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Advanced Flow Cytometry Applications in Infectious Disease and Immuno-Oncology

Introduction

Time is of the essence. Whether identifying a vaccine candidate that induces production of robust neutralizing antibodies or developing a cancer treatment with life-saving potential, speed matters. Success relies on the ability to rapidly execute a variety of assays, often with small sample volumes, in a biologically relevant, high throughput, reproducible, and cost-effective workflow.

For infectious diseases, assays related to antibody function, neutralization studies and epitope mapping are essential for development of vaccines and therapeutics. In cancer, screening clinical candidates and measuring cellular response is foundational to selecting small molecules and monoclonal antibodies for development, while elucidating T cell phenotype, effector function and secreted cytokines are prerequisites for advanced cell therapies.

Among the technologies used to characterize antibodies, cell phenotype and response, and functional insights is flow cytometry. While flow cytometry is an indispensable tool for analysis of cells and cell-based assays, it is recognized as a difficult technique requiring a great deal of expertise to master. Because the technology is so complex, many researchers choose to route samples to centralized flow cytometry core labs, which further complicates workflows and significantly lengthens the time to results.

The iQue® advanced high throughput flow cytometry platform was designed to simplify this powerful technique without sacrificing advanced assay capabilities, thus expanding access to a greater number of labs and users. The system enables rapid, high throughput protein analysis, immunophenotyping, functional assessments and profiling, including antibody screening and immune cell activation. Deeper biological insights are enabled by simultaneous measurements of cell-specific parameters—such as phenotypic changes, proliferation rates, and secreted factors—in a single multiplexed assay, using volumes as low as 1-2 µL per well. The iQue® platform utilizes a patented sampling method, which allows for the fastest sample acquisition in the industry. The platform can rapidly process data from multiple assay plates, is compatible with 96, 384, or 1,536-well configurations, and offers continuous plate loading via connection with any automation system. Since this unique sampling method uses just a few microliters of sample, this enables researchers to conserve assay reagents and precious patient-derived samples while retaining material for further downstream characterization studies.

Combined with iQue Forecyt® software, which accelerates the transition from acquisition to analysis within minutes, data can be easily visualized and rapidly interpreted without the need for data extrapolation and export, even for complex biological assays.

This white paper describes use of the iQue® advanced flow cytometry platform for a wide variety of assays essential to the development of therapeutics and vaccines to battle infectious diseases and the advancement of therapies, both conventional and complex, to treat cancer.

Infectious Disease Applications

The emergence of novel pathogens continues driving a remarkable paradigm shift in the speed at which new vaccines and novel therapeutics are developed to fight infectious diseases. Time-to-result in discovery and development is critical. Increasingly powerful high throughput analytical techniques, accessible to drug and vaccine developers at any level of expertise, are available to accelerate workflows to characterize viral biology and the host immune response.

The following research studies demonstrate use of the versatile iQue® advanced flow cytometry platform to enhance throughput and streamline data analysis for a broad range of multiplexed assays including antibody isotyping, neutralization and functional studies, and epitope mapping.

SARS-CoV-2 Studies

The speed at which the COVID-19 pandemic reached all parts of the world made development of effective SARS-CoV-2 vaccines and therapeutics a global health imperative. As an emerging pathogen, all aspects of the virus had to be elucidated along with an understanding of the humoral immune response and rapidly emerging variants with enhanced infectivity.

Mercado, *et al.,* used the iQue® platform to quantify antibody titer for neutralization studies and to gain insights into immune cell function for recombinant, replication-incompetent adenovirus vectors encoding a full-length and stabilized SARS-CoV-2 spike protein.[1] The researchers developed a series of vectors encoding different variants of the SARS-CoV-2 spike protein and evaluated their immunogenicity and protective efficacy. Effector functions, including antibody-dependent neutrophil phagocytosis (ADNP), cellular phagocytosis (ADCP), complement deposition (ADCD) and natural killer activation (ADNKA) assays, were studied. In February 2021, Johnson & Johnson was granted emergency use authorization by the US Food and Drug Administration (FDA) for the Ad26.COV2.S vaccine, which was described in this publication.

In parallel with vaccine development, researchers explored the immunological mechanisms and serological signatures that underlie the different clinical trajectories experienced by COVID-19 patients. Atyeo, *et al.,* used the iQue® platform to assess levels of antigen-specific antibody subclass,

isotype, sialic acid, galactose and Fcγ-receptor binding levels in patients' plasma samples.[2] The platform was also used for functional analysis of plasma samples to quantify ADCP, ADNP, ADCD and ADNKA. The authors observed distinct antibody signatures among individuals with different outcomes.

Dogan, *et al.,* developed SARS-CoV-2-specific antibody and neutralization assays that revealed a wide range of the humoral immune response to virus.[3] The iQue® platform was used in conjunction with a highly sensitive bead-based fluorescent immunoassay from Sartorius for measuring SARS-CoV-2 specific antibody levels and isotypes in COVID-19 patient plasma and serum. This type of highly specific and sensitive assay is essential for understanding the quality and duration of antibody response to SARS-CoV-2 and in evaluating the effectiveness of potential vaccines.

As the COVID-19 pandemic continued to expand, several variants of concern emerged. Understanding the mechanisms leading to increased transmissibility and possible immune resistance was critical to guide intervention strategies. Cai, *et al.,* explored the structural basis for enhanced infectivity and immune evasion of SARS-CoV-2 variants.[4] Using the iQue® platform as an integral part of their workflow, the researchers determined that reshaping of antigenic surfaces of the major neutralizing sites on the spike protein can lead to resistance to some potent neutralizing antibodies and result in enhanced viral fitness and immune evasion.

In addition to vaccines, therapeutics will be essential to control the COVID-19 pandemic and complete protection from SARS-CoV-2 infection may require antibodies that block viral particles attaching to host cells and others to assist in eliminating infected host cells postinfection. Atyeo, *et al.,* note that while significant effort has been invested in identifying antibodies that block infection, the ability of antibodies to target infected cells through Fc interactions may be vital to eliminate the virus.[5]

To explore the role of Fc activity in SARS-CoV-2 immunity, the authors studied the functional potential of a cross-SARS-reactive antibody derived from a SARS-CoV-2 infected individual. The authors explored Fc functional profiling of the original antibody and an engineered version to examine the role of Fc effector function on the response to SARS-CoV-2 infection. ADCP, ADNP, ADCD and ADNKA functional insights were determined using the iQue® platform (Figure 1). Distinct Fc functional profiles resulted in enhancement of disease, pointing to antibody mechanisms of action that may be detrimental when developing antibody therapeutics against the virus.

While approved SARS-CoV-2 vaccines offer robust protection from the virus, the immunologic mechanisms of protection and how boosting alters immunity are not well understood. Alter, *et al.,* profiled the humoral immune response in non-human primates immunized with either a single- or double-dose regimen of the Novavax vaccine (NVX-CoV2373). The researchers used the iQue® platform to perform isotyping assays and derive functional insights including ADCP, ADNP, ADCD, ADNKA. Study results suggested that a single dose may prevent disease, but that two doses may be essential to block further transmission of SARS-CoV-2 and emerging variants.

Ebolavirus Studies

Ebolavirus causes severe disease in humans with an average mortality rate of 50% in past outbreaks. The devastating impact of this pathogen continues to drive development of both vaccines and therapeutic antibodies. In the studies outlined below, the iQue® platform was used to characterize neutralizing antibodies against clinically relevant ebolavirus species.

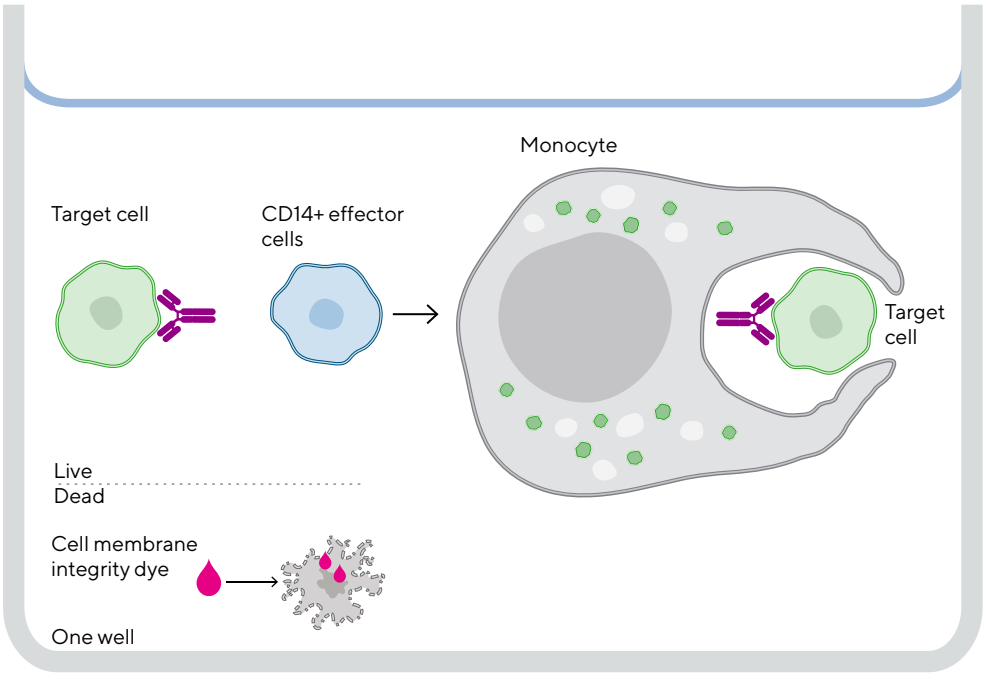
Gilchuck, *et al.,* isolated monoclonal antibodies from survivors of ebolavirus infections and identified a potent antibody which bound an epitope in the viruses' surface glycoprotein base region and effectively neutralized three different strains of the virus.[6] In a subsequent study, Gilchuk, *et al.,* analyzed the antibody repertoire in human ebolavirus survivors to identify a pair of neutralizing monoclonal antibodies that cooperatively bind the ebolavirus glycoprotein base region and glycan cap epitope.[7] The versatile iQue® platform was used in several experiments described in these publications, including evaluation of binding of antibodies to the ebolavirus glycoprotein expressed on the surface of Jurkat cells; the capacity of antibodies to inhibit cleavage of the glycoprotein, which occurs during infection; and binding of antibodies following epitope mapping using an ebolavirus glycoprotein alanine-scan mutation library.

Murin, *et al.,* further explored the mechanism behind activity of broadly neutralizing and synergistic glycan cap antibodies in concert with glycoprotein base-binding antibodies.[8] The iQue® platform was used to analyze synergistic binding of antibodies to cell-surface displayed glycoprotein and binding of antibodies to an ebolavirus glycoprotein alanine-scan mutation library. Results of the study provide the molecular basis for synergy, breadth of reactivity, and virus neutralization by potent glycan cap-directed antibodies and suggest a strategy for design of therapeutic antibody cocktails.

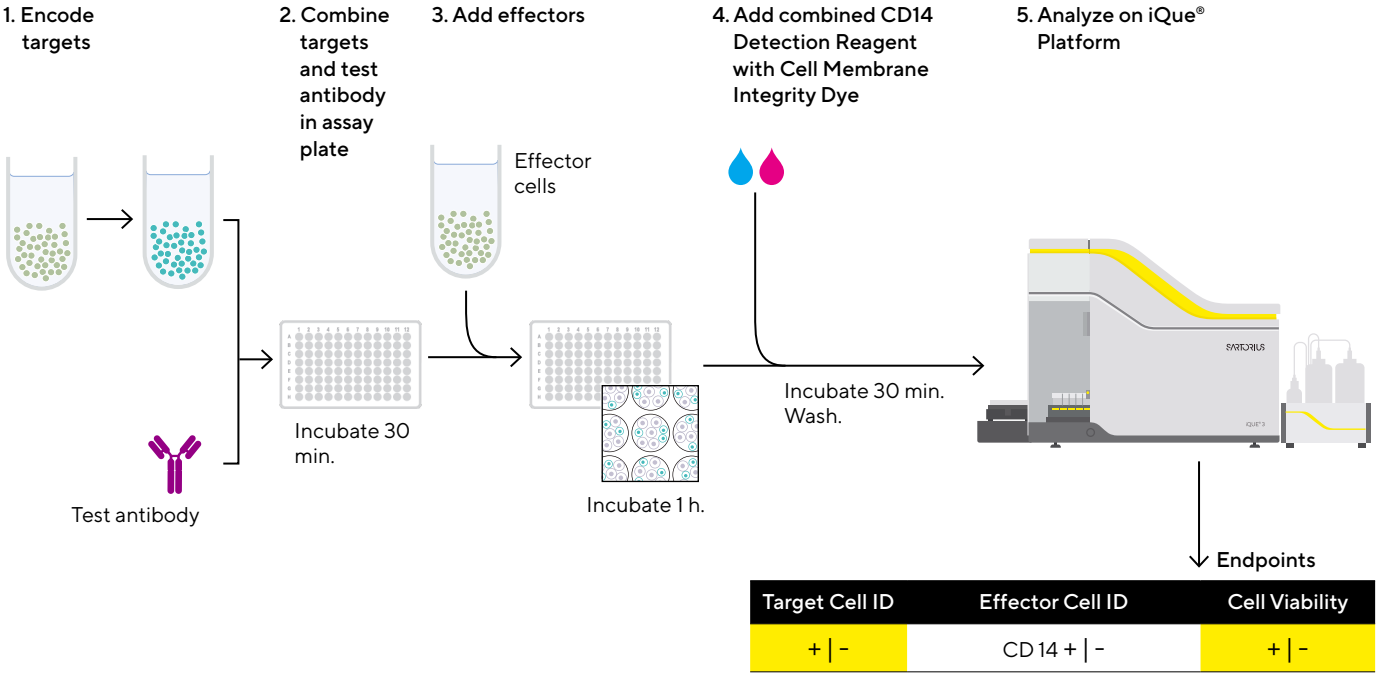
Figure 1

Rapid, Quantitative ADCP Assay Design Using the iQue® Platform

A.



B.



Note. (A) A rapid and quantitative ADCP assay design and workflow using the iQue® platform to investigate mechanisms of action in the field of antibody development is shown. Candidate antibodies can be tested for the ability to bind target cells engineered to display an antigen from a pathogen. The assay then provides a fluorescent readout for phagocytosis by monocytes, in a high throughput and multiparametric manner, and reports on cell viability, along with the ability to multiplex the readout for additional secreted proteins of interest, such as cytokines or effector proteins. (B) The streamlined workflow, with minimal wash steps, on the iQue® platform allows for rapid assay setup, acquisition, and integrated data analysis.

The utility of a protective combination therapy using two multifunctional human antibodies continues to be explored.[9] This study described development of a therapeutic cocktail comprising two broadly neutralizing human antibodies that recognize the ebolavirus glycoprotein and provides preclinical data to support clinical development for a pan-ebolavirus therapy. The iQue® platform was used to assess the binding of antibodies to ebolavirus glycoprotein and measure antibody-dependent cell-mediated cytotoxicity (ADCC).

Zika Virus Studies

Transmission of the Zika virus has been confirmed in dozens of countries with millions of cases of infection. Neutralizing antibodies to the Zika virus offer potential as both prophylactic and therapeutic agents. Long, *et al.,* demonstrated that a highly effective human monoclonal antibody has postexposure therapeutic activity against Zika infectivity in a mouse model.[10] The team used the iQue® platform to measure antibody reactivity to an epitope-mapping library generated using alanine-scanning mutagenesis.

Using Zika as a model virus, Gilchuk, *et al.,* developed and demonstrated an integrated sequence of technologies, including the iQue® platform, designed to enable rapid response for discovery of antiviral antibodies.[11] As an integral part of the workflow, the advanced flow cytometry platform was used for high throughput quantitation of monoclonal antibodies, competition binding analysis, and epitope mapping.

HIV Studies

Several critical challenges remain in the effort to fully understand the nature and progress of human immunodeficiency virus (HIV) infection. Kwon, *et al.,* used the iQue® platform as part of the workflow to define the specific subset of resting CD4+ T cells that harbor intact, replication-competent latent reservoirs of HIV-1 provirus.[12] Greater definition of these subsets would allow more specific targeting of reservoir cells and provide insight into viral persistence.

Another focus of investigation is how antibody effector functions evolve following HIV infection and how the humoral immune response is naturally tuned to recruit antiviral activity of the innate immune system. These questions were addressed by tracking the trajectory of the immune responses following acute HIV infection.[13] The iQue® platform was used in ADCD assays to better define antibody effector functions.

Broad Applicability to Other Infectious Disease Research

In addition to the pathogens described above, the iQue® platform has also been central to deriving insights into the induction of antibody effector functional responses in influenza[14] and Mayaro virus,[15] identifying new sites of vulnerability in the hepatitis C virus via alanine-scanning mutagenesis[16] and reporter virus production, epitope mapping, and neutralization in the study of dengue virus.[17]

The platform is also being used in novel applications such as viral-specific T cells (VSTs), which represent a possible treatment for viral infection after stem cell transplant.[18] To optimize production of VSTs, the authors designed a high throughput assay based on the iQue® platform to fully characterize T cell viability, function, growth, and differentiation. The system was used to measure T cell phenotype and function, including expression of memory markers, and cytotoxicity.

Hagen, *et al.,* have incorporated the iQue® platform into a workflow exploring how gut microbiota can impact responsiveness to vaccination, which has significant implications for increasing vaccine efficacy and improving global health.[19] The authors demonstrated the potential for antibiotic-driven perturbation of the microbiome to influence immune responses to vaccination in healthy adults.

Immuno-Oncology Applications

The versatility and speed of the iQue® flow cytometry system also delivers throughput advantages for the discovery and development of advanced cancer therapeutics including multispecific antibodies, antibody-drug conjugates (ADCs) and adoptive cell transfer as well as small molecules and monoclonal antibodies. The multiplexing capabilities of the platform enable drug candidate screening, phenotyping, functional studies, and cytokine measurements, along with characterization of cell subpopulations for cell therapy. The ability of the iQue® platform to enable assay miniaturization is especially critical for applications in immuno-oncology when the source material is limited, such as patient-derived cells.

Monoclonal and Multispecific Antibody Applications

Comacho-Sandoval, *et al.*, developed and validated an ADCC assay to test the efficacy and potency of biopharmaceutical products using conventional flow cytometry.[20] Based on their experience in the development and validation of bioassays under GLP-cGMP environment, they transferred this ADCC assay to the high throughput iQue® platform in which they were able to evaluate cell membrane permeability, caspase activation, and phosphatidyl serine exposure as characteristics of death on target cells in the same sample with low volume of acquisition. The authors note that these results demonstrated that high throughput technology is suitable for use in control quality environments, and that the automation provided a faster acquisition and analysis of data with precise and accurate results.

Immunotherapies targeting CD20 on the surface of tumor cells are an integral part of the care regimen for patients with non-Hodgkin's lymphoma and B-cell chronic lymphocytic leukemia. Unfortunately, disease relapse or recurrence occurs in many patients. Resistance of B-cell malignancies to CD20-targeting monoclonal antibodies is rarely a result of a loss of CD20 expression or mutations and as such, this protein remains an attractive target for therapeutic intervention. Preclinical studies have shown that a CD3 x CD20 bispecific antibody can induce T cell activation and T cell-mediated cytotoxicity towards CD20-expressing malignant B cells with high potency.[21] In this study, T cell activation and T cell-mediated cytotoxicity were measured by flow cytometry using the iQue® platform following co-culture with tumor cells.

Recruitment of native T cells by systemic delivery of bispecific "bridging" proteins has become an important modality in the fight against cancer. These molecules typically include a tumor-targeting moiety fused to an anti-CD3 recognition domain for engagement of T cells. This approach brings T cells into the proximity of tumor cells, triggering effector-driven lysis of target cells. Fierle, *et al.*, studied the potential of tumor endothelial marker (TEM-1), a cell surface antigen frequently expressed in the context of tumors, as a target for T cell-redirected immunotherapy to increase the selectivity of targeting compared to classical bispecific antibody formats.[22] The researchers used the iQue® platform for quantitation of effector cytokines produced by these novel trivalent T cell engagers.

Antibody Drug Conjugate (ADC) Applications

ADCs leverage the specificity of monoclonal antibodies to selectively target and deliver potent drugs to antigen-expressing tumor tissues. With this construct, these therapeutics have the potential to minimize the systemic side effects of their cytotoxic warheads. Success of an ADC is dependent on selection of an appropriate target on cancer cells to properly direct the therapeutic while sparing collateral damage to healthy tissue.

Raman, *et al.*, demonstrated the potential of glypican 2 (GPC2) as a possible immunotherapeutic target for a variety of cancers due to several factors including cell-surface location, tumor-specific expression, and tumor dependence.[23] A GPC2 ADC was shown to be efficacious against neuroblastoma and small-cell lung cancer via binding of a tumor-specific conformational-dependent epitope of the core GPC2 extracellular domain. In this study, the iQue® platform was used in the membrane proteome array workflow to determine whether the anti-GPC2 antibody displayed cross-reactivity with more than 6,000 human plasma membrane proteins.

Adoptive Cell-Based Therapy Applications

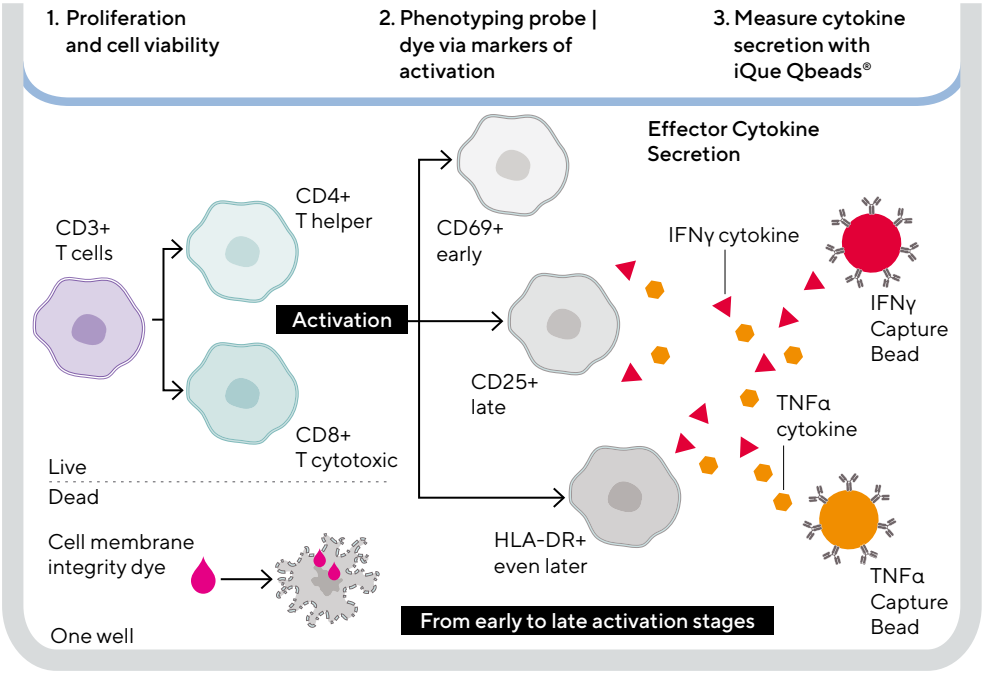
Cell-based therapies represent a remarkable leap forward in treating a wide range of challenging diseases and conditions. While these advanced modalities have delivered impressive clinical outcomes, the percentage of cancer patients that respond to this type of treatment remains frustratingly low. The potential offered by these therapies and the obstacles to their widespread application are driving a significant amount of research focused on understanding the complexity of the immune response.

Rizell, *et al.*, recently reported on the tolerability of ilixadencel, which consists of monocyte-derived, allogeneic dendritic cells stimulated with a combination of

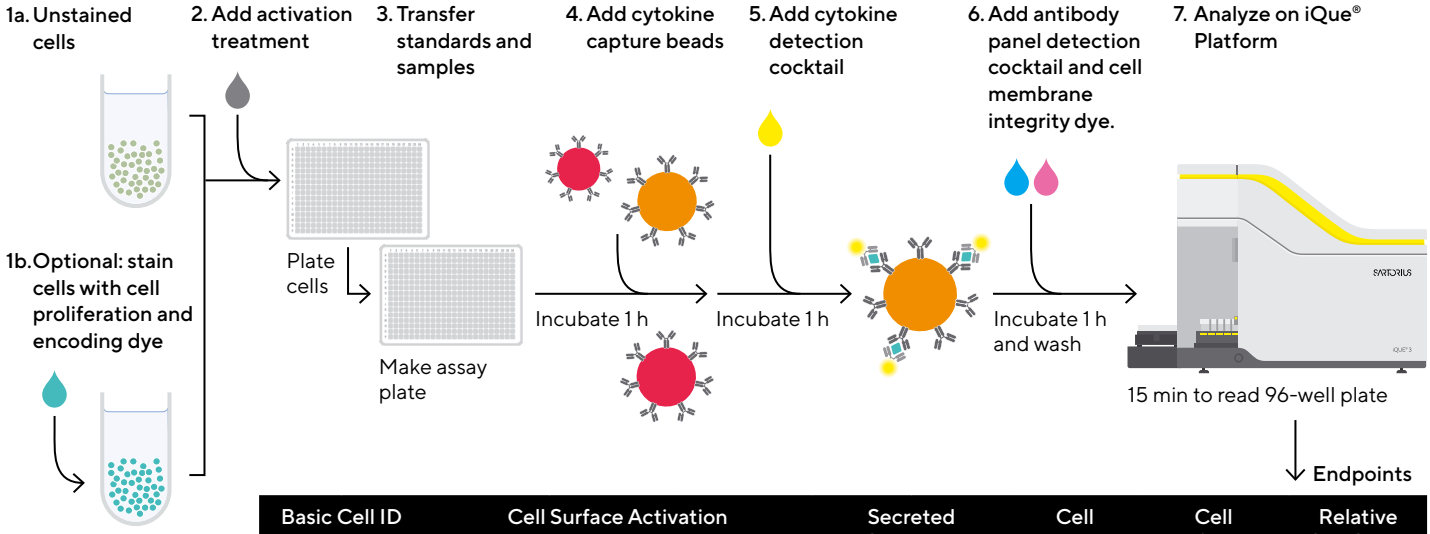
Figure 2

Measuring T Cell Activation Using the iQue® Platform

A.



B.



Basic Cell ID		Cell Surface Activation Markers			Secreted Cytokines		Cell Viability	Cell Proliferation (optional)	Relative Cell Count
		CD69 (early)	CD 25 (late)	HLA-DR (even later)	TNFα	IFNγ			
CD3+	CD4+	+ | -	+ | -	+ | -	+	+ | -	+ | -	+	+
	CD8+	+ | -	+ | -	+ | -	+ | -	+ (Th1)	+ | -	+	+

Note. (A) This describes a multiparametric assay for determining T cell activation state, immunophenotype, cell viability, and proliferation as well as the concentration of the secreted cytokines IFNγ and TNFα. These 11 metrics are collected from a 1-2 µL sample, from a single well on the iQue® platform. (B) The workflow from sample preparation to acquisition takes approximately 3 hours, along with pregated templates for simplified and visual data analysis of plates worth of data (Figure 2B).

proinflammatory factors, administered by intratumoral injection to function as an immune primer in hepatocellular carcinoma.[24] A possible mode of action for intratumorally injected ilixadencel is recruitment of alloreactive CD3+ T cells leading to a mixed leukocyte reaction (MLR) and production of cytokines that induce maturation of bystander dendritic cells. The impact of potentially coadministrated drugs, including anti-PD1 antibodies, on CD3+ T cell activation/proliferation against ilixadencel was investigated using the iQue® platform (Figure 2).

A novel CAR design with exquisite drug sensitivity, robust antitumor responses, and flexibility to enable multiplex antigen targeting or retargeting has been described by Leung, *et al.*[25] The authors believe this approach may further assist the development of safe, potent, and durable T cell therapeutics. The iQue® platform was used in these studies to measure cytokine production by modified T cells to monitor for cytokine-release syndrome, which is associated with many CAR-T responses.

Gene Transfer Applications

Common lentivirus vectors are efficient gene delivery vehicles but offer little specificity. This broad tropism limits their use for targeted gene delivery *in vivo*. To minimize engagement with off-target cells and tissues, a strategy referred to as pretargeting is being leveraged.

The approach defined by Parker, *et al.*, involves the administration of pretargeting molecules such as bispecific antibodies that bind both selected epitopes on target cells and nanocarriers, followed by administration of drug-loaded nanocarriers.[26] In their study, the authors explored how different bispecific formats may impact the efficiency of the pretargeting process. The iQue® platform was used in cell uptake assays to determine if the bispecific antibodies remained on the cell surface or were taken up by the cells. Use of tetravalent bispecifics was shown to be an important feature of pretargeting molecules and provides support for this approach as a promising nanoparticle delivery strategy. In a subsequent study, Parker, *et al.*, sought to improve specificity of lentivirus vectors using a bispecific antibody that binds both the vectors and cell receptors combined with ablation of the native receptor binding of the vector to minimize off-target transduction.[27] Coupling bispecific specificity and ablated native vector tropism synergistically enhanced the selectivity of the targeted gene delivery system. The authors believe that by abrogating the native broad tropism, this redirection strategy may enable lentivirus-based gene delivery *in vivo*, expanding beyond current *ex vivo* applications. Bispecific-mediated viral infectivity and transduction efficiency was measured by flow cytometry using the iQue® platform.

Small Molecule Applications

Small molecules continue to be a mainstay in the discovery and development of novel medicines, even as the drug industry has expanded into new modalities such as cell therapies, gene therapies, RNA interference (RNAi), clustered regularly interspaced short palindromic repeats (CRISPR), and others. The iQue® platform has been integrated into small molecule screening workflows, offering significant speed, throughput, and multiplexing advantages.

While 60% to 70% of acute myeloid leukemia (AML) patients enter complete remission after a standard induction regimen, most relapse within three years; the five-year overall survival rate is only 27%. Given these statistics, identification of novel treatment strategies for AML represents a pressing medical need.

Three recent studies incorporated the iQue® platform into workflows for screening potential therapeutics for AML. Baccelli, *et al.*, identified mubritinib as a strong *in vitro* and *in vivo* antileukemic compound, acting through ubiquinone-dependent inhibition of electron transport chain complex I (ETC1).[28] Kuusanmak, *et al.*, implemented an iQue® flow cytometry-based approach to simultaneously evaluate the *ex vivo* sensitivity of different cell populations in 34 primary AML samples to seven drugs and 27 rational drug combinations.[29] Data demonstrated that different cell populations present in AML samples have distinct sensitivity to targeted therapies. To characterize diversity in drug responses in major hematopoietic cell types, Majumder, *et al.*, developed a high throughput flow cytometry assay using the iQue® platform to enable monitoring of the dose responses of 71 oncology compounds simultaneously on multiple hematopoietic cell populations defined by surface antigen expression.[30] A comparison of drug responses in healthy and neoplastic cells (from patients with either AML, multiple myeloma, or chronic lymphocytic leukemia) showed healthy cell responses to be predictive of the corresponding malignant cell response.

The iQue® platform is also being used in a workflow to screen small molecule libraries for modulation of FOXP3 and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) in human regulatory T (Treg) cells. FOXP3+ Treg cells play an essential role in controlling immune responses in cancer and are actively being explored for their clinical potential. However, expression of FOXP3 in induced Tregs, recognized as the master regulator of Treg cells, is unstable and molecular targets involved in regulating FOXP3 expression and Treg cell function are not well defined. Drug targets capable of regulating FOXP3 expression and its downstream genes, such as CTLA4, have the potential to stabilize the Treg phenotype and function. Ding, *et al.*, have

developed an automated 384-well plate iQue® flow cytometry phenotypic assay measuring protein expression of FOXP3 and CTLA4 in human Treg cells.[31]

Inhibition of the antiapoptotic machinery of cancer cells is a promising therapeutic approach that has driven the development of small molecule BH3 mimetics, which mimic BH3 proteins by antagonizing the prosurvival function of antiapoptotic proteins, inducing apoptosis in cancer cells. To qualify as an authentic BH3 mimetic several criteria must be met including the need to function directly on the mitochondria of a cell of known antiapoptotic dependence, and directly and selectively inhibit the antiapoptotic protein with high-affinity binding. Villalobos-Ortiz, *et al.*, developed a comprehensive biochemical toolkit consisting of BH3 profiling in parallel with high throughput viability testing to validate BH3 mimetic candidates.[32] As part of this workflow, the iQue® system was used for viability testing.

Blake, *et al.*, used an iQue® flow cytometry-based assay to screen a library of more than 800 protein kinase inhibitors and identified compounds that promoted either the stability or degradation of MYC in a KRAS-mutant pancreatic ductal adenocarcinoma (PDAC) cell line.[33] Because stabilization of the MYC oncoprotein by KRAS signaling promotes growth of PDAC, a better understanding of how this stability is regulated may lead to effective therapies for a very challenging cancer.

A recent study incorporated the iQue® platform to help define the antiproliferative mechanisms of deferiprone (DFP), a hydroxypyridinone-derived iron chelator currently in clinical use for iron chelation therapy.[34] The authors found that DFP derives its antiproliferative activity largely from the inhibition of a subset of iron-dependent histone lysine demethylases (KDMs). They also identified new DFP-based KDM inhibitors that are more cytotoxic to cancer cell lines; one lead compound potently inhibited breast tumor growth in murine xenograft models. All flow cytometry-based assays were conducted using the iQue® platform.

Summary

As demonstrated by this collection of infectious disease and oncology applications, the iQue® platform represents a game-changing technology with unmatched throughput, versatility, and multiplex capabilities. When used with iQue Forecyt® software, which enables transition from acquisition to analysis within minutes, data can be easily visualized and quickly interpreted without the need for data extrapolation and export, even for complex biological assays. Whether performing functional studies to understand antibody neutralization or immune cell function in vaccine or oncology discovery and development, the iQue® platform will accelerate and streamline workflows, even for those who are not experts in flow cytometry, leading to actionable results in a compressed timeframe.

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