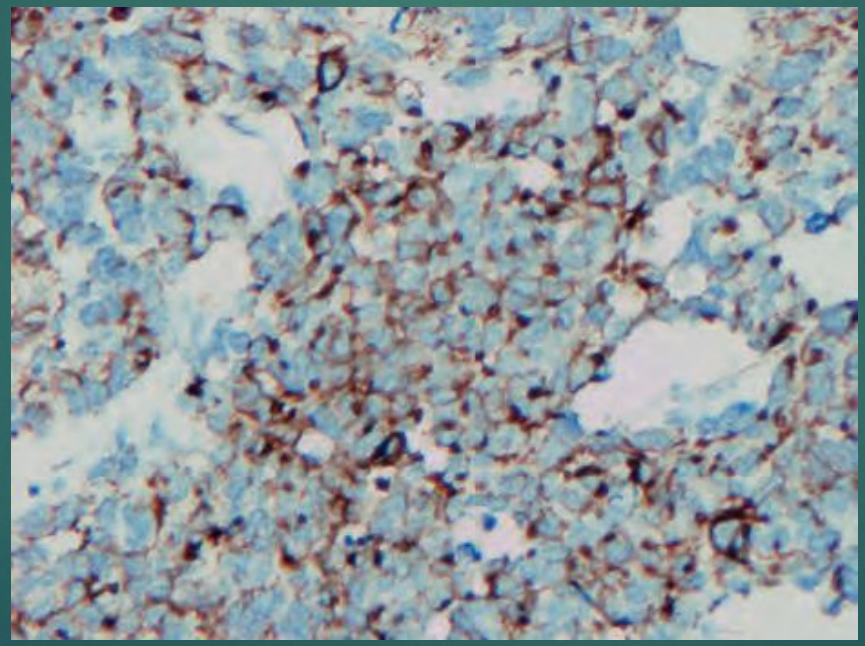


Figure A. *Malignant melanoma. The neoplastic cells show a diffuse nuclear and cytoplasmic staining reaction*

Staining

- **Stain Patterns**
- **5. Special patterns**
- These are usually associated with a certain diagnoses. An antibody will stain in a pattern that is specific to this diagnosis.

  E.g. Keratin 20 in Merkel cell ca this pattern is called a dot-like pattern.



Staining

- b. Microorganisms -the staining of microorganisms can be successfully done in the Clinical Lab.
- These antibodies are usually ASR – Analyte Specific Reagents.
- There are many infectious IHC stains available but usually Laboratories will run those that they have sufficient numbers of cases so they will have control material available.

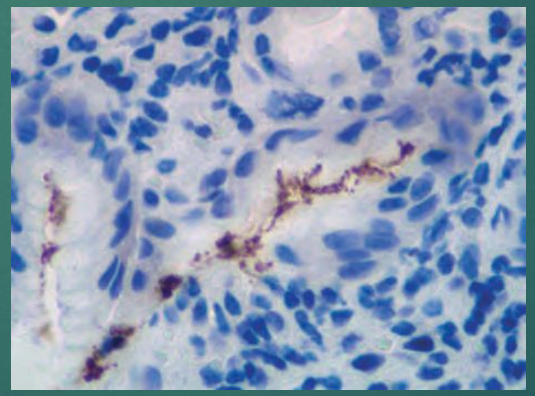
  Most common are:

  H pylori

  CMV

  HSV 1+2

  EBV – usually done by ISH (EBER)



H pylori by IHC

Staining

3. Pathology

- What antibody is used to aid in the diagnosis of what disease or inflammatory process?
- Use the resources we have mentioned as well as NSH and Vendor Teleconferences.
- Become very familiar with Pathologist's orders, for single stains and different panels

E.g. Lung carcinoma – what is it?- Adenocarcinoma, Squamous, Neuroendocrine, Metastatic?

CK 7 why is it used? What does it stain? What other keratins are helpful?

Adenocarcinoma stains – TTF and Napsin

Squamous cell ca stains – CK 5/6, P63, p40

Neuroendocrine stains – Small cell/carcinoid – Synaptophysin, Chromagranin, cd 56

Staining

- C. Components
- 1. Concentrated antibodies – are usually very high in titer or concentration
- Read your specification sheet for suggested dilution ranges and control tissues as well as suggested retrieval guidelines.
- Usually Supplied as a **liquid**
- If not may be **Lyophilized** (powdered) - needs to be re-constituted with sterile water
- Use the appropriate clone in your laboratory- Pathologist will usually suggest
- IVD (In vitro diagnosis) is needed if you are a clinical Laboratory
- RUO (Research use only)can only be used if no IVD is available –you must search for IVD first and document if unavailable.
- You must optimize the concentrated antibody by determining the correct concentration and correct retrieval process if any.

Staining

- C. Stain Components/Characteristics
  - 2. Pre-diluted antibody
  - These antibodies are pre-packaged and come at the right titer for use on your instrument and with the companion detection kits. They are not intended to be diluted.
  - They still need to be optimized and validated before putting into use.
  - More cost involved using pre-dilutes, but less tech time involved in handling.

Staining

- C. Stain Components/Characteristics
- 3. Titrations
- Titration is the dilution of a concentrate in a controlled step like fashion.
- The correct titration of an antibody is the concentration that provides the highest dilution with the best specific staining and the least background.
- There are excellent tutorials on-line, review your textbooks and your Histology class materials so you understand solutions and their preparation.

For example the dilution of a 37% Formalin solution to make a 10% solution.

For this problem you will deal in mls. With antibodies we will be using microliters (ul or lambda) and making up into ml solutions.

1ul = 1/1000ml 1 ml = 1000ul

5ul = 5/1000ml 5 ml = 5000ul

Staining

- 3. Titrations

- You will calculate the optimal dilution for your concentrated antibody in your Optimization process
- Read your specification sheet, this will tell you the protein concentration, give you a suggested range of dilutions and the retrieval solution needed for optimal visualization. Keep in mind this is done in their Laboratory using their diluent and detection systems and their instrument.
- Use a good quality diluent –usually from the Vendor that supplies the bulk of your concentrates.
- Make sure your pipettes are well maintained.
- Make dilutions up in clean plastic graduated tubes rather than directly into the rtu container to eliminate mistakes.

Staining

- **3. Titrations**

- **In practice you will start with your antibody and consider it "neat" or a 1:1 solution.**
- The **formula** for this if you are preparing one tube of a certain concentrate is:

1. $\frac{\text{concentrated antibody} \times \text{volume needed}}{\text{Divide by dilution}} = \text{amount of antibody}$

- Eg. $\frac{1.1 \times 1000\ \text{ul}}{1:50} = 20\ \text{ul of concentrate}$
- **2. Volume needed - amount of antibody = amount of diluent**

  1000 ul - 20 ul = 980 ul of diluent

Staining

- **3. Titrations**
- **If this is your range think of the dilutions this way**.
- 1:25 is 1 part antibody to 24 parts diluent
- 1:50 is 1 part antibody to 49 parts diluent
- 1:100 is 1 part antibody to 99 parts diluent
- 1:200 is 1 part antibody to 199 parts diluent
- Each one of these dilutions is half as strong as the prior.
- This is called a serial dilution and we will be showing you how to set this up.

- **Serial dilutions** minimize waste of antibody and test multiple dilutions in a controlled step-like fashion.
- If you are doing this on an instrument you will need to know the minimum amount of volume for your titration vials.
- Label all vials carefully with concentration, make up enough to be able to test your variables.
- Label all slides carefully with parameters. I like to make up a chart or explanatory sheet with all my variables.

Staining

- 3. Titrations:
- In the **optimization** process you will be determining the optimal dilution of your antibody. Most labs select 3-4 dilutions that cover the range suggested on the specification sheet and by your Pathologist..

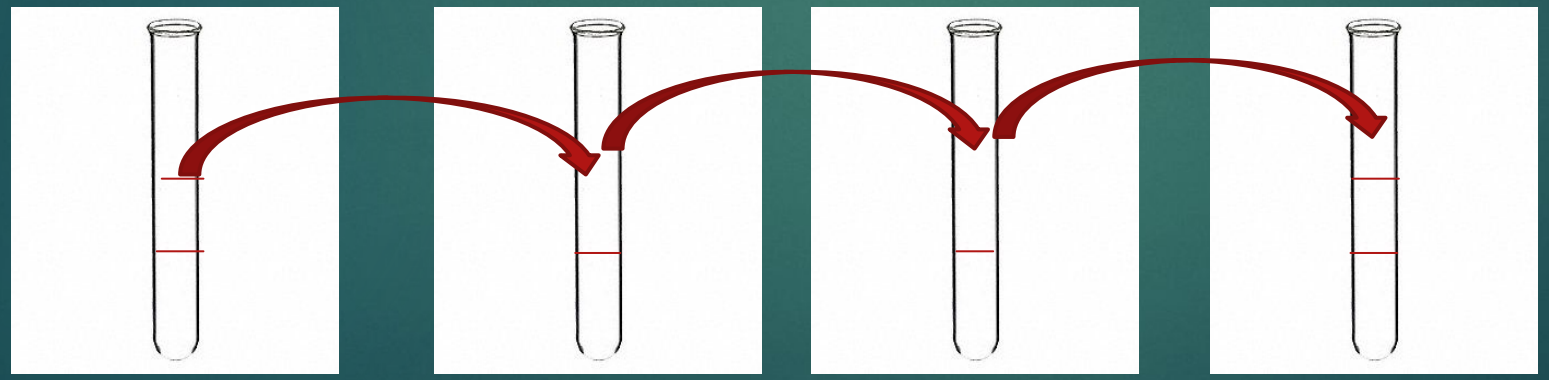
  E.g. If the specs. suggest 1:50 to 1:100, labs often start at 1:25 and go to 1:200
- Your **range of dilutions** will all be tested with the same conditions - standardized results.
- **Control tissue** used from same block and cut in sequential sections (Multi tissue controls work best- or tissue as directed by the Pathologist for diagnoses).
- Detection kit lot # same
- Test on same instrument
- Same Enzyme, Low pH, high pH lots retrieval and no retrieval if the specification sheets suggests no retrieval.
- The **results** of these tests will be reviewed by the Pathologist and he will pick the optimal dilution or further dilutions to achieve results.

Staining

3. Titrations

- We will use our example **1:25 to 1:200.**
- You will need 4 tubes or vials. We are putting 1 ml of diluent in tube 2,3,4 . Label your tubes # 1-4 with the final dilution on each. These will be transferred to your instrument vials for testing after you are done.
- **Tube 1** - 1:25 add 80ul of antibody to 1920 ul diluent to make your initial dilution for 2 ml in tube 1
- Gently mix and remove with a new pipette tip1ml and add to **Tube 2,** mix this solution it is is now 1:50
- Remove 1 ml from Tube 2 with a new tip and add to **Tube 3,** mix well, this is now a 1:100 solution in tube 3.
- Remove 1 ml from Tube 3 with a new tip and place in **Tube 4** and mix ,your solution is now 1:200, you end up with 2 ml
- You can now test all solutions with your appropriate tissue and protocols.

Tube 1 Tube 2 Tube 3 Tube 4



Staining

- 3. Titrations
- To make a serial dilution, you need to take a specific amount of diluted antibody from your first tube and add to an equal amount of diluent in the next tube. This will dilute your previous dilution in half. You can use this method and dilute out your range.

- **Diluting Pre-dilutes –** occasionally we have to use a pre-dilute that after testing appears to be too strong.
- These are intended to be used neat. It is possible to dilute the pre-dilute. Remember you will still have to validate this with recommended number of cases. Best to use same vendors diluent.
- Consider the antibody as a 1:1 solution. You will start with a limited amount of trials.
- 1:2 would be halving the concentration, you would take 1 ml of predilute to 1 ml of diluent.

$$\frac{1:1 \times 2000ul = 1000\ ul\ of\ predilute}{1:2}$$

2000 ul -1000 ul = 1000 ul diluent

Staining

- C. Components/Characteristics

- 4. Reagents
- Most clinical laboratories use the reagents that are intended to be used on the
- instrumentation and detection sysytem in use.
- The antibodies and detection developed by a Vendor are often proprietary and are manufactured to go together.
- Read all package inserts so you understand usage, storage and disposal.
- All reagents are meant to be used according to manufacturer guidelines for storage and disposal according to Regulations of Inspecting Agency and facility.
- No reagents are used beyond their expiration dates.
- Specific lot #'s are maintained.
- All reagents must be labeled with receipt, opened date and expiration date.

Staining

- C. Components/Characteristics

- 4. Reagents
- **Antibody Diluent** – use a High Quality diluent that works with the majority of your concentrates
- **Wash Buffer** – usually in kit or proprietary to instrument. May have to dilute. Needs to be pH'ed and should fall within recommended range. Need to document.
- **Detection** – usually in kit format, with or without Hematoxylin counterstain. Usually contains blocking reagent, chromogen.
- **Enhance solution** – metal solution usually proprietary to Vendor in RTU container

Staining

- C. Components/Characteristics
- 4. Reagents

- **Enzyme** – Clinical labs usually use one major enzyme, which is formulated to work with their detection systems. Protease and Proteinase K are commonly used and you have the ability to make various strengths or digest for various times.
- Other common enzymes in use are Pepsin and Trypsin.
- Most platforms today allow you to digest at room temperature or with heat.
- **De-ionized water – your water source is critical to IHC.** You must have a well maintained de-ionized water source that is cultured for contaminants. It is critical to the preparation of your buffers and the maintenance of your instrumentation
- **Retrieval solutions –** Usually a high pH and a Low pH solution that is proprietary to your detection.

Staining

- D. Troubleshooting

- This is a critical and complex process that must be addressed on a daily basis
- There are many resources available to help you address this subject.

- **Problems that can be easily resolved due to operator/tech error**. These are issues that are easy to see the root cause, and are found before the slides are handed off to the Pathologist.
- They can be easily resolved by following the correct protocol. They may still need to be addressed with staff, if repetitive. They may need to be documented - follow department policy

  Examples:
- 1. Wrong control pulled
- 2. Incomplete drying

Staining

- D. Troubleshooting

- 3. Incorrect placement of the slide on the stainer.
- 4. Incorrect placement of covertile
- 3. Incorrect test ordered and run
- These problems still necessitate repeat testing and should be looked at and discussed with staff to avoid repeat of the issue.
- More complex problems without an obvious root cause need to be looked at in a careful, step-by step manner. The problem has to be resolved to determine if further patient testing can safely happen.

Staining

- D. Troubleshooting
- Usually handled by a Technical Specialist
- All staff must understand the process and be involved in the solution.
- Develop a checklist for use.
- First look at the problem in a calm fashion.
- Describe what the issue is and talk to your Pathologist in charge of the area, they often will have insights if the problem was detected with staining patterns on control tissue or patient tissue.
- Look for other occurrences, is this a one time problem?
- Then with your checklist look at the three areas where issues occur.

- **1. Pre-analytic** – This is something affecting the tissue prior to actual IHC staining. Look at all these steps.
- Specimen id and composition – id compromised , what kind of specimen –fatty, necrotic, cauterized
- time to fixation and fixation times FIXATION IS ONE OF THE BIG ISSUES IN QUALITY OF STAINING
- Was specimen decalcified? – what type used
- In Cytology specimens alcoholic fixation can be an issue
- Processing – time, temperatures and quality of solutions
- Embedding
- Cutting – section thickness and condition

Staining

- D. Troubleshooting
- Drying time and temperature
- Selection of control – wrong control tissue chosen, wrong placement on slide
- Antibody preparation – incorrect dilution when refilling, contamination of container, condition and maintenance of pipettes. If the antibody is the issue are there other cases which had the same lot/vial?
- **2. Analytic –process of staining on instrument**
- Instrumentation- is your instrument well maintained and in working order
- Are all containers cleaned regularly
- Are solutions in correct containers and in correct instrument position, filled to proper volume
- Is de-ionized water supply good? –regular testing is required
- Look at all instrument logs for issues, warnings or error messages
- Are all consumables well maintained – covertiles – clean not scratched, slides (outdated slides) can have adhesion problems
- Staff – are all staff well versed in running and handling the day to day work? Is it a training issue problem?

Staining

- D. Troubleshooting

- **3. Post Analytic-off instrument**
- Get used to looking at slides immediately after removing from the instrument- how do they look ?
- Counterstain and coverslip if not on instrument- problem here?
- Review under the microscope and look at both your control tissue and patient tissue – Did problem affect both tissues?
- If problematic review with Technical Supervisor and Pathologist. They will have the most insight into possible problems.
- If the problem impacts patient tissue diagnosis it will need investigation and resolution
- Document all significant problems – go over your checklist and track all processes. If you have to go all the way back to specimen handling and fixation, pull the patient requisition and start at the beginning.
- Specimen tracking systems help with this process.

Staining

- D. Troubleshooting
- Remember the three main culprits when it comes to problems:
  - Fixation
  - Pretreatment
  - Instrumentation issues
- Try and recreate the issue with controls in place of patient tissue if you suspect it may be antibody or instrument issues.
- You must resolve all issues before re-testing the patient tissue on the same instrument with the same antibody.
- **Do not use a questionable instrument or antibody on any more patient samples until your problem is resolved.**
- **Instrument issues must be repaired, with non-patient samples tested before putting back into use.**
- Share problems and solutions with staff so they understand the process and its significance.
- Excellent Resources available from Vendors – Handbooks, articles, on-line tutorials.

Staining

- E. Mounting procedures
  - Different procedures require different types of mounting.
  - The two main types of mountant are Aqueous and Permanent
  - Coverslips are usually glass or the tape method.
  - It is critical that you read your protocol/procedure and are familiar with what chromogens are in use, as they often differ in permanence and requirements for cover slipping.
  - **IF** usually requires an aqueous mount because of the nature of the process
  - **IHC using DAB is permanent**. Most stain runs end in distilled water or buffer rinses.
  - Some Detection Systems require a soapy water rinse or dip to remove the "liquid coverslip" solution that is used in the staining process.
  - Once the DAB/HRP stained slides are placed in water they are dehydrated through graded alcohols to xylene similar to an H&E section, this can be done by hand or on a stainer by creating a program.
  - They are then coverslipped from xylene with a permanent mounting media using glass coverslips or the tape method.

Staining

- E. Mounting
- **HRP –AEC** and other chromogens – please read the specification sheets and protocols involved with these chromogens. They are very specific about mounting, dehydration and best practice.
- **ALK – Phos. IHC** The red with most ALK Phos. kits is considered semi-permanent and you must be very careful when you dehydrate
- Graded alcohols are usually skipped with just dips in absolute alcohols and coverslipping out of xylene.
- Best practice is drying completely out of staining and then dipping in xylene and coverslipping.
- **FISH** – usually requires a special mounting material, often containing a fluorescence counterstain which is then covered by a a glass coverslip. Slides are kept in the dark because of fading and stored at -18 to -8 degrees.
- **CISH/SISH** – there are very definite instructions for counterstaining, clearing and mounting when doing these procedures depending on chromogens. Please follow the kit/detection system/ chromogen guidelines.

Staining

- F. Preliminary Screening

- Every tech working in IHC should take the time and look at and become familiar with their stained slides under the microscope. Most Pathologists are happy to sit with you and review a case or answer staining questions.

  Printed materials help you to learn what the stained slides should look like
- Macro – look at your slide or tray of slides when you take them off the instrument
- Is there obvious staining missing –look at your controls. You should be able to detect both blue and brown, if Hematoxylin is applied on-line.
- If using a covertile, is it on correctly, is there pooled solution on top.
- Are you missing obvious tissue, is the tissue folded, are there circles on your sections where it looks like solutions are missing.

Staining

- F. Preliminary Screening

- Under the Microscope –
- Look at your slides under the microscope, confirm macroscopic issues
- Review both control and patient tissue – does it appear appropriate? Compare to references.
- Alert the Tech Specialist and Pathologist if you think there may be a staining issue. This can save TAT time on critical cases, and their feedback also helps you in the troubleshooting/learning process. Detecting an instrument, antibody or process issue early will help the department to avoid negative impact on patient results.

Laboratory Operations

15-20%

Laboratory Operations

- A. Quality Control/Quality Assurance
  - 1. Documentation Your Accrediting agencies require monitoring and documentation of all aspects of your laboratory practice. This must be up-to-date and signed. Required statistics must be done.
  - Procedures
    - There must be an up-to date Procedure Manual available to all personnel, hard copy or on-line.

    Quality control records
    - Review a copy of your Regulations for all documentation
    - Quality control records can be on-line, hard copy or both, but you must address all current regulations and records must be kept for the proper length of time.
      - Buffer testing –pH log
      - Lot to lot new antibody, detection kit, enzyme testing documentation
      - Antibody validation documentation

Laboratory Operations

- b. Quality control records
- Control tissue documentation
- Instrument Maintenance documentation both for routine issues and PM's
- Documentation of staining problems / resolutions
- Antibody vendor notifications
- Results of Proficiency Testing
- Pipette calibration documentation
- c. Personnel
- Training records – There should be departmental documentation for all people trained and working in IHC.
- There should be competencies for all tasks performed and documentation of review of existing and new procedures.
- Do all employees perform every task? Are some assigned to dilutions?
- Optimization/validation? Antibody testing?
- Documentation of antibody preparation by personnel – need to be able to troubleshoot issues- so you must document task performance.

Laboratory Operations

- **A. Quality Control/Quality Assurance**
- **Reagents/antibody lots**
- **All new lots of antibodies and detection systems, must be compared to performance of prior lots, before use on patient.**
- This must be performed according to Regulations and documented.
- Any issues must be resolved prior to use.
- This is for both concentrates and rtu reagents.
- This comparison is done on the same control tissue in use so comparisons are meaningful and usually involve multi control tissues with both positive and negative staining specimens.
- Pathologist or designee review of slides.
- This comparison is documented and the slides filed for easy retrieval and review.
- Keep current on Regulations!

Laboratory Operations

- **A. Quality Control/Quality Assurance**
- **2. Selection, utilization, and evaluation of control tissue**

- Become familiar with and follow **Regulatory guidelines**. They define what positive, negative and batch controls must be.
- Control tissue selection will be guided by your Pathologist in charge. They will direct you as to what control tissue choices/cases they want in your multi or single control tissues and what stains will be associated with these blocks.
- This should be documented in a Control Block/Tissue policy with your choices of tissues in multi block controls and what antibodies are used with these blocks
- The tissue selected should be fixed and processed in the same manner as your patient tissue

Laboratory Operations

- **A. Quality Control/Quality Assurance**
- **2. Selection, utilization, and evaluation of control tissue**
- Develop a method or process for harvesting extra tissue from the type of cases you need.
  This is tissue that is not needed for diagnosis and should not be over fixed.
- **Definitions**:
- **Positive control tissue** will confirm the proper staining of the antibody
- **Negative Control tissue** also confirms the proper staining of the antibody in the fact the chosen tissue lacks antigen
- Your controls must assess specificity of staining
- **Multi tissue controls** – are specifically chosen to both positive and negative staining of one or more antibodies.
- **Single tissue control** is a single tissue that is placed on a slide that confirms positive staining of the antibody. There may or may not be negative internal control structures present. You often see single + controls on organism stains.

Laboratory Operations

A. Quality Control/Quality Assurance

- **Definitions:**
- **Batch control** is a control that is run with a batch of patient slides being stained at the same time with the same antibody on the same instrument. A Batch control must stain appropriately, be reviewed and approved before any patient slides in the batch are read.
- **Control block preparation and use:**
- Cases are selected for control tissue use from archived tissue or recently prepared blocks, based on tissue type or diagnosis.
- The tissues are made into multi tissue blocks or single controls. The case #'s and organization of what the tissue in the block and its placement (map) is documented.
- A file or bank of control blocks are created for use.
- An **H&E is cut from this block** and reviewed by a Pathologist or designee to insure diagnostic material – this is documented.

Staining

- A. Quality Control/Quality Assurance

- Additional slides are cut from this block if it is good and all antibodies are applied/tested on these slides that would normally be ordered on this control block.
- Slides are again reviewed by a Pathologist or designee.
- If all stained slides are satisfactory this is documented, and the block can be put into use for patient testing.
- The documentation of control slides/tissues are kept according to regulations
- Any patient tissue that has control tissue that is not accurately stained is not reported. The problem must be investigated and resolved before repeating the testing.
- Labs usually keep a bank or volume of recently cut control tissue slides for daily use. These slides should be recently cut and held at room temperature no longer than a month to prevent antigen loss

Staining

- A. Quality Control/Quality Assurance
- 2. Control Tissue

- Any slides held longer should be refrigerated or placed in a freezer.
- All antigens do not maintain strength in cut tissue, some are very picky and only do well when cut fresh. Tissue cut for prognostic markers should never be stored more than a month.
- **Mounting of control tissue:**
- Control tissue slides are cut and air dried in the upright position, then stored until needed
- Most labs will mount the control tissue on the top of the patient slide, making sure it is within the borders or boundaries of the instrument (covertile size/ heating plate).
- Patient tissue is then placed below the control material and the slides again placed in an upright position and dried in an oven prior to staining on the instrument or by hand.

Staining

- A. Quality Control/Quality Assurance
- 2. Control Tissue

- All water trapped under the tissue must be eliminated or you will suffer from tissue loss or lifting.
- Drying at 60 degrees has been considered safe for most tissue types. Fatty tissue may require a longer drying time if problematic. Consult Regulatory guidelines.
- Some instruments advertise **dewaxing and baking on-line**, but most Labs have found that drying off-line with dewaxing on-line produces the best results.

- **Controls for organisms** – Many labs do IHC for several types of organisms or infectious agents.
- H Pylori being the most common
- Where to place control with organisms – on the top or bottom?
- Are you running a batch control?
- Remember it is best to use in-house controls processed and fixed in the same manner as your patient tissue.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 2. Control Tissue

- **Note about archival tissue used for controls**. Many institutions have regulations regarding the use of archival tissue. With molecular studies there are many tumor markers that are being run on the original tumor blocks years after they were made, usually to compare to a patient tumor recurrence. You do not want to exhaust tumor blocks that are not ready to be discarded if it can benefit the patients treatment.

Laboratory Operations

A. Quality Control/Quality Assurance

3. Method selection, optimization, and validation

- **Method selection**
- Your method selection for each optimization is defined by the instrumentation you have in your Laboratory. If your lab has one type of instrumentation this will define your optimization. If more than one you have to look at the parameters of both instruments and the detection systems involved.
- Detection systems you are running and strength of each HRP vs ALK. PHOS.
- Do the Paths need a red stain on the tissue for better visualization?
- Can you dewax on instrument?
- What are the times and temperatures for retrieval ?
- What are the retrieval solutions you can use – is it off instrument or on ?
- What type of tissue are you using –is it fatty, can it be protected by a covertile or is it subjected to constant washing?
- What are your time constraints in case dx – is the stain you are working on a STAT type case or routine ?

Laboratory Operations

A. Quality Control/Quality Assurance

3. Method selection, optimization, and validation

- **Method selection**
- Does the instrument counterstain
- Does your instrument allow open containers for your concentrates?
- Cost of running if two instruments
- What do the experts say –research the antibody

- **Optimization** – The process of testing your antibody(s) with the variations you have set-up with your Pathologist, to determine the best quality stain, specific for your antibody with minimal background and good reproducibility.
- Read the specification sheet, this will give you information on dilutions if you are using a concentrate and best retrieval that they used in their laboratory. Also control tissue.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 3. Method selection, optimization, and validation
- **Optimization**

- Your Pathologist will direct this process and suggest what clone he wants tested.
  Usually your Pathologist will advise you in what tissue he wants tested in the optimization.
- Remember standardization
- For this discussion we are going to look at a rtu format on one antibody clone.
- Do not make your optimization too difficult it will be easier to confuse steps and
  make incorrect choices. Make a Map of what your dilutions and retrievals will be.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 3. Method selection, optimization, and validation
- **Optimization**
- If you always test all retrievals, than set up your test that way…
- Start with the routine times on your instrument so you can run these stains as you normally do
- You will run sequential sections with the same detection kit, on the same instrument.
- If you test with no retrieval –label slide accordingly and run without retrieval.
- Enzyme –test with routine Enzyme used
- Low pH retrieval
- High pH retrieval
- You want to produce a Quality stain with the least harsh retrieval if possible.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 3. Method selection, optimization, and validation
- **Optimization**
- After this run has finished counterstain and coverslip as usual. Look at your slides under the Scope. You can often see which one is obviously better. Then you will review with your Pathologist.
- You should have an Optimization work sheet that you can document your runs and parameters on.
- Your Pathologist will select the best stain and sign-off on it.
- This protocol will then be put into your instrument and you can start the validation process, or they may suggest other parameters for testing like increase in incubation time.
- If rtu is not what is needed you will approach the optimization using concentrates and dilutions, as we discussed under dilutions.
- Most instruments have systems for testing dilutions that allow you to add a certain amount of the test antibody to the slide on-line using the same parameters as the rtu. This will require many more slides, dependent on your dilution range. Follow dilution range guidelines we discussed before.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 3. Method selection, optimization, and validation
- **Optimization**
- Testing dilutions requires very precise labeling of your slides and vials in use.
- Make up your worksheet carefully as you go.
- After slides have finished they will be counterstained and coverslipped and reviewed by the Pathologist, they will direct the process if further testing(dilutions) are needed.
- If the antibody is to be used with a red chromogen they may ask to see if with Your ALK PHOS kit in use.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 3. Method selection, optimization, and validation
- **C. Validation -** The validation process occurs once you have your optimal conditions defined for your antibody. It will be driven by your Pathologist and current Regulatory Guidelines.
- The **CAP guidelines** are specific as to how many positive and negative cases must be tested for each routine kind of antibody stain , each prognostic antibody and how many stains must be done if any changes occur in Lab conditions like instrument issues, water issues, reagent changes, retrieval changes, processing changes, etc if you are using the same clone of antibody. If you are switching clones you must do a new validation.
- The regulation should be checked yearly as they change.
- Currently it is 10 positive and 10 negative cases for routine antibodies
- 20+/20- for prognostic antibodies.
- Minimum # of cases for testing when changes occur to be determined by the Lab Director. Must be same antibody clone.

Laboratory Operations

A. Quality Control/Quality Assurance

3. Method selection, optimization, and validation

c. Validation

- The Prognostic stains like ER/PR and Her 2 neu have very specific guidelines and testing requirements that need to be strictly followed and documented.

  Other Methodology -You cannot just test the positive and negative cases they have to be compared to another proven methodology (FISH) or another labs in-use validated same clone.
- All chosen cases will be tested on the same instrument with the same detection system with the optimized antibody.
- These will be documented on a Validation Worksheet with information (case # and diagnosis) and room for Pathologist input. They will be reviewed for appropriate staining and signed off.

Laboratory Operations

3. Slide Storage

- Review your regulations and guidelines for length of time and storage requirements.
- This is inclusive of all Patient case materials – slides and blocks, your testing and validation slides, control block material and slides, Proficiency testing slides, lot-lot testing materials and other QA/QC slides.
- Often Patient slides and blocks are filed away from the lab, just because of volume issues.
- You want to keep slides that need to pulled and reviewed in your immediate area.
- Many labs keep their optimization and validation slides as long as the antibody is in-use. It is especially important for Prognostic markers.
- You need to keep other non-specified slides – for at least as long as your Inspection cycle time. For example if CAP comes every two years –you want to have all lot-lot tested slides, Control slide tests, and QA/QC issues, and CAP proficiency testing slides. This also includes all associated paperwork-documentation.

Laboratory Operations

- A. Quality Control/Quality Assurance
- 5. Troubleshooting
- This was discussed thoroughly in prior sections. You will need to troubleshoot all issues in your optimization and validation process just as in day-to-day operations.
- However, some problems may be different, in the fact mistakes can be made in making up your dilutions, which will require repeat of your process.
- Repeat, if results are the same possibly the antibody you have purchased doesn't work as well with your detection and you require a different vendor or clone.
- Sometimes it is the case materials, or controls selected, that may require additional testing, if so your Pathologist will be able to direct this process.
- Conduct your testing in a standard, well documented manner. It is essential to document failures as well as successes.
- Document issues you have with any antibody and vendor recalls or advisories. If Vendors change protocols on certain antibodies it may be a red flag that it is problematic. Remember they often buy their antibodies from other vendors and although they are supposed to guarantee the quality through extensive testing, all antibodies are not equal.

Laboratory Operations

- **B. Safety**
  - Become familiar with CAP or Accrediting Agency Guidelines. Attain a copy of your Lab's CAP Custom checklists (Histology and All Common).
  - NSH has information. Read product specification sheets and become familiar with product SDS sheets and their pictograms. Use proper PPE.
  - Read your Laboratory Safety Policies – they should be up-to date on guidelines.

1. Storage

- There must be enough storage space in your lab to store all supplies in use, in the manner the Manufacturer recommends.
- Fume hoods and flammable cabinets if needed.
- There must be enough space in your Lab to store all the slides, blocks and materials needed in your area, or they must be close enough to retrieve. Must be stored for the minimum time required by your accrediting agency.

Laboratory Operations

- **B. Safety**
  - **2. Disposal**
    - Follow all institution guidelines for handling and disposal of reagents and chemicals as well as kits, pipettes, tubes, instrument parts, plastics, glass and paper products.
    - There are different local, state and Federal regulations in play.

Laboratory Operations

- **B. Safety**

  3. Hazards

  - Become very familiar with the products in use in your Laboratory. Review their SDS sheets – Hazards are prominently marked using the pictogram system.
  - Keep in Mind there are products in use that are hazardous and follow proper disposal.
  - Chromogens are considered possibly carcinogenic and mutagenic.
  - ISH probes contain formamide which is very hazardous.
  - There are guidelines in many facilities for the disposal of alcohols.
  - Be aware that your prefill containers when marked empty by your instrument still have a dead zone and may contain regent.
  - Formalin, Xylene and other chemicals in use have very definite disposal guidelines.
  - Glass and sharps have disposal guidelines.
  - Protected Patient information must be separated from other paper waste.

Laboratory Operations

- **B. Safety**

  4. Regulations

  - Safety Regulations are mandated by Federal, State and Local Agencies.
  - Your hospital/Laboratory usually has a department that reviews these and will make their recommendations and arrangements for disposal of all hazardous and non-hazardous materials.
  - They maintain copies of SDS sheets and determine how the institution will dispose of waste.
  - They also recommend what type of spill/splash procedures and kits need to be stocked in your area as well as what safety training and PPE is needed to insure employee safety.
  - Some safety materials have outdates. They must be labeled and restocked accordingly.
  - Eye wash stations and showers must be tested according to hospital policy.
  - Laboratories using Xylene and Formalin must have monitoring to ensure ventilation is adequate and employees levels of exposure are within allowable levels.
  - Some Laboratories monitor their alcohols and have very strict disposal and handling guidelines.
  - Mandated safety testing is carried out in all institutions.

Laboratory Operations

- B. Safety

5. Procedures

- Your Laboratory Manual should detail all safety measures and disposal methods utilized and required in your institution.
- This Procedure is often kept at the administrative level in the Lab as it refers to all departments.
- All employees should be trained and informed when any procedures change.
- Make sure everyone knows where clean and contaminated areas are located, and make sure all areas are cleaned properly on a daily basis. This is for everyone's protection.
- Safety is always an important part of any accreditation and any inspection.

Laboratory Operations

- C. Laboratory Mathematics

  There are many guidelines and resources available addressing this topic. As discussed before you should be familiar and proficient in applying the correct Math skills to not only the exam but also in your daily practice. Make sure you understand concentrations, solution preparation and dilutions. Please see resource list available. You can also access many tutorials on-line.

Laboratory Operations

- D. Ancillary Equipment/Instruments (e.g., microwave, computers, pH meter, solvent recovery, hybridization chamber)
  - All equipment used in the lab must be properly maintained and cleaned according to manufacturer's and accrediting agency guidelines. Review these guidelines to make sure all necessary documentation is being done, and maintained.
  - Detailed procedures, and schedules available to the bench staff.
  - All staff should be trained to understand basic instrument operation and basic troubleshooting.
  - There should be a defined Maintenance schedule for both routine procedures on the instrument and PM's.
  - There should be a defined procedure for all thermometers and temperature recording devices, with defined temperature ranges.
  - There should be a procedure that outlines downtime processes for all equipment in use and processes.
  - Laboratory Policy for testing and Maintenance of the water delivery system which is critical to your reagent and testing quality.
  - Pipettes must be calibrated according to CAP/accrediting agency guidelines and accurately used.

Laboratory Operations

- E. Regulations
  - 1. Federal government – CMS, OSHA, CDC
  - 2. Accrediting agencies
    - These are either CLIA or agencies with deemed status (they participate and receive Medicare/Medicaid payments) like CAP or Joint Commission
    - Your Laboratory must be accredited (follow guidelines and standards) to practice Laboratory Medicine and receive payments.
    - This will include Mandatory Proficiency testing with satisfactory results and Internal and External Inspections with satisfactory results established by the testing agencies.

CAP/CLSI Guidelines

- These agencies publish guidelines that specifically address issues of testing, Quality Control and Quality Assurance in the Anatomic Pathology Laboratory.
- Affect IHC, both routine testing and prognostic testing
- Guidelines change – You need to review and address yearly
- Change protocols and procedures accordingly
- Movement toward increased standardization of fixation, tissue handling and testing
- I believe you will see these standards evolve and become more defined, to cover all aspects of
- IHC as more and more molecular tests and companion diagnostics evolve.

ANP.22750	**Antibody Validation**	**Phase II**
	The laboratory has records of validation of new antibodies, including introduction of a new clone, prior to use for patient diagnosis or treatment.	

Source: CAP checklist 2018

Resources

- https://www.ascp.org/content/board-of-certification/get-credentialed
- https://www.nsh.org/learn/qihc-prep $225
- https://www.mihisto.org/page-1378241#!/QIHC-Study-Guide/p/14147537/category=3313140 $20
- www.nsh.org – antibody database and Image bank (must have an NSH account to access)
- Phone apps – search for histology/IHC – great for antibody images
- Dako Education Guide: Immunohistochemical Staining Methods, 6th Ed.
  - *Thanks to Joel Eckstrom & Agilent for allowing us to use the images from the above resource in this presentation*
- Level 2D: Immunohistochemistry: Harnessing the Power of the Immune System
- Carson Histotechnology text 4th edition
- Dabbs Textbook
- Anatech LTD – Pamphlet on Glyoxal fixative (how other fixatives impact IHC)
- www.CAP.org
- https://www.nordiqc.org/index.php
- https://www.pathologyoutlines.com/stains.html