A Novel IHC Technology for Tissue Cross Reactivity Screening of Humanized Antibodies in Human Tissue

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Abstract

A key characterization step for candidate therapeutic antibodies is assessing their cross-reactivity with non-target epitopes in human tissue. Detecting off-target binding of a human or humanized antibody in tissues containing endogenous human antibodies requires either modifying the antibody, e.g. with a hapten tag (biotin or FITC), or using complicated detection methods. In this work, we present a novel immunohistochemical technology, provisionally designated Vector Human on Human (H.O.H.) Detection, for detecting humanized antibody in frozen and FFPE human tissues. Even in tissues with a high endogenous antibody content such as spleen, we obtained strong, specific signals and minimal background. Using several therapeutic antibodies including anti-PD-1 (Pembrolizumab), anti-HER2 (Trastuzumab) and anti-CD20 (Rituximab), we saw excellent signal-to-noise ratios, with results comparable to detection via a hapten tag. The technology works with multiple human isotypes and antibody configurations, including bivalent antibodies without Fc regions. This approach will accelerate safety testing and avoid issues associated with using modified antibodies in place of the actual drug in cross-reactivity assays.

Introduction

As part of a pre-clinical IND application for therapeutic antibodies, the FDA recommends Tissue Cross-Reactivity (TCR) studies evaluating antibody target distribution and off-target binding against a panel of human tissues[1]. Most therapeutic antibodies are either fully human or humanized, making standard immunohistochemistry (IHC) methods impractical due to high concentrations of endogenous human immunoglobulins. Indirect detection by a secondary antibody directed against human IgG cannot distinguish between the therapeutic antibody and endogenous IgG resulting in high levels of background.

There are two main methods used to circumvent this issue: indirect detection through a molecular tag (hapten), or pre-complexing of the therapeutic antibody with anti-human IgG secondary antibodies[2]. Indirect detection of a hapten label, typically biotin or fluorescein isothiocyanate (FITC), requires covalent modification of the therapeutic antibody. This labeling is typically reactive against amine groups of the antibody and can affect target binding characteristics. Also, if the label is detected directly with an enzyme-labeled antibody or other binder, there is little signal amplification. Adding tertiary or quaternary detection layers can increase background.

The second method involves pre-complexing the therapeutic antibody with an anti-human IgG secondary antibody, then using human IgG to "quench" excess secondary antibody[3]. This method suffers from the complexity of optimizing the ratio of therapeutic antibody to secondary antibody and background is difficult to effectively eliminate.

In this work, we present a modified strategy that takes advantage of a proprietary quenching reagent that effectively excludes background originating from endogenous IgG. We simplify assay development by providing ready to use reagents where only the therapeutic antibody concentration needs to be optimized, similar to a standard IHC assay. Using a highly purified, sensitive HRP-labeled antibody, we achieved high sensitivity and a high signal-to-noise ratio. This detection approach works with a range of therapeutic antibody concentrations and therapeutic antibody formats, including bivalent Fab structures devoid of an Fc region.

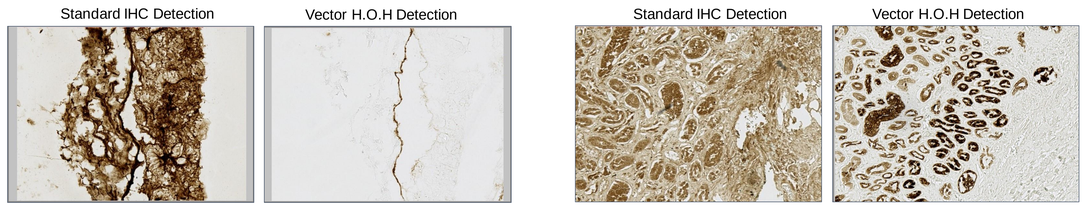
Materials and Methods

Antibodies

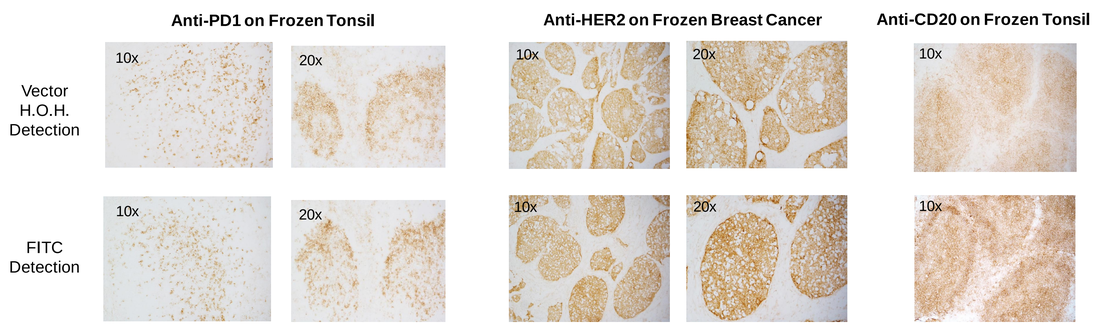
Humanized anti-PD-1 (Humanized, Pembrolizumab), anti-HER-2 (Humanized, Trastuzumab) and anti-CD20 (Chimeric, Rituximab) were purchased from BioVision (Milpitas, CA). Humanized anti-CD4 and anti-CD52 were purchased from Absolute Antibody (Boston, Massachusetts). Human anti-Desmin and anti-Cytokeratin 18/19 bivalent Fab antibodies were purchased from Bio-Rad (Hercules, CA). Anti-PD-1, anti-HER2 and anti-CD20 were labeled at Vector Laboratories with FITC to ~2-3 fluorophores per IgG molecule.

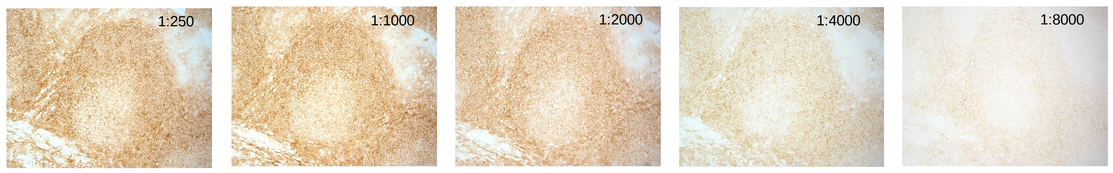
Results

Specific signal is easily distinguished with the Vector H.O.H. Detection



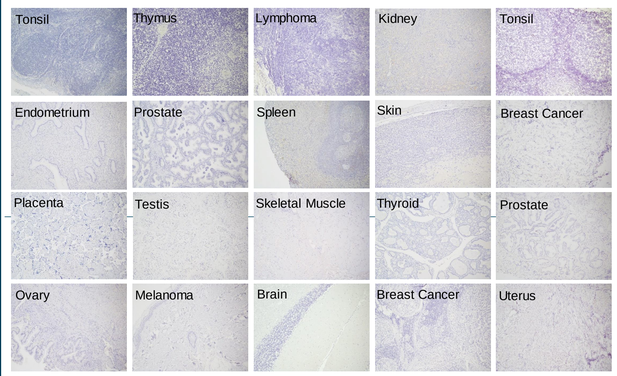
Vector H.O.H. Detection results are consistent with FITC-labeled detection





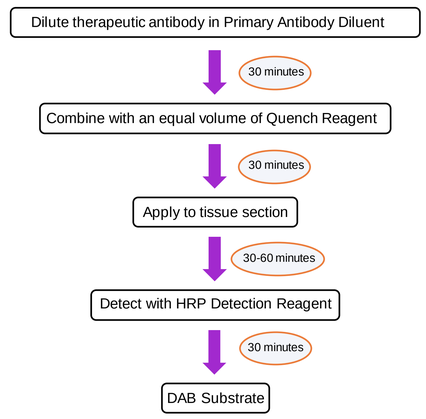
Serial sections of frozen tonsil were stained with serial dilutions of Humanized anti-CD52 and Vector H.O.H. Detection.

Vector H.O.H. Detection has minimal background in FFPE and frozen tissue



FFPE or Frozen tissues were stained with the Vector H.O.H. Detection system without primary antibody as described and counterstained with hematoxylin.

Vector H.O.H. Detection Workflow: 2.5 hours



Materials and Methods, Cont'd

Tissue Preparation
Unless otherwise noted, all reagents were from Vector Laboratories. Cryopreserved or FFPE tissue blocks along with patient data were obtained from the Cooperative Human Tissue Network at Vanderbilt University. Frozen tissue preparation: 5-micron sections were air dried, then fixed with cold acetone for 5 minutes. Sections were stored at -80°C until needed. FFPE tissue preparation: 5-micron tissue sections were deparaffinized in xylene and rehydrated through graded alcohol and retrieved by pressure cooking using Antigen Unmasking Solution, Tris-Based (H-3301).

IHC
Endogenous peroxidase activity was blocked with BLOXALL® Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution (SP-6000) for 10 minutes or 0.3% H2O2 in Methanol for 25 minutes. Sections were rinsed with PBS then blocked with R.T.U. Animal-Free Block and Diluent (SP-5035) for 15 minutes.

For the Vector H.O.H. Detection method, antibody was diluted at 2 times the final concentration in Primary Antibody Diluent and incubated at room temperature for 30 minutes, followed by addition of an equal volume of Quench Reagent and a further 30-minute incubation. The antibody solution was applied to the blocked tissue and incubated for 30 minutes. Sections were washed 3 x 5 minutes, then detected with HRP Detection Reagent for 30 minutes. Sections were washed for 3 x 5 minutes, then developed with ImmPACT® DAB EqV Peroxidase (HRP) Substrate (SK-4103) for 8 minutes. Sections were rinsed in tap water, counterstained with Hematoxylin QS (H-3404) as indicated, dehydrated through graded alcohol and mounted in VectaMount® Permanent Mounting Media (H-5000).

For detection through the FITC label, FITC-labeled antibody was diluted in R.T.U. Animal-Free Block and Diluent (SP-5035) and applied to the tissue sections for 60 minutes. Sections were washed 3 x 5 minutes and then incubated in HRP labeled anti-FITC antibody for 30 minutes. Sections were washed 3 x 5 minutes in PBS and developed and mounted as above.

Discussion

The Vector Laboratories approach to reducing background staining in IHC-based human-on-human TCR assays combines a proprietary quenching reagent with a sensitive detection system. We saw consistent staining patterns with three antibodies using this system compared to a FITC label-based method and we obtained staining patterns consistent with published data with four additional antibodies. The darker staining seen with the FITC-labeled anti-CD20 antibody may be due to the murine/human chimeric structure which has fewer available binding sites for anti-human IgG antibody than a fully human or humanized antibody. The low background achieved in this study enabled unambiguous target detection, even in challenging tissues such as tonsil. This approach works well with antibodies of different immunoglobulin concentrations, subclasses and constructs, including those without an Fc region, and greatly simplifies TCR assay development.

References

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