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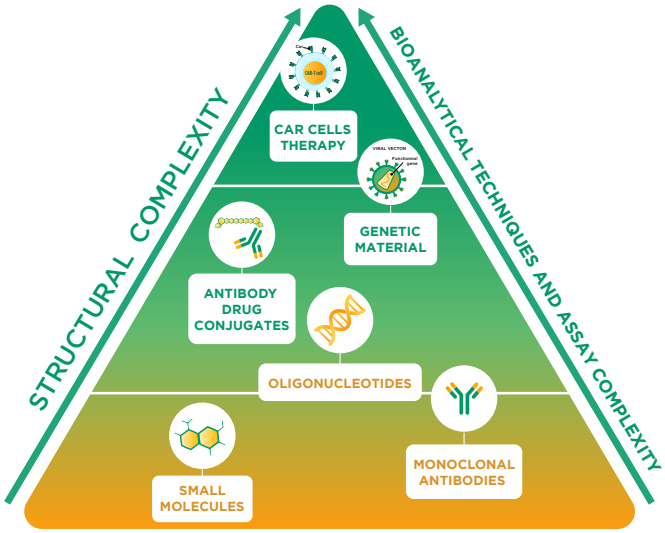
IMMUNOMODULATION ASSESSMENTS FOR CLINICAL TRIALS—SOPHISTICATED BIOANALYTICAL APPROACHES TO SUPPORT COMPLEX MODALITIES

Immunomodulatory drug development is trending toward increasingly complex modalities as science advances and more effective and safe immunotherapies are needed. Immunomodulatory drugs are at the forefront for the treatment of various types of cancer, infectious diseases, and numerous autoimmune diseases, including rheumatoid arthritis, type I diabetes, lupus, and multiple sclerosis. As the complexity of these therapeutics increases, so must the sophistication of the bioanalytical assays designed to either quantify them or measure their impact on the patient.



IN THIS ISSUE

In this issue, we review common classes of immunomodulators, bioanalytical methods used to quantify them, and their associated biomarkers. We further present two scenarios that address the complexities of bioanalysis for immunomodulators and practical considerations to ensure quality bioanalysis to inform pharmacokinetic (PK), pharmacodynamic (PD), and safety data in clinical trials.

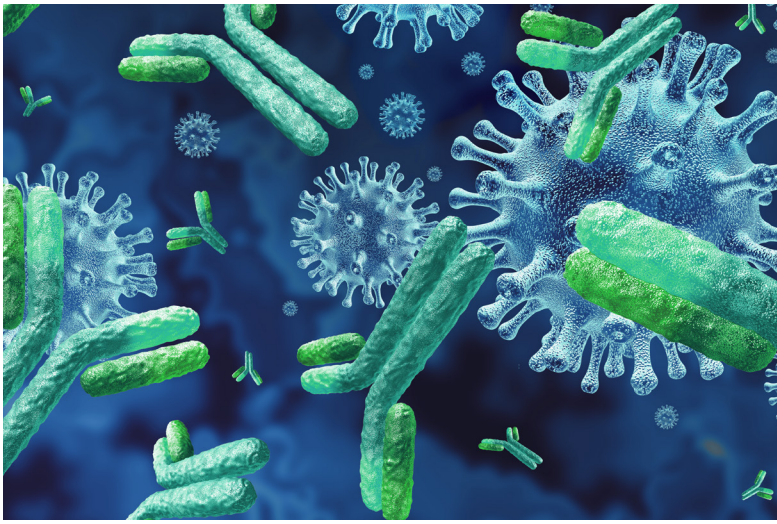
- **Scenario 1**—Effective Integration of Multiple PK and PD Endpoints in a Clinical Trial for an Immunomodulator
- **Scenario 2**—Intricate Flow Cytometry PD Assessment for an Immunomodulator

IMMUNOMODULATOR CLASSES

Immunomodulators can be broadly defined as immunostimulants and immunosuppressants, with many types of modalities in development. Some of the first classes of immunomodulators were vaccines, small molecules, and cytokines. Today, advances in understanding the involvement of the immune system in various diseases and the evolution of drug modalities have resulted in increased targeted biologics and novel gene therapy and vaccine approaches, some examples of which are described below. Each class of immunomodulator has a defined complexity and mechanism of action, thus the appropriate bioanalytical program will need to be carefully designed for the drug type as well as the intended purpose of the clinical study. Considerations include the appropriate PK and PD endpoints required for the study, and their regulatory and bioanalytical specifications.

Monoclonal Antibodies

Tumors frequently manipulate specific immune checkpoints when attempting to protect themselves by shutting down immune responses. Checkpoint inhibitors can restore and/or unleash new immune responses, promoting the elimination of cancer cells. For example, the PD-1/PD-L1 immune checkpoint pathway can neutralize T cells that normally target cancer cells. When checkpoint inhibitors block the PD-1/PD-L1 pathway, this enables T cells to regain their functionality to eliminate cancer cells. Checkpoint inhibitors are among the most successful immunomodulatory therapies developed for oncology. Monoclonal antibodies targeting either PD-1 or PD-L1 have been demonstrated to boost the immune response against cancer cells.

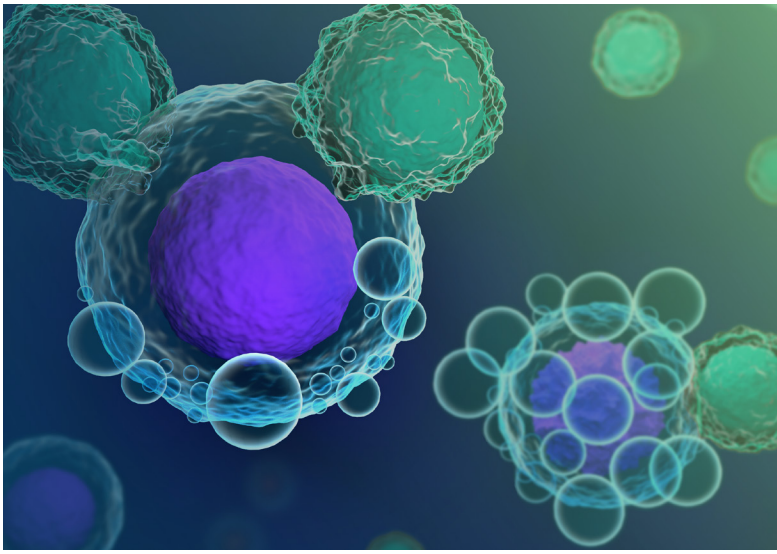


Following the success of those monoclonal antibodies, many bioengineering strategies are currently being explored, aiming to increase the therapeutic effect. For example, modifying the fragment crystallizable (Fc) region has the potential to increase the interactions with immune cells, and thus, improve mechanisms like antibody-dependent cell cytotoxicity (ADCC). Another example is glycoengineering, which aims to alter the glycosylation patterns to improve antibody stability, half-life, and effector functions.

Multifunctional Antibodies and Antibody-Drug Conjugates (ADCs)

Beyond traditional targeting antibodies, there are multiple approaches that increase the complexity of antibody modalities, including bispecific antibodies and ADCs. These approaches increase the complexity of the molecules by increasing the number of targets and, in the case of ADCs, the payload considerations, thus expanding the scope of bioanalysis required. Although traditionally ADCs are used for cancer indications, there is growing interest in extending their potential utility in inflammation and infectious diseases, and to cause immunosuppression. Generally, the preferred assay platform for ADC measurements is ligand binding assay (LBA), especially for clinical studies, owing to its high sensitivity and sample throughput. On the other hand, in the case of ADCs with cleavable linkers, the hybrid LBA-LC-MS/MS workflow has emerged as a valuable platform for assessing conjugated payload. This approach is advantageous as it is less influenced by the ADC's drug-antibody ratio (DAR), providing a more robust and unbiased analysis.

CAR-T Cells



CAR-T cell therapy is a type of immunotherapy. CAR stands for chimeric antigen receptor. In the lab, the T cells isolated from blood are modified to express a protein on their surface called a chimeric antigen receptor. The CARs can then recognize and target a specific protein on the surface of cancer cells. Given these are cell-based therapeutics, the type of bioanalysis approach differs from other modalities and adds a layer of complexity.

Vaccines

Vaccines stimulate the body's immune system to recognize and fight off specific diseases. Vaccines contain antigens, which can be weakened or inactive parts of a virus or bacteria, or they may contain a modified version of the toxins produced by the pathogen. Vaccine administration introduces these antigens into the body in a controlled manner. The immune system recognizes the antigens as foreign and mounts an immune response by activating immune cells to kill the infected cells directly or to produce antibodies that act through different mechanisms to fight the disease.

Some of the types of vaccines include:

- inactivated
- live-attenuated
- messenger RNA (mRNA)
- subunit, recombinant, polysaccharide, and conjugate
- toxoid
- viral vectors

After the initial immune response, the body creates lasting memory T and B cells for that specific antigen. If re-exposed to the actual disease-causing pathogen in the future, those memory cells can quickly recognize and respond to it, preventing illness or reducing its severity.

The bioanalytical strategy for a vaccine development is specific to each pathogen and vaccine type. Indeed, we start from a position of knowledge about the infectious disease, vaccine type and desired immunity in order to select the appropriate bioanalytical methods that will ultimately inform on the successful achievement of the primary and/or secondary objectives of the clinical study.

IMMUNOTHERAPY TRIALS—COMPLEX STUDY DESIGNS AND DIVERSE PATIENT POPULATIONS

Clinical advances in immunotherapy have revealed the need for new or adapted approaches to trial design and assessment of efficacy and safety in all phases of drug development. Some of the conventional statistical methods and endpoints used in oncology areas appear to be less appropriate in immuno-oncology, and other methods and endpoints have emerged as alternatives.

Early-phase studies that can be conducted in healthy subjects generally include biomarker, PK, and immunogenicity testing to support continued drug development, dose determination, and safety assessments. As the half-life of many immunomodulators is longer than traditional small molecules, evaluation of biomarkers, PK, and anti-drug antibodies may continue for many months, extending the patient follow-up period.

When patients are enrolled, multi-site studies are frequently necessary to ensure sufficient patients are randomized and evaluated through the trial. Multi-site clinical trials are particularly important in the field of targeted medicines, as the pool of eligible subjects will be smaller, based on requirements and inclusion/exclusion criteria.

Patient selection for specialized trials is a complex and rigorous process, requiring innovative trial designs that ensure the most appropriate subjects are selected and that appropriate bioanalysis is integrated to establish the PK and PD of the immunomodulator.

Many of these biomarker evaluations require complex processing steps (e.g., PBMC cell isolation), that must occur within a short stability window. Not all clinical sites are equipped to handle these processes, and sponsors may need to align patient sites with the bioanalytical lab's processing requirements.

In addition, the results of the bioanalytical sample analysis may be required during the study, particularly in early-phase trials, to evaluate dose escalation; therefore, a short turnaround time is required for data transfer. Careful consideration of the trial's location(s), patient access, and proximity to the bioanalytical facility are key elements of clinical trial planning.

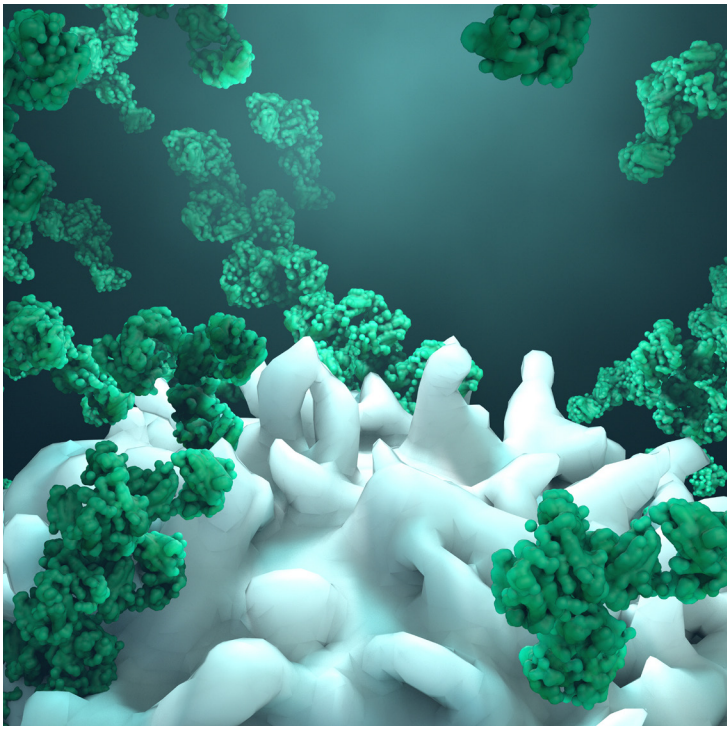
BIOANALYTICAL METHODS AND ASSAYS FOR IMMUNOTHERAPY TRIALS

Different approaches to bioanalysis are used for immunotherapy trials. Some of the most common are listed below, with their key characteristics.

ELISA/ MULTIPLEXED ASSAYS	ELISPOT	FLOW CYTOMETRY	CELL-BASED ASSAYS
Can be adapted for a wide range of applications to monitor drug levels, ADA, or biomarkers, when the matrix is in liquid form.	Can be used to monitor functional immune responses at the cellular level for a wide range of applications and drug products.	Can be used to enumerate specific immune cells through the use of multiple markers at the single-cell level for various applications, such as intracellular cytokine production, receptor occupancy, and immune-phenotyping.	Can be used for a wide range of applications such as supporting immunogenicity assessment by characterizing the neutralization capacity of the ADA response generated or pharmacological activity of the immunomodulator.
Ability to measure multiple samples simultaneously, and multiple analytes in a single sample for multiplexing assays.	Ability to measure a functional readout at the single-cell level; suitable for testing many samples simultaneously.	Can combine several markers to characterize the phenotype of the responding cells.	May be optimized and adapted for different applications during the drug development cycle, such as clinical immune monitoring.
Quantitative detection of analytes and/or qualitative in the case of ADA.	Semi-quantitative measurement of cytokine or antibody production.	Quantitative, when absolute count is considered, or qualitative readout.	Qualitative, or semi-quantitative in some cases.
Reduced sample volume and cost when multiplexing.	Lower number of cells are required per test when compared with flow cytometry.	Can detect and quantify rare populations when frequency is very low.	Can assess the entire biological activity, as opposed to a fraction of the interactions obtained by ligand binding assays.
Common biomarkers include cytokines and complement factors.	Common endpoints include inflammatory cytokine release assays. Can be used with a variety of cell types, including T, B, and NK.	Common endpoints include standard T, B, NK cell panels; with possibility for more numerous and elaborate panels when required.	
Ability to scale up for large clinical studies.			
Offers improved efficiency and throughput when compared with ELISA.			
MSD platform can provide increased sensitivity.			

BIOANALYTICAL ASSAYS

PK and Anti-Drug Antibody (ADA) Assays



PK and ADA assays are used to determine the drug's concentration over time and assess the presence of ADAs in biological samples, respectively.

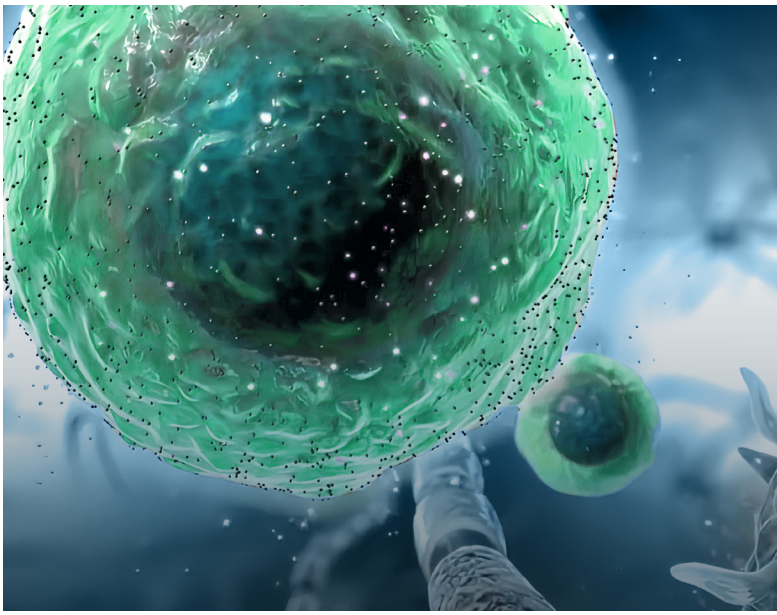
A PK profile is essential to determining the drug's dosage regimen, efficacy, and safety profile. Immunogenicity will identify any potential impact of ADA on the drug's efficacy and safety profile. ADAs can neutralize the drug's effects, leading to treatment failure and/or can be associated with adverse reactions.

Immunomodulator drugs can be simple molecules, such as monoclonal antibodies targeting immune checkpoints (e.g., PD1 and CTLA4), but are often more complex drugs, such as bispecific antibodies, CAR-T cells, ADCs, or fusion proteins. The complexity of such drugs leads to challenges during method development and validation compared to standard monoclonal antibody therapeutics. They can be composed of multiple functional domains, adding more evaluations to characterize immune responses generated to each domain of the drug during a clinical study. Therefore, access to specialized reagents, and their availability and performance are critical considerations. With some therapeutic drugs, the choice of platform is also a key factor based on the mechanism of action of the drug or the context of use. While a plate-based LBA platform, mass spectrometry approach, or PCR-based method are used to quantitate most drugs, flow cytometry may be used for CAR-T cells.

Clinical trials assessing PK or immunogenicity profiles of immunomodulatory drugs are often conducted in populations containing various cancers or autoimmune diseases. Access to corresponding matrices to perform method validation is difficult. Multiple challenges are faced in mimicking study populations due to diverse types and stages of a disease, potential co-morbidities, and prior or concomitant treatments. Additional interferences might be observed in both PK and ADA assays when using these disease matrices due to the presence of autoimmune antibodies, rheumatoid factors, or pre-existing antibodies from previous and similar therapies, or due to the composition of the drug itself.

Understanding the interactions between the drug and its target is critical, as is the level of soluble drug target present in the biological matrix, as it may cause interference in the assay and result in false positive or negative results when assessing ADAs in a patient's samples. Consequently, more consideration is required during method development to ensure the appropriate assay format and mitigation strategies are applied. Moreover, more extensive validation is required to assess cut-point, sensitivity, and selectivity (healthy vs. disease populations).

Biomarkers



Cytokines are small proteins that function as messenger molecules for regulating immune cell maturation, growth, and responsiveness. Cytokines affect the growth of all blood cells and other cells that support the body's immune and inflammation responses. Cytokines directly stimulate immune effector cells and stromal cells at the tumor site and enhance tumor cell recognition by cytotoxic effector cells.

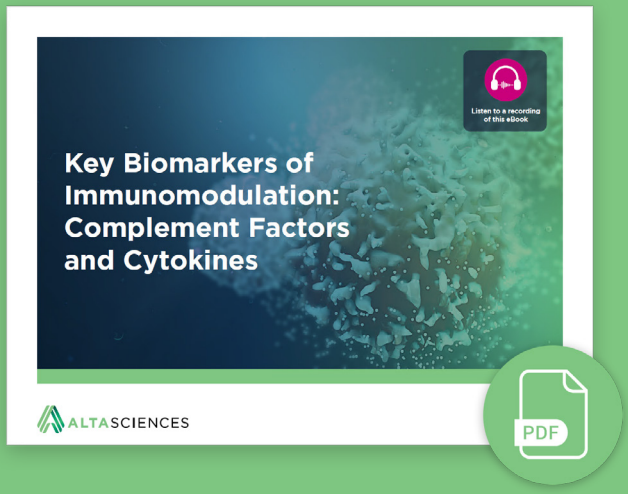
Variations in cytokine levels in various biological fluids, such as serum, blood, stool, saliva, and sweat, provide valuable information regarding immunomodulatory activity and are important biomarkers of inflammation. Abnormal or increased production of cytokines (such as during a cytokine storm) can lead to organ failure and death and must be carefully monitored.

Cytokine release assays (CRS) in which a drug is compared with a positive control antibody, especially one on the market, may be useful for putting results in context. Moreover, regulatory agencies strongly encourage comparison studies where relevant.

This assay is performed by using peripheral blood mononuclear cells (PBMCs) from a healthy donor with the study drug at different concentrations, and pro-inflammatory cytokine panels are assessed to determine whether cytokines are secreted as a result.

Panels that detect various groups of cytokines, such as subtypes of interferons (IFNs), CC and CXC chemokines, as well as interleukins, are available on the market. Panels that detect cytokines released by certain cells, such as Th1 or Th2, are also available. These assays can work with multiple matrices, such as serum, cell culture supernatants, and other bodily fluids. This allows an evaluation of the potential for cytokine storm. Analyzing multiple analytes at once gives a multiplexing power compared to the traditional ELISA, which can save time, labor, cost, and precious sample material.

For more detailed information, refer to the Key Biomarkers of Immunomodulation eBook.



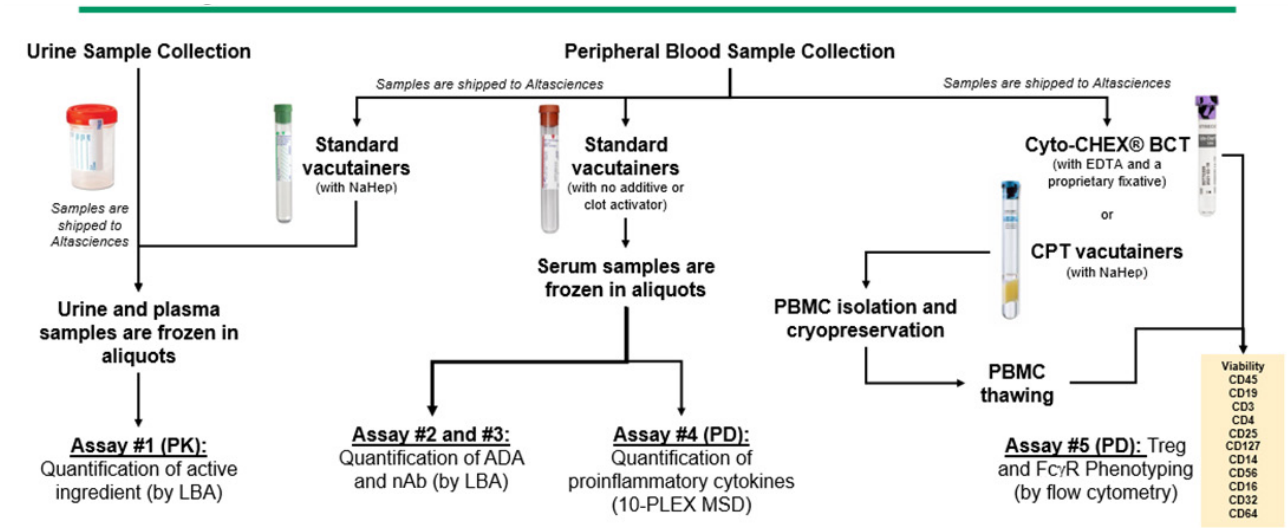
ALTASCIENCES' SCENARIOS

Scenario 1—Effective Integration of Multiple PK and PD Endpoints in a Clinical Trial for an Immunomodulator With Rigorous Sample Handling and Storage Procedures

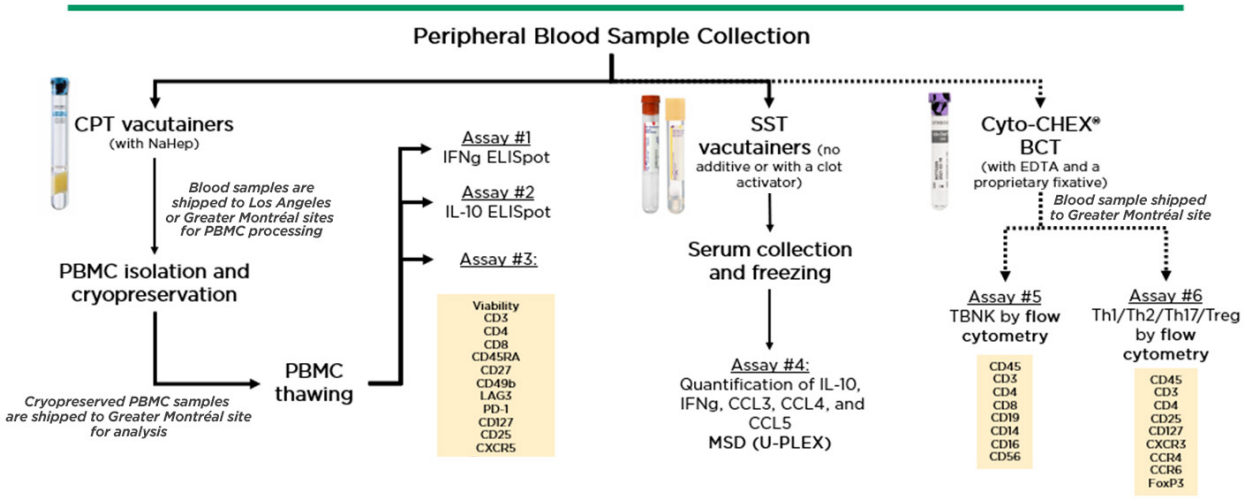
Rigorous sample handling and storage procedures ensure the integrity of samples and reliable data analysis. These procedures must be planned for in advance and are part of the master protocol.

Below are the processes of two clinical trials for which Altasciences implemented meticulous, complex sample processing for immunomodulatory testing.

Process 1. Sample Collection for PK and PD Biomarker Analysis.



Process 2. Sample Collection for Biomarker Analysis.



Considerations for Successful Sample Handling and Storage

1. **Temperature Control:** Proper temperature control maintains study sample stability.
   - Blood samples may need to be kept at specific temperatures to preserve cellular components and prevent degradation. When required for flow cytometry, specialized tubes are used in addition to temperature control.
   - Tissue samples may require immediate preservation at an appropriate temperature to maintain their molecular integrity. If the tissues are for protein analysis, storage at -80 degrees Celsius will suffice. If cells need to be homogenized for analysis, cryopreservation is required, and liquid nitrogen is utilized.
   - Cryopreserved PBMCs require storage, ideally in a liquid nitrogen tank (liquid shipper). They can also be shipped on dry ice with continuous re-supply of dry ice to maintain the temperature.
2. **Time Constraints:** The time between sample collection and processing, especially for PBMCs, should be minimized to prevent alterations in sample characteristics. Protocols should be established to ensure rapid transfer to appropriate storage conditions. Our Greater Montréal bioanalytical facility is a 15-minute drive from our Montréal clinic, and our Los Angeles clinic has its own PBMC isolation lab within its facilities.
3. **Aliquoting:** Samples should be divided into smaller aliquots whenever possible to avoid repeated freeze-thaw cycles, which can degrade sample quality. This also allows for multiple experiments without compromising the integrity of the original sample.
4. **Labeling and Tracking:** Accurate labeling of samples is crucial for proper identification throughout the trial. Each sample should be assigned a unique identifier, and detailed records should be maintained regarding sample collection, processing, and storage conditions. Barcode systems or electronic tracking databases can streamline this process.
5. **Quality Control:** Regular quality control checks should be performed to assess sample integrity and ensure that storage conditions are maintained within specified parameters. This may include monitoring the logs from the sample storage system for temperature fluctuations and conducting audits of storage facilities.
6. **Transportation:** If samples must be transported between sites (e.g., from clinical sites to central laboratories), appropriate shipping methods should be employed to maintain sample integrity. This may involve using insulated containers with temperature monitoring devices and ensuring compliance with regulatory requirements for shipping biological materials. Clinical facilities with labs located in proximity are an advantage in this area (see Time Constraints, above). Altasciences' comprehensive, kit-based service expedites and simplifies sending samples from the clinic directly to the laboratory.
7. **Regulatory Compliance:** Adherence to regulatory guidelines and requirements is essential for handling and storing samples in immunotherapy trials. This includes compliance with Good Clinical Practice (GCP) guidelines, as well as any specific regulations governing the handling and storage of biological samples in the relevant jurisdiction.
8. **Security:** Access to sample storage facilities should be restricted to authorized personnel. Security measures, such as surveillance cameras, electronic access controls, and alarm systems, may be implemented to safeguard samples.

Altasciences carefully addresses these security considerations and other measures specific to individual clinical trials to minimize variability in sample quality and ensure the reliability and reproducibility of data generated from immunotherapy trials.

SCENARIO 2—Intricate Flow Cytometry PD Assessment for an Immunomodulator: Flow Cytometry Multiple Panel Analysis

To have a complete picture of the immune system, it is fairly common to use a combination of multiple flow cytometry panels. These panels must be carefully designed to serve useful and pre-determined purposes.

Below is an example of an antibody-like therapeutic agent to be used in the context of oncology, which would lead to the design of three panels to be tested simultaneously on patient whole blood samples at multiple timepoints throughout the study.

Panel 1 – TBNKM With Absolute Count

This is a simple panel to look at the most common immune cell subsets. Typically, such panels include readouts for T helper cells, T cytotoxic cells, B cells, NK cells, and monocytes. Experimentally, this panel is very straightforward and uses a lab procedure called lyse/no-wash. It is also combined with Trucount™ Tubes, which allow an absolute count of all the immune subsets. Good synthetic blood controls are available on the market (i.e., CD-Chex Plus®, BDTM MultiCheck Control) for establishing normal reference ranges, and are excellent controls for this type of assay.

During a clinical trial, this panel establishes a baseline immune status for subjects before the therapeutic intervention and allows the assessment of any changes due to the treatment. This panel is often used as a secondary endpoint and can function as an immune safety/toxicity assessment.

Altasciences can provide an off-the-shelf TBNKM flow cytometry assay with a sample stability of 11 days.

Panel 2 – Receptor Occupancy (RO) Assay

This panel is probably the most challenging of the three assays described in this section. The receptor occupancy is a PD assessment that, in some cases, can be used for PK profiling and can go from an exploratory endpoint to a secondary endpoint. There are several ways to assess the receptor occupancy of a drug by flow cytometry, and the assay needs to be properly designed and tailored to the drug's mechanism of action. Hence, this panel tends to be small with fewer markers since the aim is to assess the binding of the drug on the immune cells, in general or in a very specific manner.

This type of assay is generally performed on fresh whole blood, creating a potential challenge with the sample's stability. Cryopreservation and subsequent thawing can impact the drug's binding to its target and create artefacts. Altasciences has expertise that delivers proper testing during method development and further characterization during method validation, making it a viable solution.

Flow cytometry is a robust platform when reporting frequency of population readouts. Our lab analysts will provide additional controls to set up mean fluorescence intensity (MFI) readouts to properly and accurately report the percentage of receptor occupancy.

Panel 3 – PD Markers

This panel will typically be larger than TBNKM and RO, with a greater number of PD markers. The objective is more exploratory and provides a more in-depth characterization of the immune responses generated while looking at the different subsets of a specific immune cell type, as well as their activation/exhaustion status. It requires tailoring that is specific to the pharmacology of the drug. For example, if we are monitoring a drug that acts on a T cell immune checkpoint, the immunophenotyping panel should be focusing on T cells and their subsets (i.e., memory phenotyping, T regulatory, etc.) as well as on broad exhaustion markers for more in-depth information (i.e., PD-1, TIGIT, CTLA4, TIM3, LAG3, etc.). When developing and validating the flow cytometry method, some biomarkers can offer the additional challenge of being intracellular markers.

Experimental control design can be quite challenging for PD marker panels. Altasciences' experts will ensure that the control can monitor, at least qualitatively, the signal for each marker. They will also analyze and address the fact that some of those markers might need an artificial expression level in a matrix that is as close as possible to the clinical sample matrix. Many commercial options are available, like Veri-Cell™ PBMC and CD-Chex Plus™, and their activated versions. Some companies offer customized synthetic control services, meaning they can be customized to obtain a specific FSC/SSC profile as well as express the markers selected (and the intensity required).

At Altasciences, we have multiple strategies for customizing the perfect controls while being embedded within the panel's method development. We have the scientific and technical expertise, as well as the flexibility to tailor a method according to our clients' needs.

ALTASCIENCES' RESOURCES

Webinars

Development of a Cell-based Assay for Dual Purposes

Overcoming Bioanalytical Challenges for PK/PD Assessment in Phase I Biologics Studies

Critical Sample Handling Processes

Poster Library

Altasciences' Scientific Posters on a Variety of Topics

Webpage

Altasciences' Bioanalytical Solutions

ABOUT ALTASCIENCES

Altasciences is an integrated drug development solution company offering pharmaceutical and biotechnology companies a proven, flexible approach to preclinical and clinical pharmacology studies, including formulation, manufacturing, and analytical services. For over 25 years, Altasciences has been partnering with sponsors to help support educated, faster, and more complete early drug development decisions. Altasciences' integrated, full-service solutions include preclinical safety testing, clinical pharmacology and proof of concept, bioanalysis, program management, medical writing, biostatistics, clinical monitoring, and data management, all customizable to specific sponsor requirements. Altasciences helps sponsors get better drugs to the people who need them, faster.