BAOJ Cancer Research & Therapy

Amadeu Borges-Ferro, BAOJ Cancer Res Ther 2016, 2: 5
2: 022

Editorial

# Oncology Research and Clinical Decisions Based on Immunohistochemistry: The Importance of the Technical Aspects That Support a Complex Method

**Amadeu Borges-Ferro***

*Lisbon Health School – Polytechnic Institute of Lisbon; Faculty of Medicine – University of Lisbon , Portugal*

Immunohistochemistry (IHC) is the group of techniques that use antibodies as specific reagents to identify and demonstrate several cell and tissue components that are antigens. This linking allows locating and identifying the in situ presence of various substances by means of color that is associated with the formed antigen-antibody complexes.

The practical value of this biotechnology area, widely used in Pathology and Oncology, in diagnostic, prognostic, theranostic and research context, results from the possibility of combining a colour marker with an antibody without causing any damage to specific binding established between antibody and antigen. This provides the microscopic observation of the target locations where the antibody and hence the antigen are present [1].

IHC is presented as a powerful means for identification of several cellular and tissue structures that can be associated with pathologies, and of the consequences, at functional and morphological level, of these same elements action.

In the last twenty years, there has been a progressive development of IHC, leading to an improvement in these methodologies for antigen demonstration in formaldehyde fixed, paraffin embedded tissues, which has contributed significantly for the diagnosis, prognosis and therapeutic guidance of several pathologies [2,3].

Nevertheless, some difficulties associated with the particularities of certain pathologies and technical limitations remain. Furthermore, the standardization of IHC has been a hard task to complete. The quality of the immunostaining depends on four main technical aspects [4].

i. Plan the full process in advance;

ii. Pre-analytical factors (emphasis on tissue fixation and processing);

iii. Antigen retrieval (AR)

iv. Sensitivity of the detection system.

Therefore, when using IHC techniques in research or clinical context, these technical aspects must be considered and addressed.

## Plan the Full Process in Advance

The planning phase must entail the evaluation of all human, physical and material resources. The right procedures for all test stages, from sample collection to the final report as well as the use of registration, monitoring and traceability methods must be in place. All associated personnel (e.g. surgeons, nurses, general operations workers, oncologists, pathologists and biomedical scientists/technologists) must be trained and competent [5]. A full validation process must be performed for each test result and quality assurance procedures must be in place throughout all technical aspects e.g. quality control of reagents namely fixative solutions, dehydrating and impregnation agents and IHC reagents. Finally, all procedures must be reviewed and updated regularly in order to keep up with the most recent advances in the field.

## Pre-analytical phase

The pre-analytical phase starts with the sample collection from the patient. Date and time of sample collection should be registered(start of cold ischemia time). All tissues must be immersed on an aqueous solution of 4% formaldehyde (10% formalin) phosphate buffered at pH 7.0 with a minimal volume of 4 times the dimensions of the tissue[6–10]. The cold ischemia time should be minimized and may extend to a maximum of 60 minutes [10–14]. Larger surgical specimens should be readily sliced and then immersed in fixative solution, for at least 24 hours with a temperature of 22 °C - 26 °C and should not exceed 96 hour of fixation time [6,7,10,12,15–17]. When grossing the specimens is not immediately possible, samples should be chilled to 4 °C, for no longer than 4 hours [10,12,15]. Samples transportation to the pathology laboratory must be done as soon as possible, under controlled conditions, respecting all safety standards for biological samples [6–10]. The surgical specimens that arrive at the laboratory not sliced and fixed, should be properly and immediately sliced and emerged in fixative solution according to the conditions set out above. The tissue processing should be automated and the reagents quality must be monitored taking into

***Corresponding author:** Amadeu Borges-Ferro, ESTeSL - Escola Superior de Tecnologia da Saúde de Lisboa, Av. D. João II, Lote 4.69.01, 1990 - 096 Lisbon, Portugal, E-mail: amadeu.ferro@estesl.ipl.pt

Rec Date: March 20, 2016, Acc Date: March 28, 2016, Pub Date: March 30, 2016.

**Citation:** Amadeu Borges-Ferro (2016) Oncology Research and Clinical Decisions Based on Immunohistochemistry: The Importance of the Technical Aspects That Support a Complex Method. BAOJ Cancer Res Ther 2: 022.

consideration the amount and type of samples that go through them [10,17]. When biological tissues must remain in the tissue processor for more than 24 hours (public holidays or weekends) a delayed start with samples in fixative (first processing station) should be performed [10,17.] The embedding of samples must be done swiftly and the paraffin blocks must be stored at 20 °C - 25 °C [7,10]. The microtomy conditions should always be kept similar for all samples. Section thickness should be 2-4μm and extension must be performed in water with controlled conductivity (e.g. distilled water). The water bath temperature must be constantly monitored at 50 °C maximum. Adhesion time and temperature must be constant, being advisable a temperature not exceeding 65 °C [10,18]. Sections for IHC, ideally, should be cut and stained as soon as possible – it is known that histological sections with more than 6 weeks can lead to antigenicity change[6,7,9,10,18–21].

## Antigen Retrieval

For the vast majority of antigens, formalin fixation will lead to a reduction of their functional affinity for the antibody (avidity) or even completely obliterate this affinity. A measurable effect that formalin fixation has on most tissue antigens is either reduced or absent immunostaining. AR, is the term used to describe the restoration of the antigen's optimal immune reactivity after formalin fixation and, frequently, consists in high temperature heating of histological sections [22,23]. In 1945, Fraenkel-Conrat and colleagues [24] conducted several biochemical studies on the interactions between formaldehyde and proteins, demonstrating that the established cross links could be destroyed by heating to high temperatures or using strong alkaline solutions. Much later, in 1991, Shi and colleagues discovered that submitting IHC slides to high temperature when immersed in specific solutions was a very important and effective step. These researchers demonstrated the great benefits of this treatment on many antigens immunostaining, which was particularly intriguing taking into account the presumed deleterious effects of high temperatures on protein antigens [25].

Although it is known that proteins denature between 70 °C and 90 °C, it appears that fixed tissues resist denaturing at these temperatures [26]. Many other researchers were able to demonstrate this phenomenon, in particular Kawai et al. They concluded that 90 °C for 10 minutes AR is more effective than 60 °C for 120 minutes [27]. In 1993, Cattoretti and collaborators, confirmed the effectiveness of this procedure and suggested alternatives to the heavy metals AR solution used previously [28]. Since then, the pH 6.0 0.01M citrate buffer became one of the most widely used AR solutions. However, later it was found there is not one AR solution that fits universally all types of antigen. Thus, several antibodies should be tested until the AR optimal conditions are found. Other very important factor is the solutions pH [29]. Some researchers have even found the pH value of an AR solution is more important than their chemical composition, particularly for nuclear and cell membrane antigens [29]. The presence of chelating agents, that promote the extraction of calcium ions affect AR and some antigens are more prone to react with certain chelating agents, usually ethylenediamine tetraacetic acid (EDTA), amongst others [30,31].

Several high temperature methods were tested such as microwave oven [25], autoclave [32–35], pressure cooker [36,37], water bath [27] and hot steam [38,39].

The AR success demonstrated that the modified protein structure induced by formaldehyde is a reversible process and that the proteins retain their primary structure.

The introduction of AR methods was undoubtedly one of the major advances in IHC – before its implementation only a small percentage of antigens could effectively be detected.

## Sensitivity of the detection system

Antibodies are proteins and, therefore, they are not naturally stained. So, it is necessary to make them microscopically visible when they are linked to the antigen. In 1941, Albert Coons and his collaborators conjugated a fluorescent marker to the antibody and this allowed the identification of the antigen [40]. However, at the time, the ratio of marker per tissue antigen was very low, and amplification was poor in most cases. The false negative rate was, then, high, and this narrowed the viability of IHC to exceptional and sporadic situations.

The ultimate goal has been, ever since, to create a stable staining method with enough intensity to eliminate doubt about the presence or absence of the antigen in the tissue [1]. Over the years abundant ways to increase the signal that is associated with tissue antigen have been developed–e.g. Simple indirect method, PAP method, avidin-biotin methods [41–46]. Some of these methods were never widely used, some others were often used back then and barely used currently [47].

Today there is a simple method that offers a higher amplification: the indirect polymer method [48,49]. The use of polymers in IHC brought 4 major advantages: higher amplification rate, lower error rate, faster turnaround time and simplicity of the method [50,51]. These systems do not have (strept)avidin or biotin and therefore the use of blocking reagents is unnecessary. The major disadvantage is its higher cost which, can be seen as a "minor" factor given its benefits [52].

Currently, there are several IHC methods that use polymers with a high signal amplification, namely the internal skeleton polymer and the enzymes micropolymer.

The internal skeleton polymer relies on a macromolecule consisting of a large central skeleton to which are attached large amounts of antibody molecules and enzymes, like horseradish peroxidase (HRP) [49]. The central skeleton molecule is dextran, a high molecular weight polysaccharide that can reach 500 kDa. Dextrans consist of a sequences of α-D-glucose units predominately linked by α-1,6 glycosidic bonds. They are manufactured from sucrose during growth of bacteria belonging to the Leuconostoc, Streptococcus and Lactobacillusgenera, all belonging to the Lactobacillaceafamily. However, most of the dextrans are synthesized by the bacterium Leuconostoc mesenteroides species. The dextran has a neutral pH, it is soluble in water and it is highly stable.

The enzymes micropolymers methods are based on the enzyme

polymerization and its association to antibodies, forming enzyme micropolymers. According to its manufacturers this approach avoids the problems arising from the use of an internal skeleton. The micropolymer, with a high density of very active enzyme, coupled to a secondary antibody generates a reagent which overcomes the steric interference that emerge from the huge dimensions occupied by the internal skeleton polymer. This method provides greater accessibility to the antigen and, due to the micropolymer's small size, it increases reagents-diffusion to the target and reduces non-specific binding.

## Conclusion

Oncology research and clinical decisions are largely based on IHC results. Considering all the multifactorial aspects that can bring bias to these results, it is essential that the IHC Laboratory procedural proficiency is established. Basic standardization and reproducibility of all methodologies, including internal and external quality control measures must be regularly applied to provide slides of diagnostic quality. The participation in IHC external quality assessments using consensual schemes such as UKNEQAS (http://www.ukneqasiccish.org/) and others, is of utter importance to measure effectiveness.

## References

1. Polak JM, Van Noorden S (2003) Introduction to Immunocytochemistry. Oxford: BIOS Scientific Publishers.

2. Swanson PE (2015) Immunohistochemistry as a surrogate for molecular testing: a review. 23(2): 81-96.

3. Werner M (1999) Tissue fixation and antigen retrieval. Rev Esp Patol 32(3): 355-361.

4. Ramos-Vara JA (2005) Technical Aspects of Immunohistochemistry. Vet Pathol Online 42(4): 405-426.

5. Arafah M, Kfoury HK, Zaidi SN (2010) HER2/neu Immunostaining in Invasive Breast Cancer: Analysis of False Positive Factors. Oman Med J 25(4): 261-263.

6. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, et al. (2013) Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. J Clin Oncol 31(31): 3997-4013.

7. Albanell J, Andreu X, Calasanz MJ, Concha A, Corominas JM, et al. (2009) Guidelines for HER2 testing in breast cancer: a national consensus of the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM). Clin Transl Oncol Off Publ Fed Span Oncol Soc Natl Cancer Inst Mex 11(6): 363-375.

8. Goldstein NS, Hewitt SM, Taylor CR, Yaziji H, Hicks DG, et al. (2007) Recommendations for improved standardization of immunohistochemistry. Appl Immunohistochem Mol Morphol AIMM Off Publ Soc Appl Immunohistochem 15(2): 124-133.

9. Gomez-Martin C, Concha A, Corominas JM, Garcia-Caballero T, Garcia-Garcia E, et al. (2011) Consensus of the Spanish Society of Medical Oncology (SEOM) and Spanish Society of Pathology (SEAP) for HER2 testing in gastric carcinoma. Clin Transl Oncol Off Publ Fed Span Oncol Soc Natl Cancer Inst Mex 13(9): 636-651.

10. Maia-Matos M, Ferro A, Teixeira A, Cristina Gavinho, Ana Cunha, et al. Consenso Tecnico para a determinacao do status da proteina HER2 por imunocitoquímica em carcinoma da mama. Saude Tecnol (#T1): 71-82.

11. Hammond MEH, Hayes DF, Dowsett M, Allred DC, Hagerty KL, et al.(2010) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med 134(7): e48-e72.

12. Yildiz-Aktas IZ, Dabbs DJ, Bhargava R (2012) The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. Mod Pathol Off J U S Can Acad Pathol Inc 25(8): 1098-1105.

13. Khoury T (2012) Delay to formalin fixation alters morphology and immunohistochemistry for breast carcinoma. Appl Immunohistochem Mol Morphol AIMM Off Publ Soc Appl Immunohistochem 20(6): 531-542.

14. Moatamed NA, Nanjangud G, Pucci R, Lowe A, Shintaku IP, et al.(2011) Effect of ischemic time, fixation time, and fixative type on HER2/neu immunohistochemical and fluorescence in situ hybridization results in breast cancer. Am J Clin Pathol 136(5): 754-761.

15. Perry N, Broeders M, de Wolf C, Tornberg S, Holland R, et al. (2008) European guidelines for quality assurance in breast cancer screening and diagnosis. Fourth edition--summary document. Ann Oncol Off J Eur Soc Med Oncol ESMO19(4): 614-622.

16. Tong LC, Nelson N, Tsourigiannis J, Mulligan AM (2011) The effect of prolonged fixation on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast cancer: a prospective study. Am J Surg Pathol 35(4): 545-552.

17. Yildiz-Aktas IZ, Dabbs DJ, Cooper KL, Chivukula M, McManus K, et al.(2012) The effect of 96-hour formalin fixation on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. Am J Clin Pathol s137(5): 691-698.

18. Thurby C, White MR, Simutis F, Gillham S, Slinker D, et al. (2009) An Improved Method of Tissue Adhesion on Glass Microslides for Immunohistochemical Evaluation by the Use of Selected Tissues from the Dog and Monkey. J Histotechnol 32(4): 198-201.

19. Henson DE (1996) Loss of p53-immunostaining intensity in breast cancer. J Natl Cancer Inst 88(15): 1015-1016.

20. Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ (1996) Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. J Natl Cancer Inst 88(15): 1054-1059.

21. Manne U, Myers RB, Srivastava S, Grizzle WE (1997) Re: loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. J Natl Cancer Inst 89(8): 585-586.

22. Boenisch T (2006) Heat-induced Antigen Retrieval: What Are We Retrieving? J Histochem Cytochem. 2006;54(9): 961-964.

23. Shi S-R, Cote RJ, Taylor CR (2001) Antigen Retrieval Techniques Current Perspectives. J Histochem Cytochem 49(8): 931-937.

24. Fraenkel-Conrat H, Cooper M, Olcott HS (1945) The Reaction of Formaldehyde with Proteins. J Am Chem Soc 67(6): 950-954.

25. Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem Off J Histochem Soc 39(6): 741-748.

26. Leong ASY, Milios J, Leong FJWM (1996) Epitope retrieval with microwaves. A comparison of citrate buffer and EDTA with three commercial retrieval solutions. Appl Immunohistochem (4): 201-207.

27. Kawai K, Serizawa A, Hamana T, Tsutsumi Y (1994) Heat-induced antigen retrieval of proliferating cell nuclear antigen and p53 protein in formalin-fixed, paraffin-embedded sections. Pathol Int 44(10-11): 759-764.

28. Cattoretti G, Pileri S, Parravicini C, Becker MH, Poggi S, et al. (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J Pathol 171(2): 83-98.

29. Shi SR, Imam SA, Young L, Cote RJ, Taylor CR, et al. (1995) Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem Off J Histochem Soc 43(2): 193-201.

30. Morgan JM, Navabi H, Schmid KW, Jasani B (1994) Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. J Pathol 174(4): 301-307.

31. Morgan JM, Navabi H, Jasani B (1997) Role of Calcium chelation in high-temperature antigen retrieval at different pH values. J Pathol 182(2): 233-237.

32. Bankfalvi A, Navabi H, Bier B, Bocker W, Jasani B, et al.(1994) Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J Pathol.174(3): 223-228.

33. Igarashi H, Sugimura H, Maruyama K, Kitayama Y, Ohta I, et al. (1994) Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. APMIS 102(1-6): 295-307.

34. Umemura S, Kawai K, Osamura RY, Tsutsumi Y (1995) Antigen retrieval for bcl-2 protein in formalin-fixed, paraffin-embedded sections. Pathol Int 45(2): 103-107.

35. Pons C, Costa I, Von Schilling B, Matias-Guiu B, Prat J, et al. (1995) Antigen Retrieval by Wet Autoclaving for p53 Immunostaining. Appl Immunohistochem 3(4): 265-267.

36. Norton AJ, Jordan S, Yeomans P (1994) Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 173(4): 371-379.

37. Miller RT, Estran C (1995) Heat-induced epitope retrieval with a pressure cooker - suggestions for optimal use. Appl Immunohistochem 3: 190-193.

38. Pasha T, Montone K, Tomaszewski J (1995) Nuclear antigen retrieval utilizing steam heat. 72: 167A.

39. Taylor CR, Chen C, Shi SR, Yang C, Cote RJ, et al. (1995) A comparative study of antigen retrieval heating methods. CAP Today 9:16-22.

40. Coons AH, Creech HJ, Jones RN (1941) Immunological Properties of an Antibody Containing a Fluorescent Group. Exp Biol Med 47(2): 200-202.

41. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG (1970) The Unlabeled Antibody Enzyme Method of Immunohistochemistry Preparation and Properties of Soluble Antigen-Antibody Complex (horseradish Peroxidase-Antihorseradish Peroxidase) and Its Use in Identification of Spirochetes. J Histochem Cytochem 18(5): 315-333.

42. Scopsi L, Larsson LI (1986) Increased sensitivity in peroxidase immunocytochemistry. A comparative study of a number of peroxidase visualization methods employing a model system. Histochemistry 84(3): 221-230.

43. Guesdon JL, Ternynck T, Avrameas S, J C Hierholzer (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem Off J Histochem Soc 27(8): 1131-1139.

44. von Wasielewski R, Mengel M, Gignac S, Wilkens L, Werner M, et al. (1997) Tyramine amplification technique in routine immunohistochemistry. J Histochem Cytochem Off J Histochem Soc 45(11): 1455-1459.

45. Vyberg M, Nielsen S (1998) Dextran polymer conjugate two-step visualization system for immunohistochemistry. A comparison of EnVision+ with two three-step avidin-biotin techniques. Appl Immunohistochem 6(1): 3-10.

46. Heras A, Roach C, Key M (1995) Enhanced polymer detection system for immunohistochemistry.

47. Vosse BAH, Seelentag W, Bachmann A, Bosman FT, Yan P, et al. (2007) Background staining of visualization systems in immunohistochemistry: comparison of the Avidin-Biotin Complex system and the EnVision+ system. Appl Immunohistochem Mol Morphol 15(1): 103-107.

48. Wong SCC, Chan JKC, Lo ESF, Amanda KC, Manson CK et al. (2007) The contribution of bifunctional SkipDewax pretreatment solution, rabbit monoclonal antibodies, and polymer detection systems in immunohistochemistry. Arch Pathol Lab Med 131(7): 1047-1055.

49. Sabattini E, Bisgaard K, Ascani S, S Poggi, M Piccioli, et al. (1998)The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. J Clin Pathol 51(7): 506-511.

50. Petrosyan K, Tamayo R, Joseph D (2002) Sensitivity of a novel Biotin-free detection reagent (PowerVision+) for immunohistochemistry. J Histotechnol 25: 247-250.

51. Kammerer U, Kapp M, Gassel AM, Richter T, Tank C, et al. (2001) A new rapid immunohistochemical staining technique using the EnVision antibody complex. J Histochem Cytochem Off J Histochem Soc 49(5): 623-630.

52. Wiedorn KH, Goldmann T, Henne C, Kuhl H, Vollmer E, et al. (2001) EnVision, a new dextran polymer-based signal enhancement technique for in situ hybridization (ISH). J Histochem Cytochem Off J Histochem Soc 49(9): 1067-1071.