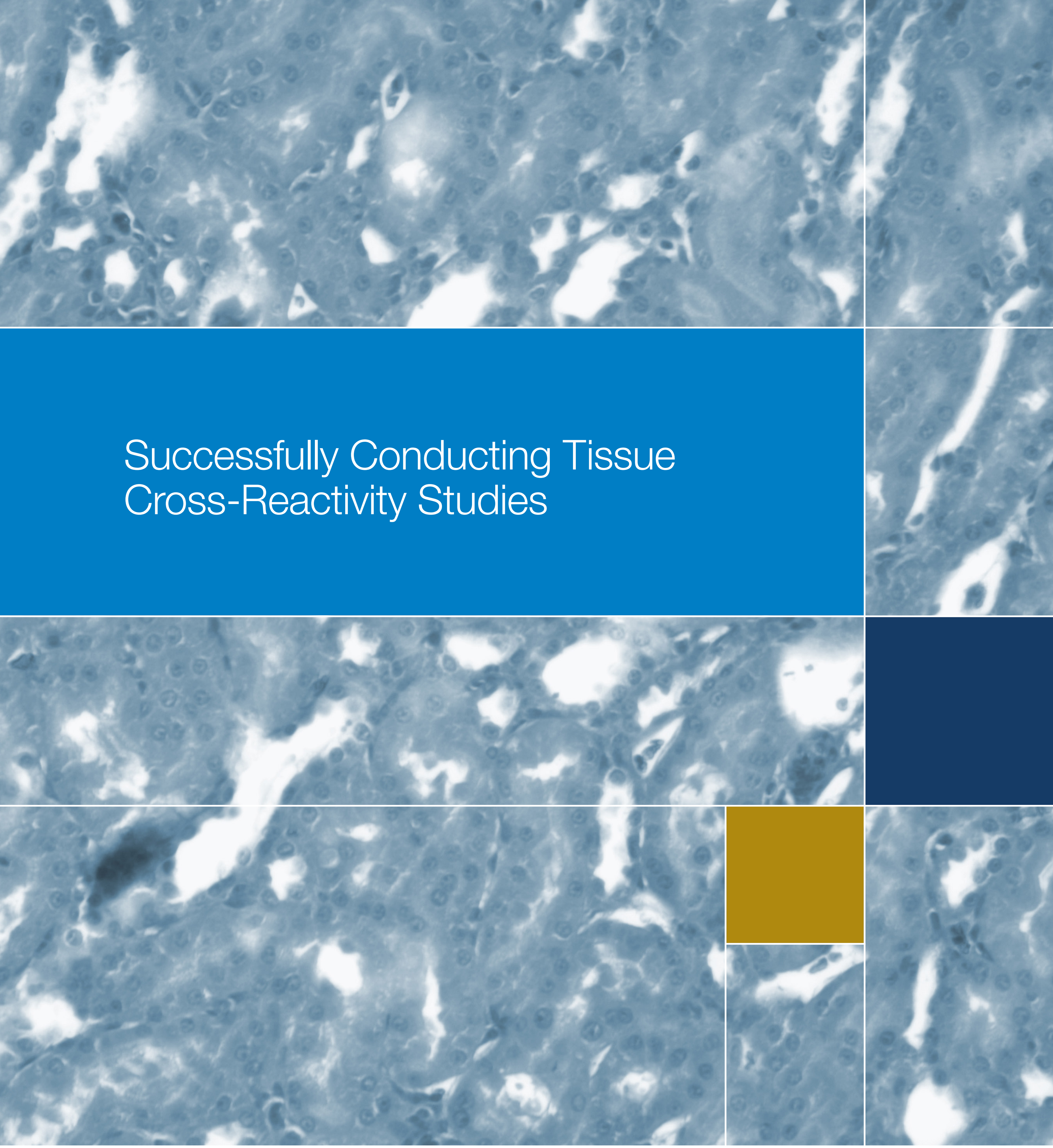


# Successfully Conducting Tissue Cross-Reactivity Studies

PRECISION for medicine

## Introduction

Developing a successful novel therapeutic is incredibly challenging and requires significant investment of both time and capital. According to a 2020 study, the average cost of bringing a single new therapeutic to market in the United States between 2009 and 2018 was nearly $1 billion, counting expenditures on failed trials.[1] Given this, it is important to identify as early in development as possible any potential issues with a new therapeutic—both to limit failures in late-stage studies and to minimize the potential for harm in clinical trials. Many of the preclinical investigations that constitute essential components of an Investigational New Drug (IND) application or Clinical Trial Application (CTA) are intended to minimize the risk of harm in first-in-human studies. For therapeutic antibodies or antibody-like molecules, preclinical tissue cross-reactivity (TCR) studies have become a key tool for gathering and assessing important data regarding on- and off-target binding.

In this white paper, we explore the role and value of TCR studies in therapeutic development. We also discuss critical considerations for developing a rigorous study that generates the robust data necessary to support preclinical decision-making and regulatory submissions.

## Background on TCR Studies

TCR studies are recommended for antibody and antibody-like molecules that contain a complementarity-determining region (CDR). These studies consist of a series of immunohistochemical (IHC) screening assays that are conducted not only to identify off-target binding, but also to detect previously unknown sites of on-target binding for a novel biotherapeutic. The presence of off-target therapeutic antibody binding in frozen ex vivo tissues is used to provide insight into potential organ toxicity in vivo. Identification of new sites of on-target binding offers the possibility of expanding the potential indications for the biotherapeutic.

Studies that compare target expression patterns between human and animal tissue can be used to rationalize organ-specific toxicities found in the preclinical species and predict how those findings might translate into potential safety issues in humans. These data may include in vivo toxicity studies and in vivo pharmacodynamic modeling studies. TCR evaluation of animal tissues may also be useful for providing supplemental information regarding potential correlations, or lack thereof, with preclinical toxicity when there is unexpected binding in human tissues.[2]

Although there is much debate on whether staining in TCR studies correlates with organ toxicity in a clinical environment, regulators do require these studies in the preclinical safety assessment package for IND/CTA submissions for most biotherapeutics.

## TCR Study Development

One of the most important aspects in designing a TCR study is the development and optimization of the IHC protocol to be used. Novel biotherapeutics are designed as drug molecules and are not inherently optimized to be ideal IHC assay development tools or reagents. This can therefore pose a technical challenge that requires a rigorous assay workup and, potentially, multiple rounds of assay development. Additionally, and crucially, a favorable dataset for a TCR study is a broadly negative result—representing no off-target binding—and therefore it is critical to validate that the assay is specific and robust before examining test tissues in order to rule out any false negative results in the TCR study.

Researchers often underestimate the length of time needed to develop a scientifically-sound IHC method, leading to study delays. Working with a TCR assay service provider who has deep expertise in IHC assay development and optimization can help to streamline the process and help ensure studies stay on schedule.

### Considerations for Study Design

TCR studies may be conducted in compliance with Good Laboratory Practice (GLP) or under non-GLP conditions. Researchers may opt to perform non-GLP tissue microarray (TMA) screening for initial assessments of biotherapeutic candidates. For example, TMA screening can be used to prioritize or eliminate candidates based on off-target binding. For regulatory submissions, TCR studies must be conducted under GLP according to the published guidance, which recommends use of Good Manufacturing Practice (GMP)-grade candidate molecule, referred to as the Test Item. The guidance calls for evaluation of the Test Item at 2 concentrations in 3 different, unrelated human donors and appropriate preclinical species. In practice, however, the majority of studies conducted evaluate a GLP-grade Test Item at a single, optimized concentration and focus primarily on generation of data in the required human tissue cohort. Where other animal species are included in these studies, the experimental approach is the same; however, these studies typically do not claim GLP compliance as they are considered to be supplementary research data.

## Considerations for Test Items

Test Items—the biotherapeutics to be administered in first-in-human studies—come in a variety of forms, some of which differ substantially from immunoglobulins in structure. Regardless of the format of the Test Item, consideration must be given to how the molecule will be detected in an IHC assay. Unlabeled human or humanized antibodies can be detected by pre-complexing with an anti-human antibody before application to test tissues. From a technical perspective, however, it is easier to work with an antibody that has been labeled with a small molecule, such as biotin or a fluorescent dye such as fluorescein isothiocyanate (FITC) or one of the Alexa fluorophores, that can be detected with a label-specific antibody. For many molecules, such labels are necessary for facilitating detection.

Biotinylation is a well-established and relatively straightforward technique for labeling biological molecules, but it requires additional avidin-biotin blocking steps in the assay protocol to avoid issues with background staining. Consequently, fluorescent labels may be preferred in a TCR study. With either label, it is important to establish the impact of labeling on the binding properties of the molecule and to ensure Test and Control Items are labeled to the same degree.

## Considerations for Control Items

Inclusion of a Control Item in a TCR study is strongly recommended.[3] The Control Item is typically a species- and isotype-matched non-immune IgG if the Test Item is an antibody, or a molecule that is identical in structure to the Test Item but binds a molecule that is unlikely to be found in human tissue—for instance, green fluorescent protein, a plant protein, or snake venom. Ideally, the Control Item is prepared in parallel with the Test Item, including any necessary labeling with biotin or a fluorescent dye, and is used to ascertain the background level and pattern of tissue binding that occurs irrespective of the CDR.

Failing to include a suitable Control Item for comparison may result in interpretation of any binding of the Test Item as specific, which can be misleading. While there are other methods for assessing binding specificity, such as preincubating with a molar excess of soluble antigen to compete for binding, these methods generally add to overall study cost and may not be feasible due to limited availability of soluble antigen.

## Considerations for Positive Control Material

Selecting a suitable positive control material is also crucial for TCR protocol development and GLP studies. Positive control material is used in the IHC assay development and to validate the Test Item binding in all of the assay runs, and ideally is a frozen tissue sample.

Frozen tissue is superior to overexpressing cell lines or other types of positive control material because it retains tissue matrix. If there are no suitable tissues that naturally express the target of interest, alternative techniques can be employed; for example, incorporation of soluble antigen into a human tissue matrix (see Table 1).

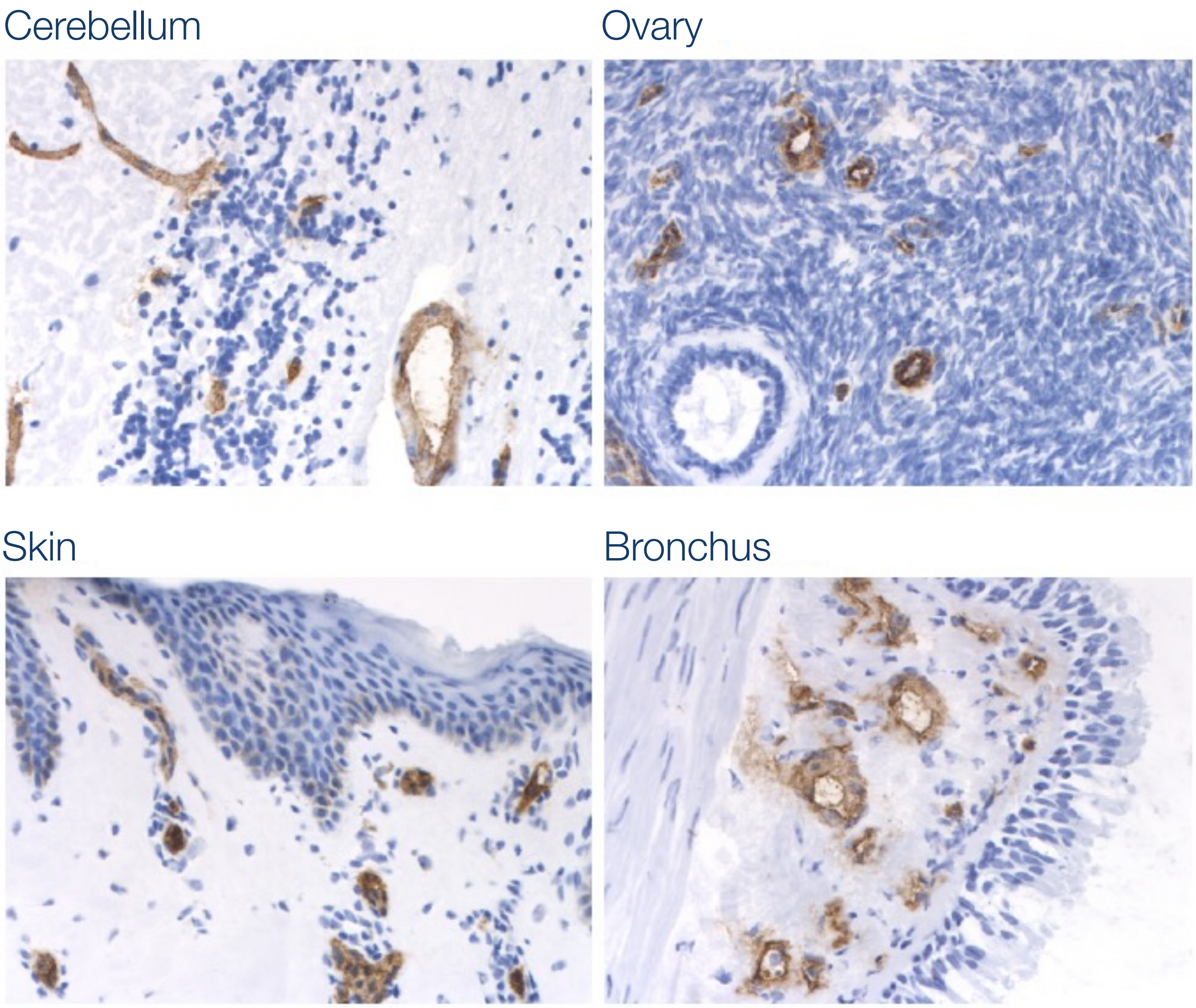
Table 1: **Alternative techniques for IHC assay development**

| Scenario | Case example |
|---|---|
| Non-human targets | When developing assays for a non-human target, such as SARS-CoV-2, where the use of frozen, infected tissues is not an option due to either availability or safety considerations, cell lines expressing the protein or epitope to which the antibody is targeted are an effective alternative.<br>Positive and negative expressing cell lines can be included in any aspect of IHC assay development. Assay conditions may need to be modified, however, when transferring to frozen tissues due to the propensity for higher non-specific binding, which can be reduced through standard approaches such as protein/serum or peroxidase blocking. |
| Modified proteins | When developing methods to detect a non-naturally occurring protein, homogenised tissue samples spiked with recombinant target protein can be created. |
| Antibody-drug conjugates | Anti-linker antibodies have been successfully used to specifically detect Test Item binding in TCR assays for antibody-drug conjugates. |
| Test Items that cannot be labeled | When a Test Item cannot be labeled, it is not possible to use an anti-label secondary antibody. An alternative approach is to pre-complex the Test Item with anti-human IgG secondary antibody before application to the tissues. |
| Multi-specific binding molecules | For bi-specific molecules, there is no regulatory requirement to study the individual binding components, just the bi-specific molecule itself. However, it is not then possible to determine if any observed binding of the bi-specific is due to binding to just 1 or both of the targets.<br>For 1 such molecule, we were asked to provide additional context and data. Thus, we generated supplementary non-GLP TCR data with commercial antibodies to each of the individual binding components to demonstrate target-specific patterns of staining. |

## Considerations for Test Tissues

FDA and EMA guidelines for the development of therapeutic antibodies and related products recommend TCR testing on a range of human tissues.[4,5] According to the FDA's Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use, frozen tissue is recommended.[4] In GLP TCR studies, the quality of the frozen tissues used is of the utmost importance. These tissues must retain antigenicity and exhibit good morphological preservation to allow adequate interpretation of staining patterns. Incorporating confirmation of tissue antigenicity into the GLP study is strongly recommended, as testing prior to the GLP study does not guarantee antigenicity once the tissue has been sectioned onto glass slides. A confirmatory assay commonly used to validate the antigenicity of tissues used in TCR studies involves immunostaining of tissues with von Willebrand Factor (vWF) antibodies (see Figure 1).

Figure 1: **Confirming tissue antigenicity with vWF immunostaining**

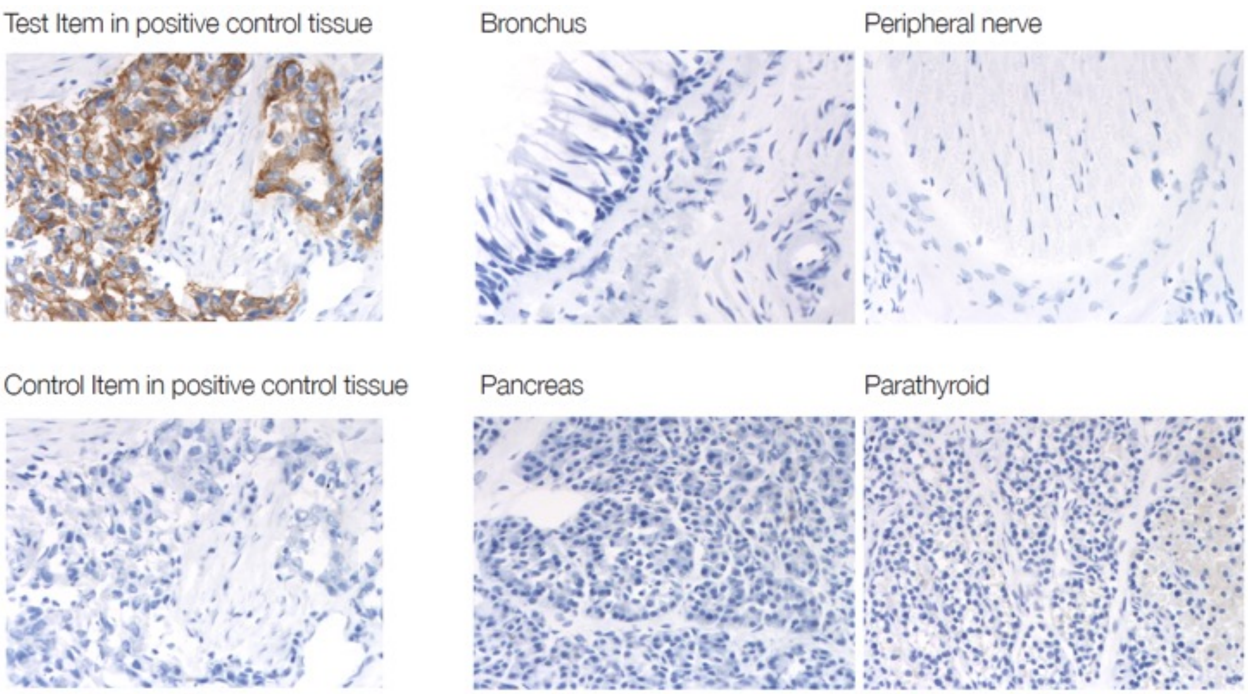



**Images show the binding of vWF antibodies (brown staining) to the vascular endothelium in frozen sections of cerebellum, ovary, skin, and bronchus.**

## Interpreting TCR Study Results

TCR study results should be interpreted by a qualified pathologist. Staining observed with the Test Item should be compared to that seen in adjacent sections that have been incubated with the Control Item (see Figure 2). Specific staining should be considered only where Control Item staining is absent or if the Test Item staining is clearly more intense than the Control Item staining. If staining is smeared or very diffuse, the pathologist will determine whether that staining is specific.

Figure 2: **Example of GLP TCR data**




**Left-hand panels show the optimized IHC assay binding of Test Item and Control Item to the positive control tissue. Right-hand panels show the absence of Test Item binding to a variety of frozen tissues.**

The cellular location of specific staining should also be factored into the interpretation of TCR study results. Staining of cytoplasm, for example, is less likely to translate into a biological effect or safety concern than membrane staining because cytoplasm is unlikely to be accessible to a biotherapeutic in vivo. Therefore, binding should be evaluated and interpreted based on the overall pharmacology and safety assessment data package.

The biological relevance of any TCR staining can only be validated when other human safety or toxicity data, such as clinical trial or post-marketing surveillance data, become available. In part, this is because TCR studies are performed on pathologically normal tissue, while patients treated with the biotherapeutic have a disease condition. The target expression profile may be differentially expressed in both magnitude and distribution in the normal and diseased states. Therefore, TCR study data must be interpreted carefully in the context of preclinical studies on a case-by-case basis to inform development decisions.

## Choosing a TCR Testing Solutions Provider

Precision for Medicine offers a unique combination of specialty lab and tissue procurement capabilities to support the development and conduct of non-GLP and GLP TCR studies. Precision for Medicine's scientific team provides customized services to meet the specific requirements of a biotherapeutic agent. As an expert in IHC assay development, Precision for Medicine has developed assays for monoclonal antibodies; mono-, bi-, and tri-specific antibody-like molecules; scFv fragments; and antibody-drug conjugates.

Precision for Medicine's 2- or 3-phase approach, outlined below, provides a cost-effective solution for making confident decisions regarding the best parameters for a study, minimizing the risk of GLP study failure. The output of our GLP TCR service is a report that is suitable for submission as part of an IND or CTA.

### Availability of Qualified Specimens

As a leading supplier of well-characterized biospecimens for research, Precision for Medicine's extensive sample inventory includes all 36 human tissue types recommended by the FDA and EMA for TCR testing (see Table 2). These tissues are fully consented for commercial research and are available from at least 3 male and 3 female donors.

Table 2: **Tissue types available for TCR testing**

| Tissue types are available from multiple male and female donors | | |
|---|---|---|
| Adrenal gland | Ileum | Prostate |
| Bladder | Kidney – glomerulus and tubule | Skeletal muscle |
| Blood cells | Liver | Skin |
| Blood vessel endothelium | Lung – bronchus and parenchyma | Spinal cord |
| Bone marrow | Lymph node | Spleen |
| Breast | Ovary | Stomach |
| Cerebellum | Pancreas | Testis |
| Cerebral cortex | Parathyroid gland | Thymus |
| Colon | Parotid salivary gland | Thyroid gland |
| Eye | Peripheral nerve | Tonsil |
| Fallopian tube | Pituitary gland | Ureter |
| Heart | Placenta | Uterus – cervix and endometrium |

All specimens undergo a 4-point inspection to qualify for GLP TCR studies:

1. Evaluation of donor clinical history to ensure experimental suitability
2. Review by board certified pathologists to validate normal morphology/pathologyy
3. Confirmation of compliance with ethical, legal, and regulatory requirements
4. Initial confirmation of tissue antigenicity

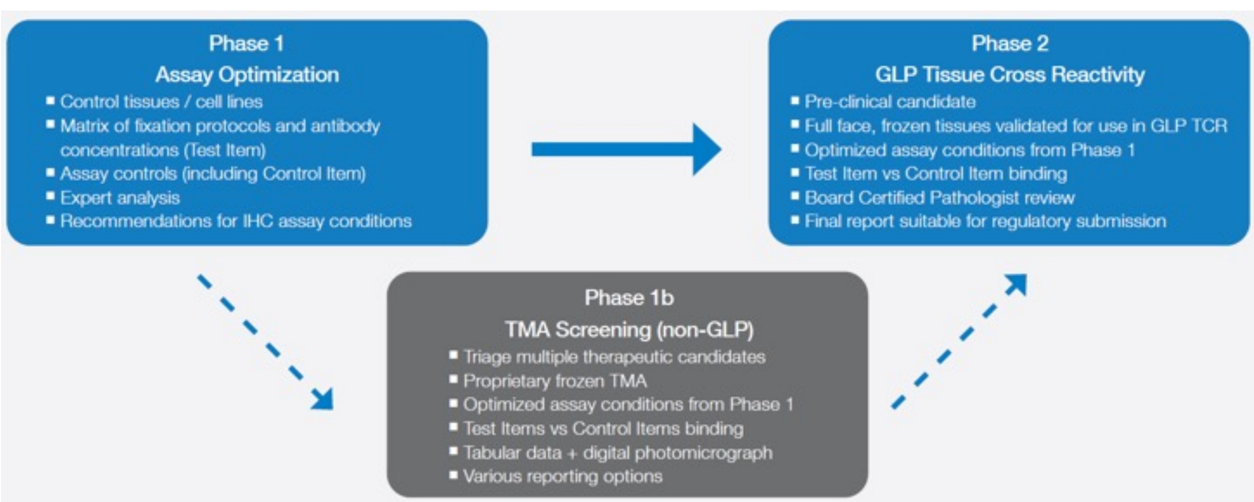
Precision for Medicine also offers a proprietary frozen TMA that can be used for non-GLP TCR studies, which can be an economical alternative for rapid turnaround on screening candidate molecules. This 3-array panel contains the 36 tissues required by the FDA and EMA, accelerating the de-selection of candidates that exhibit significant off-target immunoreactive profiles.

## A 3-Phase Approach

Precision for Medicine utilizes a 3-phase approach to TCR studies to help ensure quality data (see Figure 3):

- **Phase 1 – Assay Optimization.** The Test Item and Control Item are incubated on both positive and negative control tissues at multiple concentrations to determine the optimal specificity and staining conditions for immunohistochemical detection. The output of this phase is a report outlining the data and methodology recommendations.
- **Phase 1b – TMA Screening (non-GLP).** The optimized assay protocol is performed on proprietary frozen TMA sections, allowing efficient, cost-effective triage of multiple therapeutic candidates or earlier evaluation of molecules during lead optimization. The Phase 1b report includes methodology, tissue details, and data on the presence or absence of Test Item binding.
- **Phase 2 – GLP TCR (see Figure 2).** This assay uses full-face, frozen tissues and results undergo review by a board-certified pathologist. The report generated from this study is suitable for regulatory submission.

Figure 3: **3-phase approach to TCR studies**




Precision for Medicine also offers comprehensive strategic and scientific services, infrastructure, and technologies to accelerate clinical development and support commercialization of novel therapeutics.

## Conclusion

TCR studies are an important aspect of the preclinical development of therapeutic antibodies and antibody-like molecules as off-target binding can result in treatment-related toxicity. Performing optimized TCR assays on well-characterized, high-quality specimens under GLP conditions is essential for generating the robust data needed to manage development risk and support regulatory submissions. Working with a solutions provider that combines biospecimen availability with expertise in IHC assay development can help accelerate the development of a therapeutic antibody candidate program.

### About the Authors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Amanda is an experienced leader of scientific operations for drug discovery in both the pharmaceutical and CRO industries. She focuses on methods and approaches to provide actionable data which can directly enable biopharmaceutical research and development.

She earned her PhD, focused on drug metabolism, from the University of Cambridge.


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Since completing her PhD and a post-doctoral position at Royal Veterinary College, London, Rebecca has amassed nearly 20 years of experience working in the Biotech & CRO industries. Initially having focused on the development of human primary cell-based assays, she transitioned into Molecular Pathology where she now leads the design and execution of all types of immunohistochemical assays and other molecular pathology techniques.

## References


1. Wouters OJ, McKee M, Luyten J. Estimated research and development investment needed to bring a new medicine to market, 2009-2018. *JAMA*. 2020;323(9):844-853.
2. U.S. Food and Drug Administration. Guidance for Industry: S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals. Published May 2012.
3. Leach MW, Halpern WG, Johnson CW, et al. Use of tissue cross-reactivity studies in the development of antibody-based biopharmaceuticals: history, experience, methodology, and future directions. *Toxicol Pathol*. 2010;38(7):1138-1166.
4. Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use, Docket No. 94d-0259. February 28, 1997.
5. Guideline on Development, Production, Characterisation and Specifications for Monoclonal Antibodies and Related Products, EMEA/CHMP/BWP/157653/2007. Adopted December 18, 2008.

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