Immunohistochemistry

Immunology + Histology + Chemistry

The Principles of IHC

The Good, The Bad and The Ugly

The Good

Appropriate block, well processed, well fixed excellent 3 mu section.

Excellent IHC demonstrating expected profile, and confirms original suspicion

The Bad

Appropriate block, well processed, well fixed excellent 3 mu section.

Excellent IHF staining with unexpected results.

Repeat, reassess, recheck and seek help through colleagues or outside consultation

The Ugly

Inappropriate block, not well processed of fixed

Cannot be sectioned properly

IHC results are unreliable or erratic

No diagnostic value

Waste of time and resources

In a Nutshell

- Formalin fixed, processed, paraffin embedded Tissue
- Cut at 3 Microns
- Baked in 42 degree oven
- Stained using 3 step indirect streptavidin method
- Sent to Pathologist for interpretation and diagnosis

Simply Stated

IHC is based on the principle of Antigen Antibody Reaction.

Immune System

The Immune system can be divided into two subsystems.

The innate Immunity system recognizes microbes or foreign cells that do not belong in our bodies.

The acquired immunity system causes a production of antibodies against antigens.

Definitions

Antibody – protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target.

Antigen – a molecule that stimulates the production of antibodies.

Avidin – protein that binds biotin strongly, forming an irreversible bond.

Biotin – Water soluble B complex vitamin.

Enzyme – a protein that catalyzed a chemical reaction.

Epitope – part of a foreign organism that is being recognized by antibodies.

Factors that influence IHC success

- The avidity of antibodies of the antigen
- The specificity of for the antigen
- How the structure of the epitopes on the antigen are altered during the technique
- How easily the antibody can reach the antigen
- The quality of the secondary reagent

Avidity

Measure of the total strength of the interaction between the antibody and the antigen

Affinity

A measure of how tightly the antigen binding site of the antibody interacts with it`s cognate epiope on the antigen

Labelling Method

Three Step Indirect Streptavidin method

1. Primary Antibody against the tissue antigen.
2. Secondary (which will recognize the primary) will be tagged with biotin.
3. Third layer will be a streptadivn enzyme complex allowing the streptadivn to recognize the biotin.

Dab will be used to precipitate a colour reaction.

The benefits of this system is that the sandwich effect of layers gives a stronger and bulkier signal .

Rule 3

Don`t believe everything you read, hear, or see.

A Case in point

A series of technical developments in IHC have created sensitive detection systems. Among them is the enzymatic (Horseradish peroxidiase) developed by Avrameas and colleagues which in the presence of a suitable colorogenic substrate system, allowing visualization of the labeled antibody by orthodox light microscopy. (Dabbs 2002)

OR

A brown colour (Dab) is added to the IHC system so that we can see the antigen antibody reaction under microscope. (Green 2007)

FORMALIN PARADOX

Formalin is our best fixative to date, but during the fixation process it forms methylene bridges which mask antigen sites. We have to unmask the antigen sites so that we can produce the antigen / antibody reaction necessary for IHC.

Paradox #2

Antigen/ Antibody reactions are heat sensitive and sections should not be incubated above 60 degrees Celsius. Due to the fact that we may loose Antigenicity, But during Antigen retrieval (AR) sections are boiled at 95 – 100 degrees Celsius to unmask Antigen sites.

Antigen Retrieval

AR is the concept of recovering lost immuno reactivity through exposure to heat or enzyme.

Paradox #3

Not all Antibodies need AR.

Some Antibodies need Heat induced epitope retrieval (HEIR).

Some Antibodies need Protealytic induced epitope retrieval (PEIR)

HIER

- Heat induced AR
- Buffers of various ph values used
- Sodium citrate ph 6.0
- EDTA ph 8.0
- Heated to 95 to 100 degrees Celsius
- Short 8 minutes
- Mild 30 minutes
- STD 60 minutes
- Extended 90 minutes

The object is to obtain optimal AR with minimum time as this process is very harsh on tissue, and too much will result in tissue damage.

PIER
Proteolytic Induced AR

Enzymes are used to break down the cross link proteins formed during formalin fixation.

Examples:

Pepsin
Trypsin
Pronase
Protease
Proteinease K

In our laboratory we use Protease and Proteinease K

AR

Not all antibodies need AR

All antibodies are different

Each antibody has to be assessed on an individual basis to determine the best AR.

Remember

Simply an Antigen / Antibody reaction

Monoclonal Antibodies

Advantages

More specific and less background staining

Disadvantages

More sensitive to tissue fixation

Polyclonal Antibodies

Advantages

Less sensitive to fixation

Disadvantages

Can cause higher background staining

Primary Antibody

- Antibodies are like people
- All are individual
- All share similar characteristics
- Some share many characteristics
- Some share few characteristics
- Some are totally different

Examples

CK7 – Cytoplasmic

ER – Nuclear

CD3 – Membraneous

CD68 – Cytoplasmic and Membraneous

Calretenin – Cytoplasmic and Nculear

Primary Antibody Dilution

- Most are predilute by the manufacturer
- Some have to be diluted to suit the individual laboratory
- Use manufactures recommended dilution and adjust for your laboratory

Primary Antibody Validation

When a new Antibody is introduced to the laboratory it has to validated

The validation process involves

Antibody dilution

AR requirements

Primary Antibody incubation time

Positive controls

Negative controls

Comparison to known patient results

IHC Antibodies

There is no

ONE SIZE FITS ALL

There is no

ONE SIZE FITS MOST

Protocol

Every antibody has it's own protocol

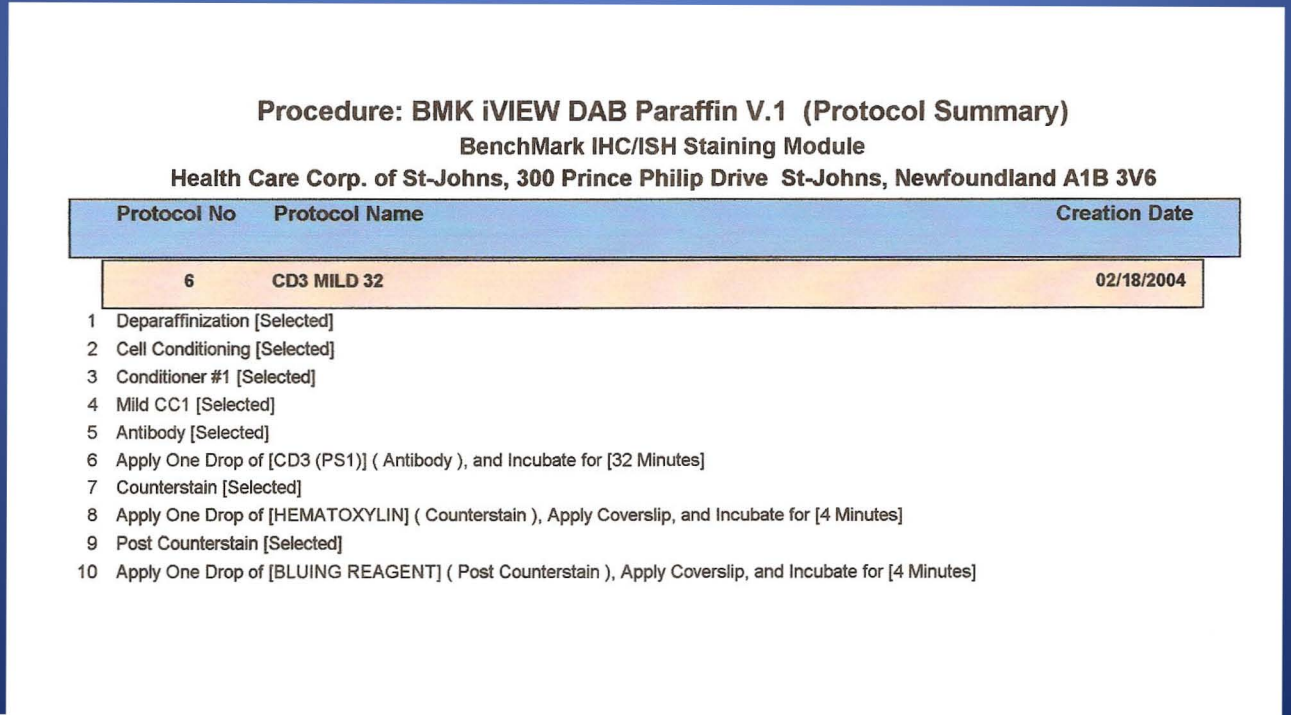
A protocol is a recipe – unique series of steps which are followed to achieve a desired result, each time and every time.

Once the parameters are determined the protocol is set and should not be altered unless the antibody or clone changes

Sample Protocol

Anti CD3 Antibody has been considered the best all-round T cell marker

Remember Just an antigen – antibody reaction



Procedure: BMK iVIEW DAB Paraffin V.1 (Protocol Summary)

BenchMark IHC/ISH Staining Module

Health Care Corp. of St-Johns, 300 Prince Philip Drive St-Johns, Newfoundland A1B 3V6

Protocol No	Protocol Name	Creation Date
6	CD3 MILD 32	02/18/2004

1 Deparaffinization [Selected]
2 Cell Conditioning [Selected]
3 Conditioner #1 [Selected]
4 Mild CC1 [Selected]
5 Antibody [Selected]
6 Apply One Drop of [CD3 (PS1)] (Antibody), and Incubate for [32 Minutes]
7 Counterstain [Selected]
8 Apply One Drop of [HEMATOXYLIN] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
9 Post Counterstain [Selected]
10 Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]

Protocol # 6 : CD3 MILD 32 (02/18/2004)

Procedure: BMK iVIEW DAB Paraffin V.1

BenchMark IHC/ISH Staining Module

Health Care Corp. of St-Johns, 300 Prince Philip Drive St-Johns, Newfoundland A1B 3V6

Step No	Procedure Step
1	***** Select EZ Prep *****
2	***** Start Timed Steps *****
3	***** Mixers Off *****
4	Warmup Slide to 75 Deg C, and Incubate for 4 Minutes
5	Apply EZPrep Volume Adjust
6	Incubate for 4 Minutes
7	Rinse Slide +
8	Apply EZPrep Volume Adjust
9	Incubate for 4 Minutes
10	Rinse Slide +
11	Apply EZPrep Volume Adjust
12	Apply Coverslip
13	Warmup Slide to 76 Deg C, and Incubate for 4 Minutes
14	Rinse Slide +
15	Apply Depar Volume Adjust
16	Apply Coverslip
17	Disable Slide Heater
18	***** Mixers On *****
19	[Short - 8 Minute Conditioning]
20	Rinse Slide +
21	Apply Medium Cell Conditioner #1
22	Apply CC Long Coverslip
23	Warmup Slide to 95 Deg C, and Incubate for 8 Minutes
24	[Mild - 30 Minute Conditioning]
25	Apply Medium Cell Conditioner #1
26	Apply Coverslip
27	Warmup Slide to 100 Deg C, and Incubate for 4 Minutes
28	Apply Coverslip
29	Apply Cell Conditioner #1
30	Incubate for 4 Minutes
31	Apply Coverslip
32	Apply EZPrep CC Volume Adjust
33	Incubate for 4 Minutes
34	Apply Coverslip
35	Apply Medium Cell Conditioner #1
36	Incubate for 4 Minutes
37	Apply Coverslip
38	Apply Cell Conditioner #1
39	Incubate for 4 Minutes
40	Disable Slide Heater
41	Incubate for 8 Minutes
42	Rinse Slide +
43	Adjust Slide Volume
44	Apply Coverslip
45	***** Select Reaction Buffer *****

Protocol # 6 : CD3 MILD 32 (02/18/2004)

Procedure: BMK iVIEW DAB Paraffin V.1

BenchMark IHC/ISH Staining Module

Health Care Corp. of St-Johns, 300 Prince Philip Drive St-Johns, Newfoundland A1B 3V6

Step No	Procedure Step
46	Warmup Slide to 37 Deg C, and Incubate for 2 Minutes
47	Rinse Slide +
48	Adjust Slide Volume
49	Apply One Drop of I-VIEW INHIBITOR, Apply Coverslip, and Incubate for 4 Minutes
50	Rinse Slide +
51	Adjust Slide Volume
52	Apply Coverslip
53	Apply One Drop of [CD3 (PS1)] (Antibody), and Incubate for [32 Minutes]
54	Rinse Slide +
55	Adjust Slide Volume
56	Apply One Drop of I-VIEW BIOTIN Ig, Apply Coverslip, and Incubate for 8 Minutes
57	Rinse Slide +
58	Adjust Slide Volume
59	Apply One Drop of I-VIEW SA-HRP, Apply Coverslip, and Incubate for 8 Minutes
60	Rinse Slide +
61	Adjust Slide Volume
62	Apply Coverslip
63	Rinse Slide +
64	Adjust Slide Volume
65	Apply One Drop of I-VIEW DAB and One Drop of I-VIEW H2O2, Apply Coverslip, Incubate for 8 Minutes
66	Rinse Slide +
67	Adjust Slide Volume
68	Apply One Drop of I-VIEW COPPER, Apply Coverslip, and Incubate for 4 Minutes
69	Rinse Slide +
70	Adjust Slide Volume
71	Apply One Drop of [HEMATOXYLIN] (Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
72	Rinse Slide +
73	Adjust Slide Volume
74	Apply One Drop of [BLUING REAGENT] (Post Counterstain), Apply Coverslip, and Incubate for [4 Minutes]
75	Rinse Slide +

As you can see from the extended version of the protocol, there is no such thing as a simple antigen - antibody reaction.

There are many factors which can affect the final result.

IHC GOAL

To ensure run to run reproducibility, evaluated against in-house controls, days, weeks, and months apart.

Factors affecting IHC detection threshold

- Biological variation
- Sample collection
- Fixation processing
- Section thickness
- Section pre-treatment (AR)
- Antibody type or clone
- Antibody dilution
- Antibody incubation time
- Antibody incubation temperature
- Sensitivity of detection kits
- Histochemical reaction

The greatest factor affecting IHC detection threshold is

Fixation and Processing

(90% of IHC staining problems)

Formalin Fixation – Processing Results in

- Variable morphology and cell content
- Variable shrinkage and Hardening
- Variable masking – destruction of epitopes
- Variable porosity
- Variable basophilic – acidophilic reactions
- Variable intensity of stain

Can lead to variable success – failure of IHC techniques

Rapidly Changing Technology

- One step direct conjugate method
- Multi step detection PAP (Peroxidiase Antiperoxidiase)
- Avidin biotin conjugate ABC
- Biotin – Steptatavidin (B-SA)
- Almpification – Tyramide
- Polymer based Labelling

These systems give greater sensitivity when combined with sensitive Antibodies

Polyconal Rabbit

Monoclonal Mouse

Rabbit Monoclonals

IHC is the future

- IHC has revolutionized the classification and diagnosis of tumors.
- No longer are we dependent on histology alone or on special stains.

Having just said that

- The most special stain/test still done today is a well fixed, well processed, well sectioned, thin, no knife tracks, H&E or (PAS) stained section to be viewed under the light microscope.

Basics

How to use IHC

- Morphology decides “what is the lesion”
- Benign vs. Malignant
- Differential diagnosis (DDX)
- IHC provides lineage confirmation
- IHC standard of care in surgical pathology
- Benign cells have characteristics distribution of protein epitopes which tipically/usually are carried with them into neoplasia.
- Rules and guidelines have been developed
- Sometimes tumors do not by the rules

Morphologically similar tumors are not necessarily antigenically identical

Sometimes tumors do not play by the rules

Sometimes tumors do not read the literature

IHC should confirm what you already know taking into consideration the patient history, H&E stain, along with special stains.

IHC is only one piece of the puzzle

10 Commandments of IHC

- 1. Start with a reasonable differential diagnosis (DDX)
- 2. Never use 1 antibody in a DDX
- 3. Use a panel
- 4. Sensitivity must be high
- 5. Use monoclonal antibodies when possible
- 6. Control should match slide
- 7. Use your own database
- 8. If results are weird investigate further
- 9. If a beginner or a problem arises GET HELP
- 10 Send uncommon/rare cases to an expert

Summary

Points to Ponder

IHC is a fantastic tool to aid in the diagnostic process. When used in conjunction with H & E Morphology, patient history, to confirm what you already know.