CONTROLS IN DIAGNOSTIC IMMUNOHISTOCHEMISTRY (dIHC)

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CONTROL! CONTROL!
YOU MUST LEARN CONTROL!
ISSUES ADDRESSED

• Why use controls?
• What type of controls?
• What tissues are to be used?
• How to interpret & Trouble shooting with controls
• How to monitor performance of controls
• Standardisation of controls
• How to prepare control blocks?
• Controls in manual & automated system
• Chart of Negative and positive controls
• References
Why use controls?

To ensure

1. The antibody stains the right antigen - SPECIFICITY
2. The antibody stains at the required level of intensity - SENSITIVITY
3. The performance characteristics of the antibody are optimum for use in clinical diagnosis
4. Consistency (reproducibility) of results
5. Exclude artifacts introduced in the protocol
6. Control variation in conditions of the test environment
7. Control variation of parameters of the tissue tested

RESULTS ARE ACCURATE AND VALID
REPORTS HAVE CLINICAL RELEVANCE
Controls check on

- Less successful or too dilute antibody /RTU
- Insufficient epitope retrieval
- Insensitive detection systems
- Platform problems

Provided a validated and optimised protocol is used
SENSITIVITY - AE1/AE3
SPECIFICITY – CD15
Eg. CEA – Non specific staining
Types of controls

1. Antigen (tissue controls)
   - Positive controls
   - Negative controls
     - Negative antigen control
     - Negative reagent control
   - Endogenous tissue background controls

2. Antibody controls
   - Isotype controls
   - Absorption controls

3. Internal controls / External controls

4. Batch controls / Onslide controls
Positive controls

• Use tissues expressing the antigen of interest and that have been previously tested for the same antigen
• Confirms the presence of the antigen
• Demonstrate (validate) that the protocol is able to detect the antigen of interest
• Sensitive at the level of clinical relevance
• A range of controls must be used from weak to strong staining
• Three tier system of sensitivity
• Weak staining control is necessary to differentiate from negative staining and will help to prevent false positives
Negative controls

1. Negative tissue control
   • Tissue - without the antigen of interest and subjected to the same protocol
   • Demonstrates that the IHC test has specificity
   • Identify non specific staining and false positive staining reactions
   • Non specific staining - staining of normal or abnormal cells that are not part of the intended use
EMA – Smooth Muscle
• False positive staining
  - Specific
    Cellular site expected to stain negative BUT stains positive
    Eg. ER negative tumour
  - Pseudo specific
    Cellular site expected to stain negative stains positive
    Eg. Synaptophysin
• If there is a positive staining in the negative control the patient result is invalid
2. Negative reagent control

- Tissue containing the antigen of interest with out application of primary antibody

- Evaluate non specific staining / non epitope binding of all of the other components used in the protocol

- Confirms lack of cross reactivity of secondary antibody with antigen
Eg. Neprilysin CD10 negative reagent control
Synaptophysin
Labelled Streptavidin-Biotin system

No antibody
Labelled Streptavidin-Biotin system:
*Neg. reagent control mandatory*

Synaptophysin
Polymer based system
Endogenous control

• Endogenous peroxidases and proteins may give rise to background staining
• Binding may occur due to hydropic interactions, ionic interactions and hydrogen bonding
• Kidney, liver, vascular areas red blood cells, lysosomal membranes
• Affects the chromogenic detection step
• Block with hydrogen peroxide
• Examine tissue under LM prior to commencement for any chromogenic signal
Endogenous control Eg.

FOX A2 Blocked & unblocked with goat serum
Isotype control

• Used to confirm the specificity of the primary antibody

• Primary antibody is replaced with non immunogenic immunoglobulin of the same isotype. Eg. IgG1, IgG2a, IgG2B, IgM

• Demonstrate if the primary antibody is interacting with the antigen binding site – paratope or if due to a non specific interaction with the immunoglobulin molecule
Absorption control

• Check if the primary antibody binds the specific epitope of the antigen of interest

• Primary antibody is first inactivated by preincubation with the immunogen (purified peptide parts) and then used in place of the primary antibody.

• Antigen : antibody ratio 10:1 to fully saturate the antibody and incubate at 4 °C
Internal tissue controls

• Intrinsic or built in controls use tissue elements in patient samples that is evaluated for the expression or absence of certain epitopes
• Internal control evaluate the preanalytical and analytical phase
• To be used in conjunction with external controls
• Not to be used in isolation
External controls

• External controls are recommended over internal controls.
• External control can be run with every batch.
• On-slide external controls are recommended.
• IC and EC together can provide useful information.
Positive on slide internal and external control
Selection of tissues for controls

- Use normal tissue as far as possible
- Appendix, tonsil, liver, pancreas
- High expressers – appropriate antibody
- Low expressers - appropriate sensitivity
- Non expresser – appropriate specificity
- Tissue with only high expressers will not detect - a poorly calibrated assay
  - Reduced number of antibodies in a optimally calibrated assay
Standardisation of control tissues

• Uncontrollable variables in the analytical phase
  - Sample preparation
  - Section thickness
  - Variability of protocol in different environment
  - Variability in chromogen
  - Tissue heterogeneity
  - Variation in pathology reporting

• ICAP – IHC critical assay performance indicators
Standardization of Negative Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Panel

Emina E. Torlakovic, MD, PhD,* † † E. Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),§∥∥∥
John Garratt, RT,† † † Blake Gilks, MD, FRCPC,† † † Elizabeth Hyjek, MD, PhD,*
Merdol Ibrahim, PhD,† † † Rodney Miller, MD,‡ ‡ ‡ Soren Nielsen, HT, CT,§§∥∥∥
Eugen B. Petcu, MD, PhD,§ Paul E. Swanson, MD,¶∥∥ Clive R. Taylor, MD, PhD,‡ ‡ ‡
and Mogens Vyberg, MD§§∥∥∥ AIMM 2014, 22:241

Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

Emina E. Torlakovic, MD, PhD,* † Soren Nielsen, HT, CT,‡ ‡ ‡ Glenn Francis, MBBS, FRCPA,
MBA, FFSc (RCPA),∥∥⊥∥∥ John Garratt, RT,† † † Blake Gilks, MD, FRCPC,† † †
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Paul E. Swanson, MD,¶∥∥∥ Xiaoge Zhou, MD,*** † † † Clive R. Taylor, MD, PhD,‡ ‡ ‡ ‡
and Mogens Vyberg, MD§§∥∥∥ AIMM 2015, 23:1
Interpretation - Invalid assay

- Inappropriate positivity
- Inappropriate negativity
- Non specific positivity
  - RBC
  - All pigments
  - Plasma
  - Necrotic material
  - Others
Table 5  Summary of required characteristics of any reference standard that would provide a basis for accurate quantification of IHC on FFPE tissue

<table>
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<tr>
<th>Immunohistochemical reference standard: requirements</th>
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<tr>
<td>It must be subjected to all of the same rigors of sample preparation (ischemia, transport, fixation) as the “test” tissue</td>
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<tr>
<td>It must be integrated into all steps of the test (assay) protocol, including evaluation of the result</td>
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<td>It should contain a known amount of the reference standard protein</td>
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<td>It should be universally available to all laboratories performing the assay</td>
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<tr>
<td>It should be inexhaustible and inexpensive</td>
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Taylor CR. *Quantitative In Situ Proteomics--*Cell Tissue Res. 2015; 360:109-120.
Potential new controls

• Cell culture lines controls
• Bioengineered control tissue have known and quantified levels of protein expression
• 3D Faux tissue controls
How to prepare control blocks

USE OF MICROARRAY TISSUE TECHNIQUE FOR ANTIBODY VALIDATION IN IMMUNOHISTOCHEMISTRY
Sosai CSP, Mendis R, Adikaram L, Pathirana A, Kodithuwakku C, Meedeniya N, Lokuhetty MDS
Lanka Hospitals Diagnostics, Colombo, Sri Lanka
Controls in manual vs automated
Automated platforms

• Superior to manual staining
• Use of robotic arms has improved time based reagent deposition compared to manual
• Consistency of staining
• Standardization of procedure
• 30% cold areas
• Biomolecule degradation / slide oxidation – Reduced staining intensity can occur
Conclusion

• The IHC test must be validated for the protocol and platform
• In practice use positive and negative controls
• Use reagent negative control if using ABC/LSBC
• Use three tier expressers so you don’t mis the weak expressers
• Use www.
Recommended Antibody Resource
Web Sites

• http://www.proteinatlas.org (last accessed May 16, 2016)
• http://www.antibodyresource.com (Cambridgeshire, UK; last accessed May 16, 2016)
• http://www.antibodyregistry.org (last accessed May 16, 2016)
• http://www.biocompare.com/antibodies (South San Francisco, California; last accessed May 16, 2016)
• http://www.antibodypedia.com (Solna, Sweden; last accessed May 16, 2016)
Control tissues

- Immunohistochemistry Positive Control Tissue List - IHC World
- www.Nordicqc.org

2. Novus biologicals.novusbio.com
3. Literature Review and Geisinger Experience. Standardization of Diagnostic Immunohistochemistry
