

Specificity and standardization of immunohistology

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The extraordinary specificity of the antigen-antibody reaction has led to the development of immunohistology for the localization of a wide variety of molecules in cells and tissues. As a matter of fact, there exist no single and defined protocol of cellular immunolabeling. In contrary, protocols vary largely due to the experimental goals and the technical possibilities. Fundamental immunohistologists are aware that careful attention has to be paid to all preparative steps because the success of an immunostaining experiment depends on both the quality of the employed reagents and the appropriate histological specimens. Even if all reagents have been optimally prepared, cellular immunostaining may be confusing, mainly due to destroyed antigenic epitopes in the course of histological specimen preparation, but also due to the fact that cells of a certain histogenesis will not stably express antigens at any time.

The preparation of highly specific immune sera (and very often the use of purified antibodies) is a *conditio sine qua non* in immunohistology. Apart from contaminating antibodies (elicited by impurities of the antigen preparation used for immunization), heterophile antibodies, naturally occurring antibodies and cross-reactivity can be a major problem of the method. Antibody synthesis may be induced non-specifically, i.e. antibodies directed against a defined antigen arise following exposure to an unrelated antigen. Epstein-Barr virus infection, for example, results in polyclonal B cell stimulation and a large repertoire of antibodies including heterophile antibodies. Heterophile antibodies are often of unknown origin and arise as multispecific antibodies during the early immune response.

In theory, antibodies are highly specific, and for each antigen there is a corresponding antibody. A given antibody, however, may cross-react with a second (unrelated) antigen. This occurs when two different antigens share an identical epitope or when antibodies specific for one epitope bind an unrelated epitope possessing similar chemical and structural properties. For example, cross-reactivity is the basis for the presence of natural blood group antibodies (isoagglutinins) induced in an individual by exposure to cross-reacting microbial antigens present on intestinal bacteria; cross-reacting antigens induce the formation of antibodies in individuals lacking similar antigens on their red cells.

The challenge of specificity and reproducibility

From the beginning of selective cell labeling by use of fluorochrome conjugated antibodies until today with all the further developments in immunohistology, histologists are called for a number of control reactions to prove the specificity of their staining reactions. This very special technology with its great opportunities is technically complex. For many reasons, immunohistological reagents have been prepared by the user himself, so he was almost able to reflect critically his results. Now, with the vast number of antibodies and detection methods

being commercially available and with the demand for ready-to-use reagents, the behaviour in research or diagnostic laboratories has changed considerably.

Since our early studies with immunofluorescence (more than 30 years ago) which was followed by many different immunoenzyme techniques for research and patient diagnostics, pitfalls in immunostaining are well known to us. Reagent preparation is always part of special know-how and sophisticated laboratory work. Today, histologists (mainly in surgical pathology) are less familiar with preparative and analytical immunochemistry. This is linked with considerable drawbacks: due to the lack of standardized products and procedures in applied immunohistology, reproducibility of results and among different laboratories is open to questions. This makes reporting and interpretation of immunohistological findings sometimes doubtful.

No aspect of the immunohistological technique has to be ignored, i.e. from the moment of reagent preparation to specimen collection until the final microscopic work (KUHLMANN WD et al. 1970; KUHLMANN WD et al. 1974). The multiple facets of reagent preparation, tissue sampling and the selection of histochemical staining methods to approach adequate specificity were treated in some detail (KUHLMANN WD 1977; KUHLMANN WD 1984). Furthermore, in all chapters of this website dealing with both reagent preparation and tissue sampling, the importance of controls is stressed.

Since immunohistology can detect markers of tissue specificity and specific markers of cell-type differentiation as well reflecting histogenesis and, also, the expression of disease related functional proteins, this technique has become a valuable tool to assess protein expression. Potentially, this technique has the power to provide diagnostic, therapeutic and prognostic information for personalized medicine. For confidence in immunohistochemistry it is necessary to perform appropriate quality controls. This is quite well accepted (Standards NCCLS, 1997; MAXWELL P and MCCLUGGAGE WG, 2000; O'LEARY TJ, 2001; PACKEISEN J et al., 2002). Yet, immunohistology still lacks of standardization; the methods are dominated by a range of poorly controlled variables. Consequently, disparate results in literature regarding the relationship between biomarker expression and patient outcome are to be expected which decrease the credibility of many studies (MCCABE A et al. 2005).

Efforts in standardization

With all the newer developments and innovations in the last ten years, immunohistology has become more and more common in histopathology. In practice, however, immunohistological reagents are often applied just as "special" stains (much comparable to classical histological stains). Because histologists are more interested in morphology and not so in adequate care of the reagents by avoiding the rigors of quality assurance, extensive quality control procedures, reagent validation and documentation as is conventional in the clinical laboratory are not applied. Consequently, a high level of disagreement of histopathologic diagnoses may be found in observer studies.

In the 1990's, problems of quality in diagnostic immunohistochemistry became obvious. The status of quality assurance, quality control and standardization in immunohistochemistry was reviewed by the Biological Stain Commission (BSC). An *Immunohistochemistry Steering Committee* (IHSC) was established, and a strategic plan was presented by the BSC. In the meantime, several reports and approaches to standardization have been published (TAYLOR CR 1992; TAYLOR CR 1992; TAYLOR CR 1994; TAYLOR CR 1998; O'LEARY TJ et al., 1999) TAYLOR CR 2000; O'LEARY TJ, 2001; SHI SR et al. 2005; SHI SR et al. 2007; TAYLOR CR

and SHI SR, 2008). Reading of these papers is strongly recommended to all those who are involved in immunohistology.

The BSC *Quality Assurance and Standardization Program for Immunohistochemistry* is very ambitious. Despite of some progress, the whole project is still in development. Three primary divisions have been made: (a) “Antibodies and Reagents”; (b) “Technical Procedures”; and (c) “Interpretation and Reports”. Even if this division is quite arbitrarily (recognizing that these three components of immunohistology are intrinsically linked together), a degree of separate analysis appears necessary for practical reasons. Finally, these jobs of standardization have an incremental approach in three phases (TAYLOR CR 1992; TAYLOR CR 1994)

- **Phase I, guidelines and standards**

- *Antibodies and reagents*: standardize specifications provided by the manufacturer (package insert)
- *Technical procedures*: establish general guidelines for all aspects of the immunohistochemical procedure, including minimum required controls
- *Interpretation and reports*: develop guidelines for format and minimal of immunohistochemical report

- **Phase II, testing and proficiency programs**

- *Antibodies and reagents*: develop uniform internal testing programs for all reagents by manufacturer: utilizing standard test substrate (e.g., multiple tissue block)
- *Technical procedures*: expand existing proficiency testing programs: design to test effectiveness of stain protocol in use in the lab, using standard test substrate
- *Interpretation and reports*: expand existing proficiency testing program: design to test interpretative abilities, using prestained cases on Kodachromes

- **Phase III, external reference testing laboratory; qualifications**

- *Antibodies and reagents*: develop external testing program by “reference Laboratory” allowing submission of reagents for certification
- *Technical procedures*: qualifications/training program for histotechnologists and immunohistochemistry lab supervisors
- *Interpretation and reports*: qualifications/experience/lab utilization levels for pathologists

In 1998, the Food and Drug Administration of the United States (FDA) issued the rule for classification of immunohistological reagents and kits (Code of Federal Regulation, Food and Drug Administration, Medical Devices, 1998; Classification/Reclassification of Immunohistochemistry Reagents and Kits (21CFR part 864. Doc. No. 94P-0341), Federal Register 63: 30132-30142).

With respect to this FDA rule, immunohistological stains will fall into three classes

- **Class I**: these stains are recognized as “special” stains that are adjuncts to conventional histopathological examination. Stains used by pathologists in the classification of neoplasms are in this category
- **Class II**: those immunohistological stains that are not directly confirmed by routine histopathologic controls (and have no morphologic correlates), but do have accepted scientific validation are class II; examples cited include ER/PR (estrogen and progesteron receptors)

- **Classe III:** Those remaining immunohistological stains that are “new” or do not have acceptable scientific validation are class III

For detailed commentary see CR TAYLOR 1998, *Biotech Histochem* 73, 175-177, *Report from the Biological Stain Commission: FDA Issues Final Rule for Classification/Reclassification of Immunohistochemistry (IHC) Reagents and Kits.*

Validation of staining procedure

The goal to standardize immunohistochemistry includes a number of elements. A single procedure does not exist because of the complexity of the technique with its different staining approaches and the multiple reaction steps. When the histologist has established his protocol, the whole procedure is validated each time including “positive” and “negative” controls with anticipated patterns, f.e. by the use of tissue microarrays. New reagents should be always compared with those which are in actual use and which work successfully.

Laboratories have to control and confirm that the reagents (purchased or made by themselves) perform as supposed, e.g.

- **Antibodies:**
 - Primary antibodies
 - Linking/bridge antibodies
- **Labeled reagents:**
 - ABC reagents, PAP complexes, avidin/streptavidin
- **Substrates and buffers:**
 - Chromogens, other substrate reagents and buffers including those for washing and dilution
- **Other components:**
 - Blocking proteins
 - Counterstains

Apart from the employed reagents, all histotechnical procedures need also careful controls. Such controls include at least the following preparation steps,

- **Tissue processing:**
 - Tissue sampling
 - Fixation, dehydration
 - Embedding (paraffin etc.)
- **Antigen retrieval:**
 - Retrieval buffers
 - Retrieval procedures (HIER, PIER)
- **Blocking of background:**
 - Inhibition of endogenous enzymes
 - Inhibition of background due to interaction of reagents with tissue
- **Choice of immunostaining procedure:**
 - Conjugated antibodies vs. unconjugated methods
 - Avidin-biotin methods, PAP, others
- **Chromogens, substrates:**
 - Selection according to special needs
 - Enhancing steps

- **Counterstaining, mounting:**
 - Selection of appropriate dye
 - Staining procedure
 - Dehydration and mounting in resinous medium or use of an alternative method

Daily quality materials should include “positive” and “negative” controls to provide informations on specificity and performance of the selected immunostaining procedure

- **Positive control (for antibody specificity) with known expected result:**
 - Non-patient tissue containing the antigen to be detected in order to control the antibodies in use and to control the incubation procedures. It is necessary to use tissues being fixed and embedded as the patient samples
- **Negative control (for antibody specificity) to detect cross-reactivity:**
 - Tissues expected to be negative for the antibodies in use; tissues must be processed in the same way as the patient sample
- **Negative control (for non-specificity) to detect background reactivity:**
 - Tissue of patient is reacted with unrelated antibodies; incubations are done with diluents and all subsequent reagents of the selected immunostaining procedure

The above notions reflect general rules and protocols. Some experience and practical steps can be directly derived from many publications (f.e. MAYERSBACH H, 1959; KUNKEL HG et al., 1963; NOVIKOFF AB et al., 1972; PETRUSZ P et al., 1976; KUHLMANN WD, 1977; WOOD GS and WARNKE R, 1981; PETRUSZ P, 1983; KUHLMANN WD, 1984 STRAUS W, 1987; ELIAS JM et al., 1989; AREVALO JH et al., 1993; GRUBOR NM et al., 2005). Application details with relevant references are given in chapter *Reagents*.

Selected publications for further readings

Mayersbach H von (1959)
 Kunkel HG *et al.* (1963)
 Kuhlmann WD *et al.* (1970)
 Novikoff AB *et al.* (1972)
 Kuhlmann WD *et al.* (1974)
 Petrusz P *et al.* (1976)
 Kuhlmann WD (1977)
 Wood GS and Warnke R (1981)
 Petrusz P (1983)
 Kuhlmann WD (1984)
 Straus W (1987)
 Elias JM *et al.* (1989a)
 Taylor CR (1992a, 1992b)
 Arevalo JH *et al.* (1993)
 Taylor CR (1994)
 Taylor CR (1998)
 O’Leary TJ *et al.* (1999)
 Maxwell P and McCluggage WG (2000)
 Taylor CR (2000)
 O’Leary TJ (2001)
 Packeisen J *et al.* (2002)
 Grubor NM *et al.* (2005)

McCabe *et al.* (2005)
Shi SR *et al.* (2005)
Shi SR *et al.* (2007)
Taylor CR and Shi SR (2008)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>