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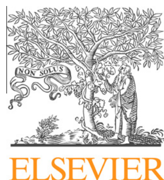

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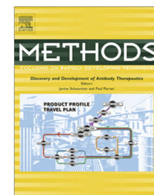

Antibody validation of immunohistochemistry for biomarker discovery: Recommendations of a consortium of academic and pharmaceutical based histopathology researchers

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This article is dedicated in the memory of Neil Gray and Chris van der Loos, both of which were trusted colleagues and friends in the histopathology community. They will be sadly missed. Neil died on 31 July 2012 and Chris on 26 Nov 2013.

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ABSTRACT

As biomarker discovery takes centre-stage, the role of immunohistochemistry within that process is increasing. At the same time, the number of antibodies being produced for "research use" continues to rise and it is important that antibodies to be used as biomarkers are validated for specificity and sensitivity before use. This guideline seeks to provide a stepwise approach for the validation of an antibody for immunohistochemical assays, reflecting the views of a consortium of academic and pharmaceutical based histopathology researchers. We propose that antibodies are placed into a tier system, level 1–3, based on evidence of their usage in immunohistochemistry, and that the degree of validation required is proportionate to their place on that tier.

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1. Introduction

The term "biomarker", in its broadest sense, defines any biological or physiological entity that is used to identify disease, guide targeted therapy or monitor for re-occurrence. In histopathology, immunohistochemistry (IHC) is routinely used for diagnosis [1] and with the advent of the patient selective cancer therapy trastuzamab for breast [2] and gastric cancer [3], or demonstration of C-kit for gastrointestinal stromal tumours targeted therapy [4], it is being used as a decision making tool to ascertain those patients who are most likely to benefit from treatment. Furthermore, the possibility of using antibodies to detect specific EGFR mutations as a guide for the administration of EGFR-targeted therapies in non-small cell lung cancer could result in IHC being used as a quick and cost effective replacement for the DNA sequencing based methods presently employed [5].

The development of an IHC biomarker can begin at the same time as the association of gene expression with a disease points toward development of a drug. Thus microarray data, next-generation sequencing and sometimes *in situ* hybridisation can provide the targets for biomarker selection and point to the need to either select or make an antibody to that target [6]. Whether 'home grown' or selected from existing commercial offerings it is of critical importance that the biomarker antibody is validated as specific for its target and of sufficient sensitivity to allow IHC demonstration over the required dynamic range demanded by the pathology it will be used to identify. The chief benefit of early validation is that the IHC based biomarker can be used with confidence during the drug development process to assist in understanding the target

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better, to segregate pathologies most likely to benefit from therapy and potentially to become the method by which this selection is made in the clinical setting. In the wider context of research pathology where IHC is frequently employed, thorough antibody validation will ensure that quality reagents are used. Regrettably, information supplied in many academic publications [7] and contained in commercial data sheets is not sufficient to allow confidence to be built into an antibody and on-going validation is required [8]. Thus, time needs to be expended by others to make good the information gap, a process that is inherently inefficient when an antibody is shown to be unacceptable for use. The purpose of this guideline, similar to guidelines published recently on tissue microarrays [9] and the efforts of the Clinical Laboratory Standards Institute for standardisation [10] is therefore to provide a tiered approach to the validation of an antibody for use as an IHC biomarker in formalin-fixed, paraffin-embedded (FFPE) tissue and to promote this being undertaken before it is used as a biomarker of disease. These guidelines are equally applicable to the validation of an antibody for use in frozen tissue IHC and wholemount *in situ* staining protocols. A summary of the key features of these guidelines is contained in Table 1.

2. Steps to validation

2.1. Step 1: Understand target

It is vital before attempting any validation that a full literature review of the target is undertaken. Firstly, this will build a picture of when and where expression is to be expected and, should IHC have been attempted previously, can point to antibodies that could be evaluated. Secondly, where post-translational modifications or splice variants have been described, this information can be used to predict detection of multiple bands in Western blotting and thus antibodies that would have been rejected as "non-specific" will be kept. Databases such as OMIM [11], Uniprot [12] or Genecards [13] are particularly useful for gathering such information. Note, however, that online resources that are based on mRNA expression can provide spurious results, since the levels of protein and mRNA do not always correlate [14,15]. The biological relevance of the target is important, as this can give an indicator on the likely sub-cellular localisation of the target and as a consequence any non-specific interactions can be identified. For example, a transcription factor is likely to have a nuclear localisation and therefore a cell surface staining pattern would be spurious.

2.2. Step 2: Identify cell and tissues

Control material is critical to the validation and can take several forms. Positive and negative cultured cells, identified through the literature search, can be used for Western blotting, flow cytometry (for membrane bound targets) and the preparation of FFPE cell blocks for IHC [16,17]. When selecting positive and negative control cell lines it is of great value to determine the expression of the required biomarker using more than one assay format e.g. confirming positive or negative biomarker expression of cells by flow cytometry before using as an IHC control. This builds confidence in the expression profile of control cell lines and forms part of the validation process. It is important when FFPE cell blocks are made, that cells are spun down lightly to retain cytology, fixed in the same buffers and times as tissue controls, suspended in agarose and processed in the normal manner to mimic the tissue that they are validating. The standardisation of this process is equally essential, since a variety of fixation regimes and processing methods are referred to in the literature. Once individually processed, the creation of a cell line microarray (CMA) [18,19] can assist not only in determining reaction across multiple cell lines, but also as a quality control check of the IHC once the biomarker antibody has been validated [20]. Using cell lines in this manner has already been validated in the clinical setting [21].

The use of cell culture lines can also prove beneficial particularly where they can be manipulated by transfection to introduce different 'dose' levels of the target in otherwise weakly positive or negative cell lines. As transfection efficiency rarely reaches 100%, a proportion of the cells should remain negative or weakly stained for the target in question which can be useful in differentiating IHC signal from background noise. This also remains the case where RNA is used to knockdown positive cell lines. Indeed, a recent confocal study on cell lines has demonstrated that in 765 proteins studied using 75 antibodies that siRNA silencing can be effective in 80% of cases [22].

Whilst cell lines can provide an indicator of the expression, particularly in comparison to non-IHC methods, the use of positive/negative control tissue is essential for the full validation of the antibody and evaluation of non-specific binding to other tissue components. The literature and online reviews should point to potential positive and negative tissues. However, as expression levels are often modulated in disease then it may be important to include a range of pathologies and, preferably, matched normal tissue. Prior to selecting positive material, the quality of the tissues needs

Table 1
Step-by-step guide to validating an antibody.

Step	Special considerations
Understand target	Full literature review Note the biological relevance and expected sub-cellular localisation
Identify cells and tissues	Identify or create positive/negative cell lines Identify positive/negative control tissues Check the quality of the control material with standardised antibodies and ensure it matches the quality of the test material
Choose an appropriate IHC method	Use a commercially prepared and validated kit Test multiple retrieval conditions to optimise the staining Consider the use of automation
Identify level of validation required	Identify which tier of validation is appropriate Tier 1: Well known antibody with high quality literature evidence Tier 2: Well known antibody used in an alternative species or unvalidated tissue Tier 3: Unknown antibody. Inconsistent/No literature evidence
Control of IHC	Use the same controls that were used for validation when performing test samples Test antibody in at least one other non-IHC method Use negative controls, such as omission of primary antibody or isotype-matched controls to identify any background staining
Publication of results	Include appropriate control material either within publication or as supplementary material MISFISHIE guidelines should be used

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