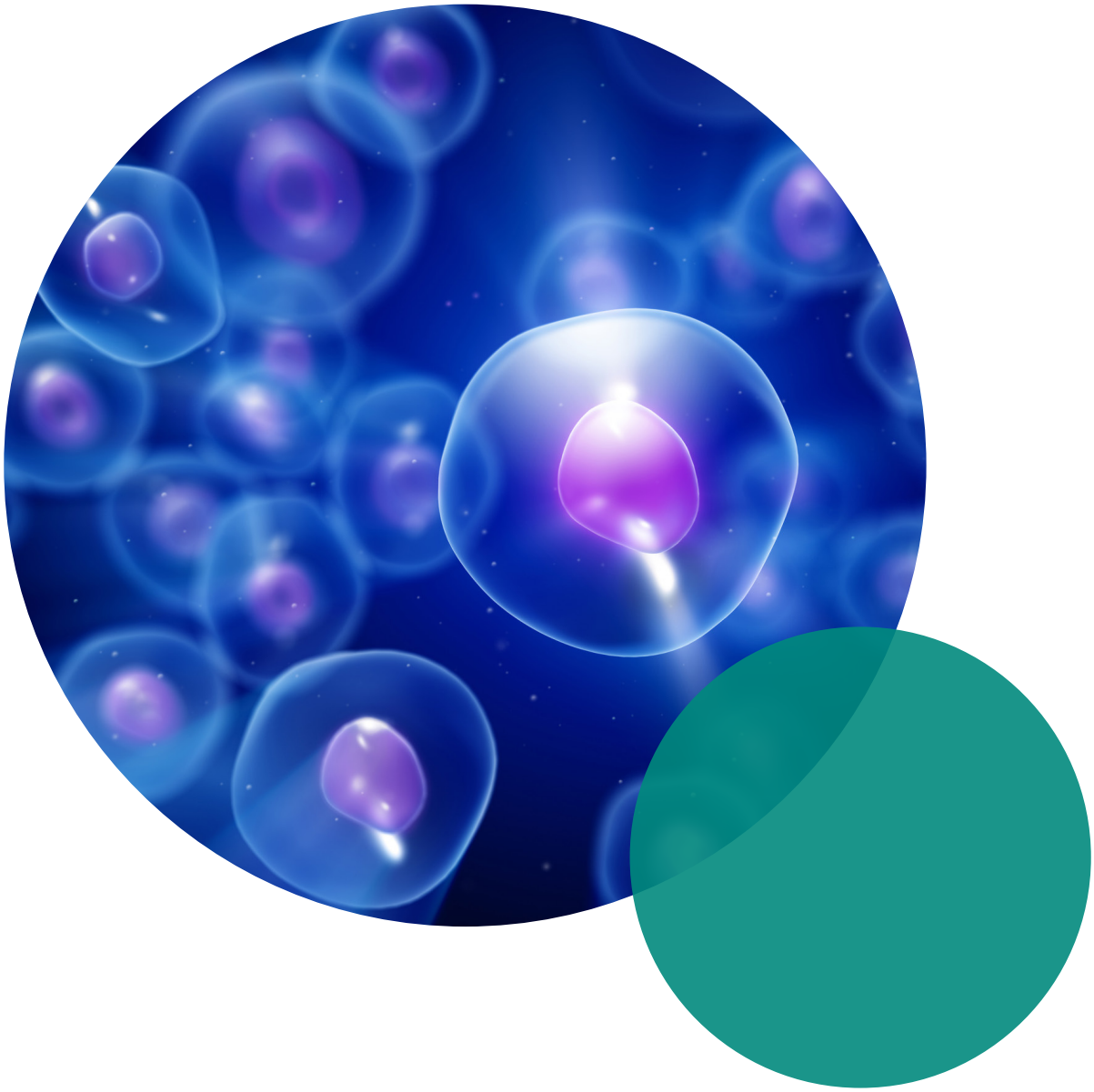
Driving Clinical Trials Forward:
The Benefits of Establishing a Strategic Partnership for Flow Cytometry



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Introduction – The critical role of flow cytometry in clinical trials assessing targeted therapies

Targeted therapies for use in clinical studies, such as those in immuno-oncology (I-O), are the future of pharmaceuticals. They are the source of medical breakthroughs and the sales of these new medicines are rapidly increasing. Critically assessing the efficacy and safety of a targeted molecule in I-O trials is essential. Flow cytometry is a sophisticated technology that can provide specific information on how biologics and other targeted therapies interact with cells from a treated subject in clinical trials.

Flow cytometry is a technology for cellular analysis, providing clinically relevant and essential information during the clinical trial testing phase of new drug candidates. With laser technology and single cell analysis, flow cytometry precisely measures how specifically and efficiently a targeted therapy binds to its intended cellular receptor. In this way, flow cytometry can measure the proportion of available target receptors to which a drug candidate binds. This assessment may correlate with a study drug's therapeutic potential and clinical efficacy. In addition, flow cytometry can determine how the treated cells or test subjects are responding to treatment by measuring the presence of other molecules in biological matrices, such as blood or tissue samples. These capabilities make flow cytometric assays invaluable for characterising and evaluating preclinical research, demonstrating proof-of-concept, and monitoring patient outcomes in clinical trials.

Many standardised flow cytometric assays have been validated for common tests, and are off-the-shelf, for example quantifying immune cells such as T, B and natural killer lymphocytes. However, detecting the presence of molecules, such as bi-specific monoclonal antibodies or checkpoint inhibitors, studied in a number of I-O trials, that target specific receptors, requires custom flow cytometric assays specific to these molecular targets.

Given the cost, time and expertise needed to develop and qualify robust internal capabilities in flow cytometry, many sponsors find strategic partnering with a clinical research organisation (CRO) the best solution. A partner with the scientific expertise, global resources and mission commitment required to effectively develop and uniformly execute flow cytometric assays can help drive successful clinical trials forward, while increasing the overall efficiency of a pharmaceutical sponsor's drug development program.

Effectively deploying complex flow cytometric assays in global studies requires an infrastructure of laboratories capable of ensuring comparable results for tests, regardless of where and when they are done. In addition to sample collection and transportation monitoring, this global capability also requires significant investment, expertise and quality control experience to create and maintain.

This paper outlines best practices in developing and implementing flow cytometric assays for clinical study samples. Examined also are the benefits of adopting a strategic partner in a CRO to perform these complex assays. An important consideration in selecting a CRO is selecting one with the expertise to support targeted therapy development, by providing a customised approach to flow cytometry. A CRO which has the global reach to support clinical trials around the world is also paramount.

A customised approach to flow cytometry

Customised assays used in research and early clinical trials are major factors in improving the efficiency of targeted therapy development. Because of the specificity of these customised assays, they can powerfully predict the performance potential of targeted compounds in vivo. Three types of custom flow cytometric assays and how they can be used to predict and assess targeted therapy performance are detailed below.

1. Receptor occupancy assays

The potential effectiveness of drugs and biologics that target cell receptors can be assessed using receptor occupancy (RO) assays, which measure how efficiently the compound binds to immune cell surface receptors. The classic approach to RO flow cytometry assays is to develop two monoclonal antibodies that bind to a target protein: one that is non-competitive, meaning it binds to all target receptors even if they are already occupied by the test compound; and one that is competitive, meaning it only binds to target receptors not occupied by the test compound, since both the test compound and competitive antibody bind the same epitope. Both the non-competitive and competitive antibodies are tagged with different fluorochromes.

When added to a cell sample that has been treated with the test compound, the non-competitive antibody attaches to all target receptors, and these can be counted using flow cytometry to determine the total number of potential receptors in the sample. The competitive antibody binds only to unoccupied target receptors, and dividing this number by the number of total target receptors reveals the proportion of receptors bound by the test compound (see figure 1).

An alternate method involves sequentially treating the test sample with tagged competitive antibodies and antibodies that bind to the target compound bound to receptors, directly measuring bound and unbound receptors (see figure 2). In general, the higher the proportion of receptors bound, the greater the potential efficacy of the compound.

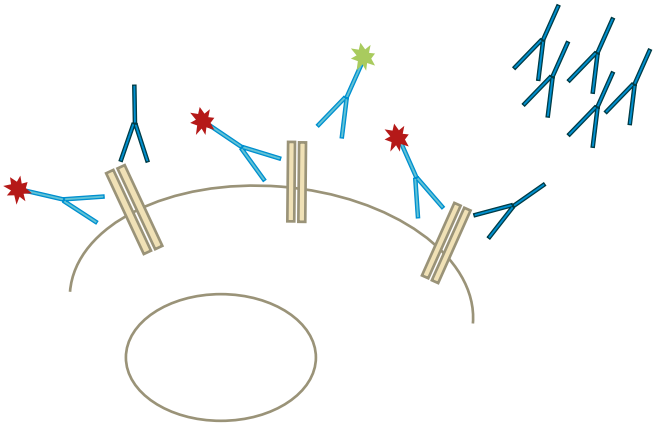


Figure 1. Determination of Receptor Occupancy Using Competitive and Non-Competitive Antibodies. A cell membrane receptor serves as the target for two different antibodies, one that is non-competitive ( ) and one that is competitive ( ) for the epitope recognised by the test drug ( ). A saturating dose of unconjugated test drug can be used as a pretreatment to bind all the target epitopes and determine maximal binding. Ideally, this binding is similar to that obtained with the non-competitive antibody.

Running RO tests on samples treated with different concentrations and exposure times in drug development and preclinical trials can generate useful pharmacodynamic information on which compounds are likely to be successful. Flow cytometry RO tests in clinical trials can be used to inform go-no-go decisions in early clinical trials and on dosage levels in later trials to assess clinical effectiveness. An analysis of clinical trials using flow cytometry between 2006 and 2018 found that these methods were most often used in phase 1, 1 / 2, and phase 2 studies. Since every target of a test compound is receptor-specific, a customised RO assay is required for every compound tested.

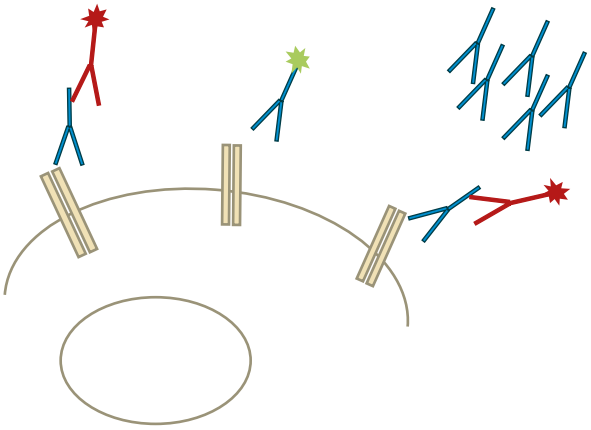


Figure 2. Receptor Occupancy Assay Experimental Design Using A Direct Detection Approach. A cell membrane receptor serves as the target for an antibody that is competitive ( ) for the epitope recognised by the test drug ( ). Wherever free target epitopes exist, competitive antibody will bind. Unconjugated bound study drug can be detected with a secondary antibody ( ). A saturating dose of unconjugated test drug can be used as a pretreatment to bind all target epitopes and determine maximal binding. Free venus bound receptor is quantitated and percent receptor occupancy is determined.

2. Immunophenotyping assays

Binding a targeted compound to one cell type may trigger intracellular reactions that affect other immune cells and immune responses, which can be either therapeutic or potentially hazardous. Therefore, immunophenotyping assays are often run concurrently with RO tests to detect these effects.

Immunophenotyping involves identifying a range of markers for immune activity in peripheral blood, bone marrow or tissue samples. These include the presence and quantification of sample characteristics including T cells, B cells and other white blood cell types with specific biomarkers. Like RO assays, these tests use antibodies tagged with fluorescent molecules that bind to specific molecular targets, allowing cells to be identified and counted by type. Many of these assays are standard and commonly used, though custom assays for identifying specific antigens may also be required.

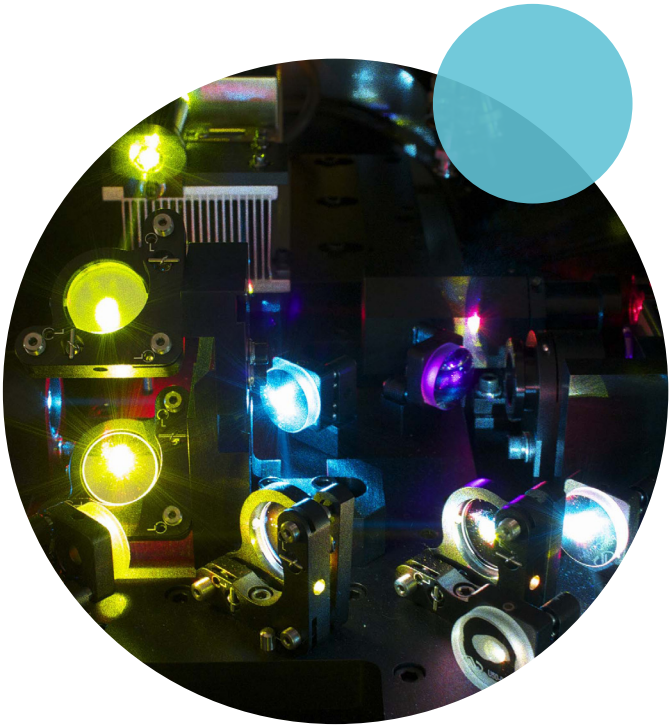
Summary of types of Flow Cytometry Assays Developed and Validated by ICON

- Receptor occupancy assays, customised per drug
- Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) assays
- Rare event enumeration – Myeloid Derived Suppressor Cells, Dendritic Cell Subpopulations, Blasts
- Ultra-rare event enumeration – Circulating Tumor Cells, tetramer assays for antigen-specific T lymphocytes
- T cell maturation phases – naïve, central memory, effector memory, effector
- B cell maturation phases – naïve, switched memory, non-switched memory, double negative
- *In vitro* stimulation and quantification of intracellular cytokines [IL-2, -4, -5, 13, -17, γ-IFN]
- Intracellular small molecule measurement [antibody-drug-conjugate] correlating to relative amount of drug delivered per cell
- NFκB phosphorylation assays
- p38 phosphorylation assays
- MACS bead separation of purified leukocytes
- Basophil assays
- Eosinophil assays
- Custom RUO flow cytometry assay approved by New York State Department of Health (NYSDOH) for use as a clinical diagnostic assay for patient enrolment [medical decision]

3. Functional assays

Functional assays seek to characterise not only the presence of specific immune cells and factors, but also how they may be induced by a candidate compound. Subjects are treated with the compound, and flow cytometry is used to assess changes before and after treatment in immune cell subpopulations. For example, intracellular cytokine production can be measured which can provide an indication of magnitude of response. This gives additional insight into the pharmacodynamics and potential efficacy and side effects of candidate compounds.

Taken together, RO, immunophenotyping and functional custom flow cytometric assays are powerful tools for developing targeted therapies. However, they do entail highly technical processes and clinical experience which are outlined below.



Best practices for developing and implementing flow cytometric assays in clinical development programs

Due to the complexity and highly technical nature of developing custom flow cytometric assays and performing standardised assays, many sponsors choose to outsource these functions to experts in the field. Regardless of whether assay design and execution are outsourced or undertaken internally, a close working partnership between product researchers, clinical study design and operations, regulatory and flow cytometry experts is essential for success.

Communication, early, often and ongoing

Open communication about the goals, methods and technical details of an assay program between developers and test experts is critical to matching assay approaches to specific study needs. Whether the need is for consistent execution of a panel of a previously determined standardised assay; refinement, validation and execution of an existing custom assay; or conceptualising and developing new custom assays, establishing a common understanding early makes the entire process easier and more efficient.

Because clinical development programs are iterative, with details and goals shifting as results and evidence accumulate, ongoing communication and collaboration are needed to ensure assays address changing needs. For example, differences between the health populations used to develop the assay and the actual study population characteristics may require adjustments to the assay protocol or interpretation framework as a compound advances through clinical trials. Also, depending on whether the assay will be used for research only, clinical trials, or clinical treatment, various state and national guidelines, typically those promulgated by the US Food and Drug Administration and the New York State Department of Health must be considered.

For portfolios with multiple products, it is helpful to take a strategic communication approach throughout all product development stages. This can ensure that tests run during early development provide the necessary information to inform clinical trial design, and inform decisions about which treatment candidates or programs have the best chance of success.

Robust and use-appropriate assay method validation processes

Demonstrating the scientific integrity of a study, not to mention winning regulatory approval, requires the presentation of methodologically and statistically reliable clinical evidence. Because flow cytometric assays provide evidence supporting product development, test programs must be designed to ensure they address relevant research and clinical questions in a way that will pass scientific and statistical scrutiny. It is also necessary to ensure assays are validated for the intended use of the results, such as for exploratory, clinical diagnostic for patient management during a trial, or as a companion diagnostic test for drug treatment.

Keep in mind that the evidence threshold differs according to the use of an assay, with its stringency increasing as patient risk increases in the development process. For example, assays for research-only use may be validated for use across laboratories and study sites with an initial demonstration of 20 percent to 30 percent precision on standard cell samples.

At the other end of the patient risk spectrum, however, flow cytometric assay requirements are more stringent. Any assay used to support patient treatment decisions, whether in clinical trials or in vitro diagnostic tests for monitoring clinical treatment, must undergo a more rigorous assessment across test laboratories.

For all flow cytometric methods to be implemented in clinical trials, validation processes should include:

– **A detailed plan** including experiment outlines and goals, such as timeline and specific types of cells or phenotypes that will be identified; intended assay use and any associated evidence acceptance criteria; descriptions of assay methods employed; and any equipment, reagents, test cell lines or other standardised supplies needed.

– **Quantitative assessments** of assay characteristics, including feasibility for measuring theoretically relevant parameters; biologic variability across sample sources when needed; precision of measurement; stability of samples and consistency of results over time and across sites; and statistical validation of results.

– **Documentation** of all processes, methods, instrumentation and materials sufficient to reproduce results.

Note that determining an appropriate assay for a particular clinical application is as much an art as it is a science. Experts with extensive experience developing, optimising and validating complex cell based methods that can troubleshoot issues as they arise are an invaluable asset for moving development programs and clinical trials forward.

Robust data reporting, analysis and validation processes

Similarly, demonstrating assay data integrity and significance are essential for showing study scientific integrity and passing regulatory muster. Details of data collection methods, raw data results and statistical frameworks, assumptions and calculations should be outlined to ensure reliability, and developed and documented to ensure they meet requirements.

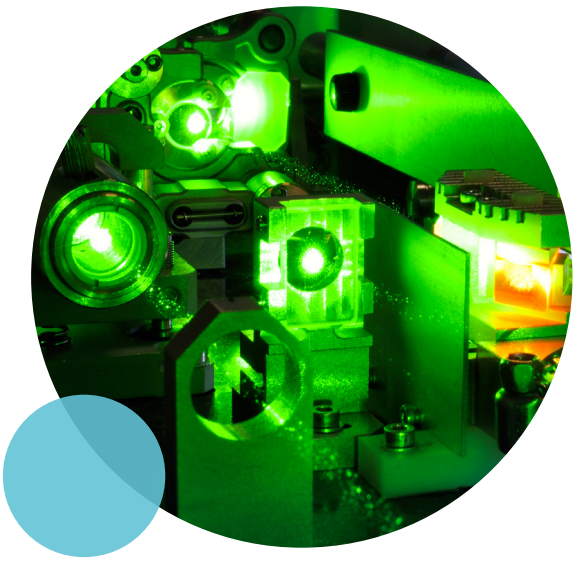
Quality control

Documenting quality control is critical for demonstrating assay reliability. Aspects that must be documented include:

– **Process quality control.** This includes ensuring appropriate sample collection, timely transportation, and any necessary temperature or other environmental control required to ensure sample integrity. It also includes ensuring processes for preparing and analysing samples are uniform at labs around the world.

– **Fluorescence quality control.** This entails ensuring that fluorescent tags necessary for identifying cells or cell-associated biomarkers are consistent over time and across global laboratories, and that antibodies for intended targets are reliable and specific.

– **Customised assay quality control.** Ensures that assays and processes – such as any special cell line, sample preparation and quantitative or qualitative evidence – required to measure or demonstrate a particular effect is developed, and scientifically and statistically validated as needed.



Case Study: Sponsor partners with ICON R&D to develop antibody drug candidate

The Challenge

A sponsor with exclusively small-molecule experience purchased a monoclonal antibody drug candidate. Customised cellular assays were needed to assess the drug and were required for clinical trial testing.

The Solution

With no experience in developing flow cytometry assays, the sponsor turned to ICON's Research and Development Department (R&D). Drawing on decades of staff experience and accredited facilities, ICON R&D developed and validated assays that enabled demonstration of proof-of-concept, mechanism-of-action and receptor binding.

In addition, a robust, cell-based biomarker assay and standard operating procedures were developed in compliance with regulatory standards for use in clinical trials. The test was implemented in central laboratories in the United States, Ireland and Singapore in support of global trials over several years. A custom QC process was developed to monitor the fluorescence intensity of key custom reagents whose stability was unknown.

The Outcome

Continuous communication to address data review for multiple trial cohorts provided high-quality data sets to the sponsor, suitable for regulatory submission. The client was highly satisfied with the results as the therapy moved toward approval.

Benefits of adopting a strategic flow cytometry partnership

Ensuring that the highly technical aspects of flow cytometric assays are suitable for specific development purposes is both valuable and difficult – particularly for sponsors with limited experience developing targeted therapies. A strategic relationship with an experienced partner can help sponsors capitalise on the benefits of flow cytometry expertise in developing high-value products at lower cost and in less time than developing a comparable internal capability.

In searching for a capable partner, ICON has the expertise and flexibility to build strong scientist-to-scientist relationships and long-lasting partnerships with sponsors. ICON provides not only assay experts with unparalleled experience and expertise, but also established regional laboratories and sample handling capabilities for supporting basic research and global clinical trials.

ICON as a Strategic Partner:

- Deep expertise to successfully develop and validate custom flow cytometric assays
- Broad experience identifying and meeting sponsor assay needs
- Uniform execution of tests across the globe
- Enhanced efficiency of clinical research and development program

Benefits of expanding fluorescent channels:

More colors + more information = better results

The selection of lasers has expanded, allowing up to 10 varying wavelengths to be used. Additionally, the number of fluorescent detectors used to capture light reflecting from cell samples has grown from one or two in early instruments to over 30 today.

Expanding the available fluorescence emission spectrum aids in resolution and helps distinguish fluorescent signals from background fluorescent noise. This greatly increases the ability of a flow cytometer to differentiate multiple phenotypic features, enhancing their usefulness in characterizing the multiple effects of drug candidates.

ICON routinely provides, at its global laboratories, the use of eight-color flow cytometers, and can now also offer global flow cytometric capabilities of measuring 25 fluorescence colors, using optimized assay panel designs. This 25-color capability significantly accelerates the ability to make more measurements simultaneously, which is especially important with limited specimen sample types. Multicolor analyses also improves the signal resolution, allowing for more accurate and sensitive measurements of low to rare cell populations. The expansion of instrumentation capabilities to detect up to 25 colors also allows our globally-standardized flow cytometers more precise identification of critical immune cell populations that are modulated by I-O targeted therapies.

Conclusion

Many sponsors prefer to outsource customised assays, requiring a partner with the expertise to consult the needs of their trial. The R&D Department at ICON seeks to cultivate a partnership with the sponsor and provide scientific expertise that enhances and helps drive the success of their clinical trials forward.

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For information on how ICON can be a strategic partner in facilitating clinical trials with flow cytometric testing contact Thomas W Mc Closkey Ph.D. at ICON R&D in Farmingdale, NY. Further information may be found at https://www.iconplc.com/ services/laboratories/global-central-laboratories/research-and-development

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