Advanced Flow Cytometry Handbook

A Guide to Advanced Flow Cytometry Assays and Workflows
First Edition

Simplifying Progress

SARTORIUS

Built to Empower: iQue® Advanced Flow Cytometry

About the Cover
Flow cytometry uses the color spectrum to identify and separate cell populations based on their unique fluorescence signatures. This enables a comprehensive understanding of cellular function and disease pathology, making it an essential tool in biomedical research, vaccine and drug development, and clinical diagnostics. Advanced techniques allow for high-throughput analysis at faster speeds than ever before.

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- Flow Cytometry by Sartorius
- Applications for iQue® Advanced Flow Cytometry
- iQue® Advanced Flow Cytometry Resources

Simplifying Progress

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Chapter 1 | Introduction

Built to Empower: How Advanced Flow Cytometry Simplifies Multiparametric Cell Analysis

What Is Flow Cytometry?

Flow cytometry is a powerful, laser-based method for analyzing the physical and chemical characteristics of individual cells in a mixed cell population. The standard flow cytometer consists of three main components: a fluidics system, an optical system, and an electronic system. The fluidics system directs the sample stream into a narrow, single-cell suspension. As the cells pass through laser beams in single file, the optical system measures specific properties like size, granularity, and fluorescence. The electronic system is responsible for digitizing and processing the photocurrent from the detector and saving the data for subsequent analysis using sophisticated software. Applications where flow cytometry analysis is used include antibody discovery, immune cell function, cytokine profiling, and cell health monitoring.

Compared to alternative methods, such as microscopy-based analysis, enzyme-linked immunosorbent assay (ELISA), western blotting, and mass cytometry, flow cytometry offers several key advantages. The overall speed of data generation is one major benefit compared to techniques like microscopy, ELISA, and western blotting, which involve manual analysis of individual samples and may require longer processing times. Unlike microscopy-based analysis, which often relies on subjective visual interpretation, flow cytometry provides objective and quantitative data on multiple cellular characteristics, including cell size, granularity, and the expression of specific markers or antigens.

The ability to perform multi-parametric analysis of tens of thousands of cells rapidly and accurately has made flow cytometry a staple technique in many research workflows. Multiplexed, multiparameter screening assays are foundational to the drug discovery process. By collecting more information, more rapidly—whether the focus is antibodies, small molecules, or phenotypic screening—researchers can identify the promising therapeutic candidates with greater confidence as they progress through the discovery and development stages.

Common Types of Flow Cytometry Analysis

Flow cytometry is used to analyze various types of samples, particularly those containing cells or particles.

- **Cell suspensions:** Analysis of different cell types, including immune cells, stem cells, cancer cells, and other cell types that are prepared by dissociating tissues or by isolating cells from blood, bone marrow, or other sources.
- **Blood samples:** Analysis of different cell populations in blood samples, such as white blood cells (leukocytes), red blood cells (erythrocytes), and platelets.
- **Tissue dissociates:** Analysis of tissue that is enzymatically or mechanically dissociated into single-cell suspensions for cellular composition, phenotype, and functional characteristics.
- **Subcellular particles:** Analysis of fluorescently-labeled subcellular particles, including vesicles, micro-particles, and liposomes for composition, size distribution, and other relevant parameters.
- **Apoptotic cells:** Distinguishing apoptotic cells from viable cells based on characteristic changes in cell surface markers, nuclear staining, and cell size.
- **Cell cycle analysis:** Assessment of proliferative status and cell cycle distribution by measuring DNA content using DNA-intercalating dyes.
- **Immunophenotyping:** Characterization of different immune cell types using antibodies specific to cell-surface markers.

Running a Flow Cytometry Experiment

The first step of running a flow cytometry experiment is sample preparation, which varies depending on the sample type and the experiment. Generally speaking, the sample is first isolated and purified to obtain a pure and homogeneous cell population for analysis. Washing steps with a buffer (e.g. phosphate-buffered saline) may be used to remove any residual fixative, debris, or media components. This helps to minimize background noise during analysis.

Labeling of the cells or particles with fluorescent probes, such as fluorochrome-conjugated antibodies, dyes, or other specific markers allows for identifying the desired cellular features or components—multiple staining panels can be used to analyze different markers simultaneously. Validated mix-and-read assay kits covering common biologics discovery and immunological applications can simplify this step.

Next, the prepared sample is injected into the flow cytometer, where it flows in a single file through a narrow nozzle or flow cell, creating a focused stream. While in flow, the cells cross the paths of one or more lasers that emit light at specific wavelengths. The illuminated cells interact with the laser light in two main ways: forward scatter and side scatter. Forward scatter measures the light that is scattered in the same direction as the laser beam, providing information about the size of the cells or particles. Side scatter measures light scattered at right angles to the laser beam, which gives information about granularity and internal complexity. Additional data is collected from other fluorescent labels attached to the cells.

The detectors in the flow cytometer convert the detected light signals into electrical signals, which are then analyzed and processed digitally. This information provides valuable insights about cell viability, cell cycle distribution, DNA content, protein expression, and various functional characteristics.

Limitations of Traditional Flow Cytometry

While flow cytometry is immensely useful for extracting insights from heterogeneous cell samples, there are drawbacks to conventional cytometers that impact time to results. The technique can be quite complex for researchers to integrate into their workflows, limiting direct access to the technology and requiring the use of centralized core labs with adequate expertise, which can slow the pace of discovery. This is partially due to non-intuitive systems that are difficult to set up and optimize prior to each experiment. Sample preparation can be another rate limiting step, often requiring long protocols and large sample volumes.

The multiplexing capabilities of flow cytometry provides a clear advantage over ELISA assays; however, low-throughput flow cytometry instruments fall short in the biopharmaceutical setting, where high throughput technologies are needed to quickly identify candidates with therapeutic potential. This paired with the high sample and reagent consumption makes it significantly more expensive to perform large screens on a traditional flow cytometry system.

Lastly, the single most challenging step of a flow cytometry experiment is the data analysis. Flow cytometry data often consists of high-dimensional data sets, making it essential to have appropriate analysis tools and deep knowledge on how to set gates to identify specific cell populations accurately, compensate for spectral overlap between fluorochromes, and apply appropriate statistical analysis methods. Performing these tasks typically involves multiple software tools and many disjointed steps.

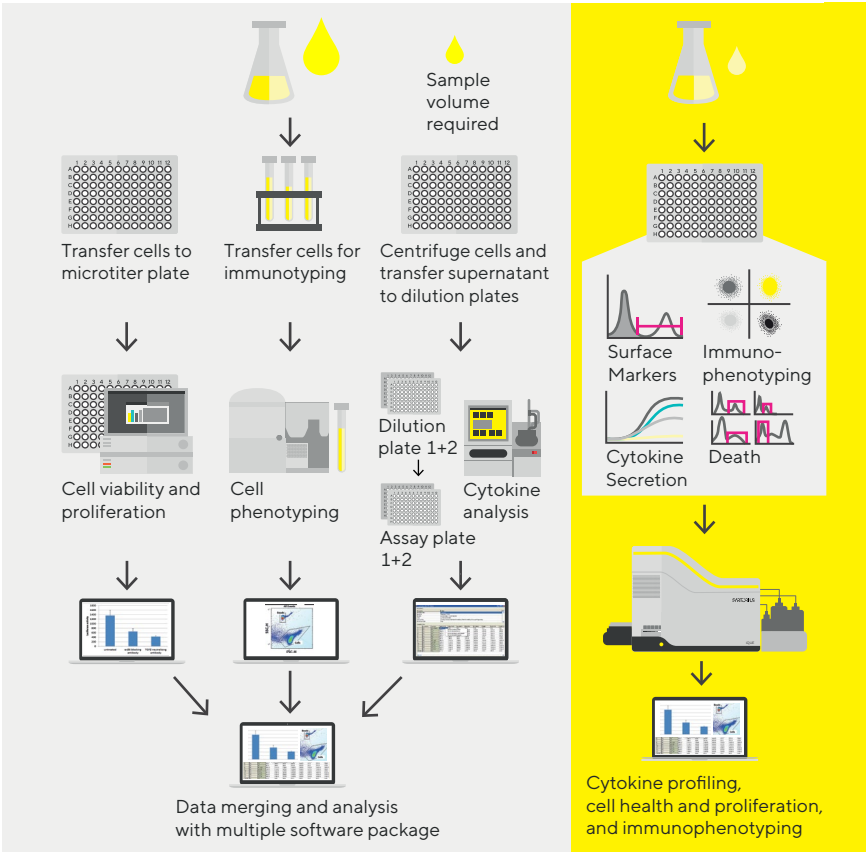
What Is Advanced Flow Cytometry?

The iQue® Advanced High-Throughput Flow Cytometry Platform re-imagines the standard flow cytometry workflow through combined innovations in both the system and software. The result is an equally powerful but versatile alternative, designed to offer advanced assay capabilities with the ease-of-use and cost effectiveness desired by research laboratories. Importantly, the technology is designed to be accessible, empowering every scientist to run an experiment and analyze the data with confidence.

The key benefits of advanced flow cytometry are:

- **Ease of use:** intuitive instruments with more automation
- **Speed:** faster workflow from setup and sampling to data analysis
- **Miniaturization:** saves precious samples and cuts reagent cost
- **Insight:** powerful software helps interpret complex datasets
- **Content:** higher multiplexing means more insights per cell

The iQue® Advanced High-Throughput Flow Cytometry Platform



Simply put, the iQue® Platform delivers more biological insights, faster and with greater ease. Multiplexed, high-throughput measurement of cell-specific parameters, protein analysis, immunophenotyping, functional assessments and profiling, including antibody screening and immune cell activation, deliver a breakthrough amount of data per well, with unprecedented speed.

A unique superpower of the iQue® Platform is the ability to combine both cells and beads in the same assay, allowing simultaneous assessment of biologically-relevant secreted proteins and cytokines. The iQue Qbeads® are available as both pre-set and customizable options for analyzing disease-relevant secreted proteins on the iQue® Advanced Flow Cytometry Platform. The beads function based on a "sandwich" principle to capture distinct proteins, enabling multiplexed quantitation of biological parameters for up to 30 secreted proteins in a single sample. A more detailed discussion of iQue Qbeads® can be found in **Chapter 3: Secreted Protein Quantification**.

The patented sampling method allows for the fastest sample acquisition in the industry; it can process a 96-well plate in as little as 3–5 minutes, or a 384-well plate in as little as 15–20 minutes, which is at least three times faster than the sampling speeds on other flow cytometers. Miniaturization on the iQue® Platform, reduces sample volumes to as little as 1 µL per well, helping to conserve precious samples and assay reagents. The platform rapidly processes data from multiple assay plates and is compatible with 96- or 384-well configurations, with options to connect to automated systems for plate handling.

Transformed Data Analysis and Compliance Workflow

A notable difference when using the iQue® Platform is the speed and ease of going from raw data to publication quality figures. This is thanks to the integrated iQue Forecyt® software, which essentially replaces the multiple software packages used with traditional data analysis workflows. These tools speed up the transition from acquisition to data analysis within minutes, allowing data to be easily visualized and rapidly interpreted without the need for data extrapolation and export even for complex biological assays.

The iQue Forecyt® software offers an intuitive and user-friendly interface, making it easy to navigate and perform data analysis tasks. Many routine tasks, such as identifying, and gating of subpopulations are fully automated based on user-defined criteria. This saves time and helps to improve data integrity as it reduces the number of manual steps required.

Real-time visualization capabilities of the iQue Forecyt® software allow users to view histograms, dot plots, scatter plots, or other graphical representations while the data is still being acquired to assess data quality, identify population characteristics, and make informed decisions during analysis. With advanced visualization tools like the panorama view and heatmaps, it is easy to quickly scan entire multi-well plates, or multiple plates, for the wells of interest.

Importantly, for laboratories working in drug development, quality control (QC), or manufacturing, the iQue Forecyt® software supports 21 CFR Part 11 workflows. iQue® users can easily meet the requirements of regulatory agencies with a secure and traceable electronic record-keeping system, export-ready custom audit reports, electronic signatures, and other security or accountability features.

Applications of Advanced Flow Cytometry

To efficiently characterize disease states and to develop a clear picture of how single cells respond to a stimulus, quantitative measurement of multiple biological readouts is required. iQue® Advanced Flow Cytometry applications provide streamlined protocols and validated mix-and-read assays kits for the assessment of multiple cell types and a range of cellular parameters in a single well.

- **Cell health:** Advanced flow cytometry techniques are widely used in cell health monitoring to assess the viability, functionality, and overall condition of cells. For example, fluorescent dyes, such as Annexin V, can be used to distinguish live cells from dead cells, or the progression of apoptosis in response to a drug.

- **Secreted proteins:** iQue Qbeads® Plexscreen allows researchers to analyze cytokines and other secreted proteins on the iQue® Platform. The protein-bound beads are individually detected as distinct events based on their physical and fluorescence properties, allowing simultaneous analysis of multiple biological parameters in a single experiment.

- **Cell line development:** High-throughput flow cytometry screening is commonly used in cell line development to identify cells expressing specific markers, desired functional attributes, or screening libraries of recombinant protein variants.

- **Biologics discovery:** By labeling target cells with fluorophore-conjugated antibodies, flow cytometry allows the evaluation of antibody binding specificity, affinity, and functional activity. These analyses are commonly used to identify high-affinity antibodies and characterize antibody-antigen interactions.

- **Immune cell models:** Immunophenotyping is one of the most common uses of flow cytometry. By staining immune cells with fluorescently-labeled antibodies specific to different markers, flow cytometry can identify and quantify various immune cell subsets, such as T cells, B cells, natural killer (NK) cells, dendritic cells, and macrophages.

- **Cell therapy:** Advanced flow cytometry plays a crucial role in various aspects of cell therapy, as a valuable tool for characterization, quality control, and monitoring of therapeutic cell populations. In chimeric antigen receptor (CAR)-T cell therapy development, for example, it allows for the determination of CAR expression levels and evaluation of functional properties, such as cytokine secretion and cytotoxicity. Flow cytometry is also used to detect and monitor target antigen expression on cancer cells.

About This Handbook

This handbook is designed to be the go-to reference for both new and seasoned flow cytometry users who are interested in the unique capabilities of advanced flow cytometry. Those using traditional systems will learn how routine workflows can benefit from the speed, throughput, and multiplexing capabilities offered by the iQue® Advanced Flow Cytometry Platform. Labs already familiar with the iQue® Platform can rely on this regular publication to learn about new reagents, assay kits, and software releases. Importantly, this handbook will feature the latest Sartorius publications so readers can stay up to date on the latest applications with advanced flow cytometry in both popular and emerging fields.

For further supporting information (videos and literature), see Appendix.

Chapter 2

Advanced Flow Cytometry Cell Health Assays

Measurements of cell health parameters are essential for studying the effects of drugs, culture conditions or genetic modifications on cell growth or viability. Such studies are used to rank compounds in drug discovery screens, identify off-target toxic compounds, as well as to investigate the cellular changes that underlie disease pathologies.[1] In order to assess cell health and viability, a variety of assays have been employed, such as ATP assays, LDH assays, cell imaging and vital dyes used in flow cytometry.

The advantage of using the iQue® Advanced Flow Cytometer for these types of measurements is the speed of sample processing and small sample requirement in combination with easy-to-use reagents and clear assay methodologies. In pharmaceutical applications, the high-throughput capabilities on the iQue® Platform allow easy integration into drug discovery workflows at an early compound assessment stage. Many of the individual reagents can be combined to provide the user with multiple assessments from a single well, providing more biological insights from a single sample. Alternatively, multiple cell types can be combined in one well with a single compound to increase the information output per sample (e.g., cross-species testing).

In this chapter, we will detail the use of advanced flow cytometry in combination with validated dyes and methods for quantifying important basic cellular measurements. The example data sets highlight the ease of use and speed of acquisition of the iQue® Platform, which together lead to simplified data analysis and increased throughput.

Advanced Flow Cytometry and Analysis Approaches

The iQue® suite of cell health assay reagents enable the detection and analysis of cell proliferation, cell viability, cell cycle, and apoptosis. Cellular samples stained in 96- or 384-well formats are automatically acquired and analyzed using the iQue Forecyt® software to generate data that can track cell health and reveal concentration-dependent responses which are used to calculate EC_{50} or IC_{50} values.

Proliferation and Encoding Dyes
Cell growth can be measured over several cell divisions using a range of fluorescent dyes in both adherent and suspension cell types. These are single dyes that have a dual purpose for both tracking proliferation and encoding. Encoding dyes can be used to measure cell type-specific growth rates or marker expression in co-cultures. This is done by staining cells with different concentrations of the dye to establish a unique fluorescence intensity.

Viability
Determining cell viability is crucial when assessing a cell's response to treatment, for example during drug discovery. The status of a cell in terms of being live or dead can be measured in a number of ways with different reagents targeting different mechanisms which indicate that the cell is compromised. When performing intracellular immunophenotyping by flow cytometry, a fixable viability dye is critical to preserve the staining pattern after fixation, and to properly identify cell populations.

Cell Cycle
The cell cycle encompasses the series of events necessary for a cell to grow and divide. Because the cell cycle plays such a fundamental role in cell biology, dysfunction of the cell cycle can lead to diseases including cancer and neurodegeneration. Cell cycle profiling of specific subpopulations is an important endpoint across the drug discovery process, from primary screening to toxicity profiling.

Apoptosis
Apoptosis is the process of programmed cell death, where cells undergo specific shutdown and digestion mechanisms. It can be triggered as a defense mechanism against toxic events or executed by cells that are no longer needed. There are numerous methods by which apoptosis is initiated and clear markers that can be assayed to determine if a cell has become apoptotic.

Proliferation and Encoding Dyes

Measurement of cell proliferation is a valuable endpoint in studies of cell growth and differentiation. The ability to proliferate is often a key indicator of cell health or a measure of the cell's ability to produce a functional response. These assays are a cornerstone of cancer therapeutic discovery, developmental biology, and drug safety research. Analysis of the sustained signaling pathways that underlie the progression of tumors, for example, accounts for >12,000 manuscripts in PubMed, the majority of which use cell proliferation analysis to evaluate tumor cell growth.

Proliferation measurements can be made in a number of ways. Simple, but crude, methods involve quantification of total cell number. Cell number is typically estimated by staining for total DNA content or monitoring the increase in metabolic activity. These techniques only approximate proliferation because they do not capture information about divisional history or the identity of proliferated cells. More sophisticated techniques detect new DNA synthesis as verification that proliferation took place. This method is more accurate as it is independent of the population size, which may not change due to turnover. These techniques, however, are not suitable for long-term studies that span many generations. Importantly, many methods are not amenable to high-throughput screening due to complex workflows, numerous wash steps, and large volume requirements.

iQue® Advanced Flow Cytometry Proliferation and Encoding Assays at a Glance

The iQue® Cell Proliferation and Encoding Dyes are designed for ease-of-use, multiplexing and high-throughput screening. The straightforward workflow can measure cell proliferation or can encode a cell population. Each dye has been specifically titrated for robust signal stability, while maintaining multiple open channels for additional endpoints.

Compared with other cell proliferation dyes, iQue® reagents offer these unique advantages:

- Suitable for short and long term studies:
  - Minimal cytotoxicity and stability for long term studies (up to six generations)
  - Encoder function optimized for short-term studies, such as antibody binding and cell-mediated cytotoxicity assays
- Fast linear response; there is no fluorescence intensity gap between the first and second generation of cells, enabling detection of proliferation in less time
- Robust and flexible solution for labeling from two to four fluorescent channels

The iQue® Cell Proliferation and Encoding Dyes are comprised of three spectrally distinct, proprietary dyes (V/Blue, B/Green, and R/Red) that can be multiplexed together, and with other iQue® reagents. The permeable dyes enter the cells, bind to either primary amine groups or to glutathione (depending on the specific dye) and then fluoresce.

Use As a Proliferation Dye

The iQue® proliferation assay functions on the principle of dye dilution. Cell proliferation is detected and quantified based on the halving or "dilution" of the loaded dye after each round of cellular division. Through subsequent cell divisions, daughter cells retain approximately half the fluorescent label of the parent cells (Table 1). Proliferated cells are distinguished from non-proliferated cells by decreased fluorescence intensity. To quantify cell proliferation, the number of proliferated cells can either be directly counted or calculated as a ratio of total cells and expressed as a percentage.

The iQue® Cell Proliferation and Encoding Dyes are designed and packaged to enable batch staining of cells in advance of plating into an assay plate. A straightforward dye loading protocol can be completed in ~30 minutes on standard laboratory hardware. Once cells are stained, they can be plated into microtiter plates and directly utilized in screening assays (Figure 1). No additional manipulations are necessary for the proliferation endpoint—representing a screening friendly, no-wash, plate-based protocol.

	Dead Cells	Latent Cells	Proliferating Cells
Generation 0			
Generation 1			
Generation 2			

Table 1

Assay Principle for the iQue® Cell Proliferation Dye

Note. Proliferating cells will have decreasing amount of dye, corresponding to lower fluorescence intensities. Dead or latent cells will maintain the initial dye intensity, which enables easy discrimination between proliferated and non-proliferated cells.

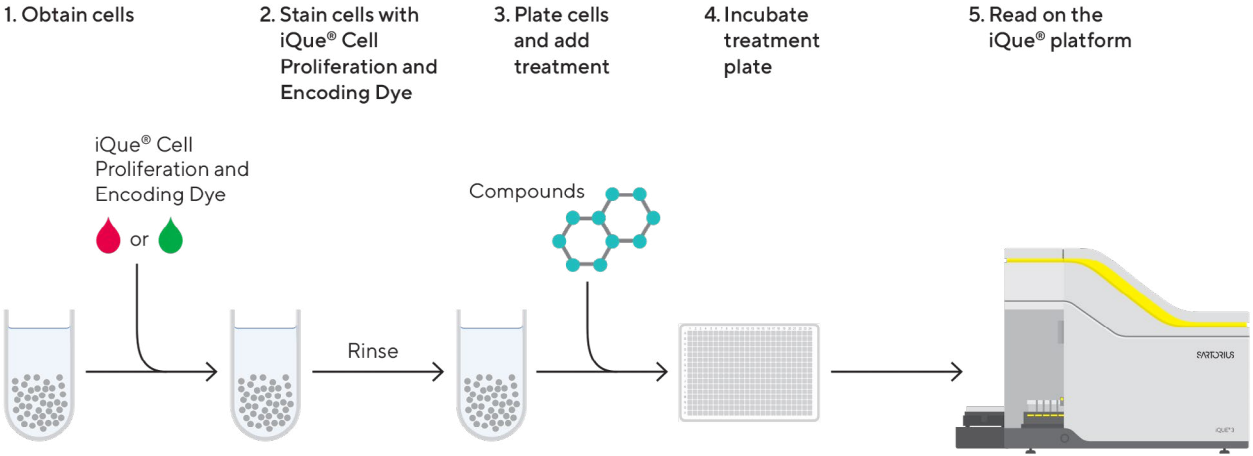


Figure 1

iQue® Cell Proliferation Dye Workflow

Note. Once a master stock of cells is prepared and stained in batch, cells can be directly added to assay wells. After the compound treatment and incubation is complete, samples can be directly analyzed without additional wash steps.

Use of Reagent as an Encoding Dye
Fluorescent cell encoding is a technique where different cell populations are labeled with different intensities of a fluorophore (Figure 2). This establishes a unique signature of fluorescence intensity that enables multiplexing of different cell types in a single well. The iQue® Encoding reagent uses the same dye as the iQue® Proliferation reagent. However, additional dye dilutions are prepared before staining to facilitate labeling of cells at different intensities.

The cell populations are then washed to remove the residual dye before they are combined for downstream assays. The addition of an unstained control yields four unique staining intensities. The iQue® Cell Proliferation and Encoding Dye has been extensively tested for screening applications using both adherent and suspension cell lines.

Example Results
iQue® Cell Proliferation Dyes produce a linear response in dividing cell lines, ≥ 6 generations. To demonstrate that iQue® Cell Proliferation Dyes can identify multiple generations of dividing cells, Jurkat cells were stained with iQue® Cell Proliferation and Encoding Dye (B/Green). Starting on the day cells were seeded (Day 0), cells were sampled daily and analyzed on the iQue® Platform (Figure 3). On each successive day, the median fluorescence

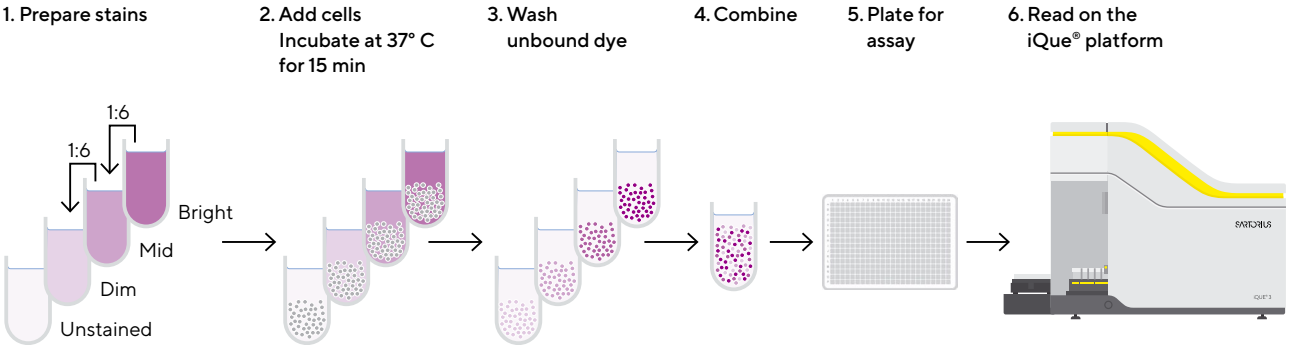


Figure 2
iQue® Encoding Workflow
Note. The master stock of dye is serially diluted to create additional dye concentrations, corresponding to Bright, Medium, and Dim staining intensities. Cells are prepared and stained in batch before being mixed into a single-cell suspension. The differentially-labeled cells can be used directly in binding and other short-term assays.

intensity (MFI) decreased. When the fluorescence intensity distributions of cells are overlaid, distinct separation is observed between daily measurements up to Day 8 (Figure 3A). Summarizing the data as MFI and plotting on a log-log scale shows linear response, as is expected for cultures dividing at a constant rate (approximately 24 hours for Jurkat cells) and with dye dilution of 50% at each generation (Figure 3B).

Summary

The example data shows that proliferation can be quantified using the iQue® Cell Proliferation and Encoding Dyes with simple workflows, ideally matched for high-throughput screening. When used in combination with an iQue® Platform the iQue® Cell Proliferation and Encoding Dyes enable multiplexing with antibodies and other dyes to yield a wide range of biological endpoints. Multiplexing can improve confidence in proliferation results and provides a richer description of the effects of your treatments, all while reducing costs. The encoder function offers an easy way to combine several cell lines in one well to further maximize data output.

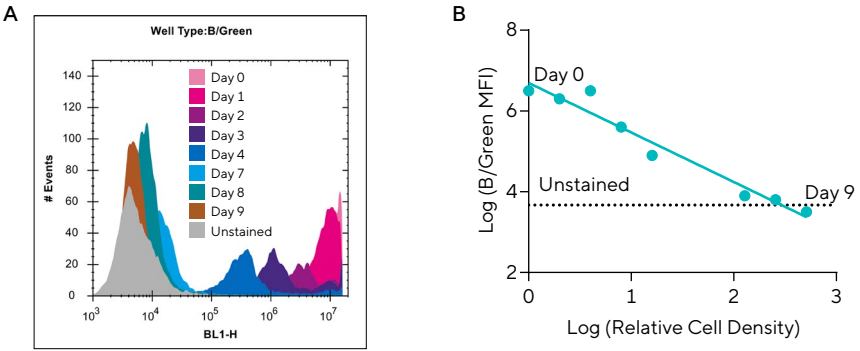


Figure 3

Tracking Cell Proliferation

Note. Jurkat cells stained with iQue® Cell Proliferation and Encoding Dye (B/Green) were sampled for up to 9 days, passaging cells on Days 2, 4, 7 and 9, to keep cells in log growth phase. The dye was able to distinguish the extent of proliferation between each day with visually distinct peak separation up to 8 days. The relationship between cell density and median fluorescence intensity (MFI) is linear across 9 days. Relative cell density was calculated as follows: cell density was normalized against the density at Day 0 and adjusted for the dilution factor on days that cells were passaged.

In summary, the iQue® Cell Proliferation and Encoding Dyes offer:

- Accurate measurements of proliferation, easily discriminating up to 6 generations of cells
- Easy multiplexing with a wide range of dyes and cell types in one well
- Optimized workflows for high-throughput screening

Viability Assays

Viability is an essential metric for determining the presence of toxic compounds during drug discovery workflows. There are two classes of common reagents that are used to assay cell viability. One class measures cellular activity or organelle function as surrogates for viability. The second class are membrane-impermeable reagents that use reagent exclusion to determine (or measure or monitor) cell membrane integrity as a measure of cell health. Loss of cell membrane integrity is a fairly late-stage event and is thus subject to less uncertainty compared to other reagent mechanisms where the cellular dynamics are still in flux and there is the potential for the cell to recover.

iQue® Cell Membrane Integrity Dyes are comprised of membrane impermeable reagents able to determine cell viability based on reagent exclusion as a measurement of cell health (Figure 4). A range of excitation and emission dyes enable flexible multiplexing with additional reagents, offering users a no-wash assay workflow, minimal cytotoxicity up to 48 hours after reagent addition, and robust signal stability with optimized titrations.

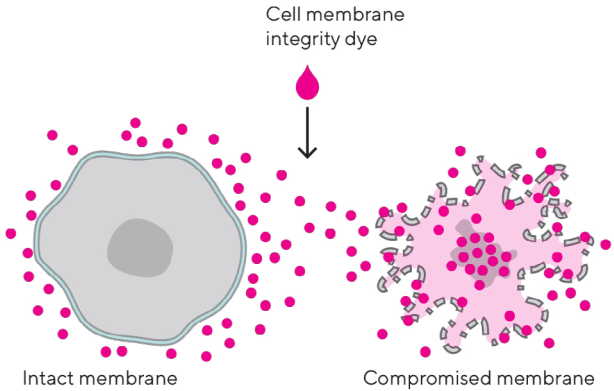


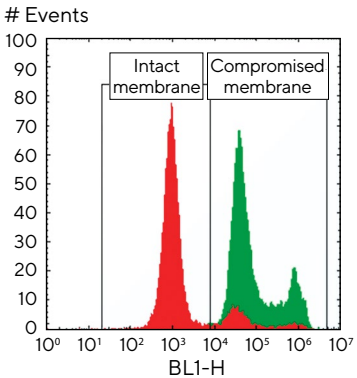
Figure 4

iQue® Cell Membrane Integrity Dye Assay Principles

Note. Cells with intact membranes can exclude the cell impermeable reagents and remain non-fluorescent. Once the membranes become compromised, the reagent enters the cell and binds to DNA by intercalation, creating a detectable fluorescent signal.

The data shown in Figure 5 demonstrates the shift in fluorescence seen in cells with these reagents. For both dyes, intact cells exclude the dye and display a lower fluorescence intensity. Cells treated to have a compromised membrane display a higher fluorescence intensity and identify the lower viability cell population. When multiplexing with other reagents, users can choose to gate on the viable or non-viable cells populations.

B | Green Cell Membrane Integrity Reagent



B | Red Cell Membrane Integrity Reagent

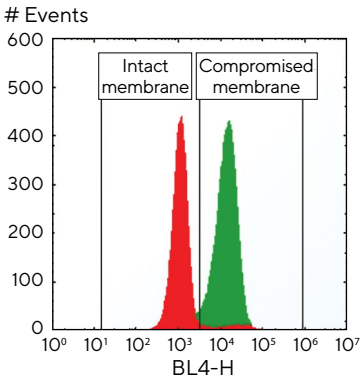


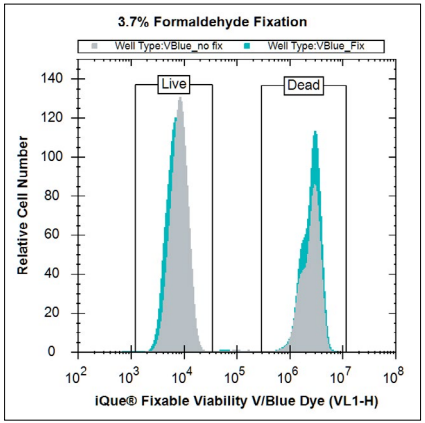
Figure 5
Identification of Cell Populations with Intact Membranes and Compromised Membranes Using the iQue® Cell Membrane Integrity Dyes

Fixable Dyes

The iQue® Fixable Viability Dyes are validated to quantify live and dead cells post fixation; permeabilization and intracellular staining of markers (immunophenotyping); and cytokines without any loss of intensity. These dyes are cell membrane-impermeant and covalently label cell-surface and intracellular amines, resulting in dim surface staining of live cells and highly fluorescent staining of cells with compromised membranes. The kits are compatible with formaldehyde and methanol-based fixatives.

Figure 6 shows representative histograms of staining with iQue® Fixable Viability Dyes. Figure 6A shows two samples of live and heat-treated (65 °C for 10 minutes) Jurkat cell mixtures that were stained with iQue® Fixable Viability (V/Blue) Dye. One sample was fixed with 3.7% formaldehyde (teal), the other sample was not fixed (grey). Figure 6B shows a mixture of live and heat-treated (65 °C for 10 minutes) Jurkat cells that were stained with iQue® Fixable Viability (R/Red) Dye, and fixed with 100% methanol. Both sets of data show there is a good separation of live and dead populations and no loss in signal post-fixation.

A



B

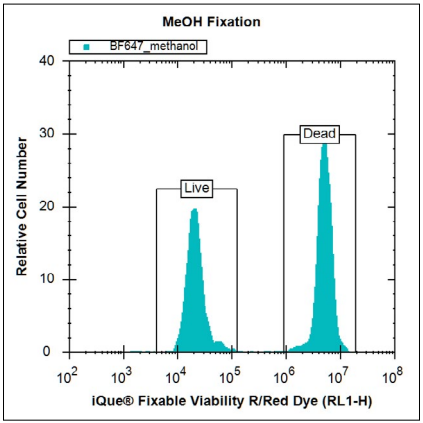


Figure 6

Representative Histograms of Live and Heat Treated (Dead) Jurkat Cell Staining with iQue® Fixable Viability Dyes

Note. (A) One sample was fixed with 3.7% formaldehyde (teal), the other sample was not fixed (grey) or (B) fixed with 100% methanol.

Cell Cycle Assessment

The cell cycle consists of four distinct phases: G0/G1 phase (growth), S phase (synthesis), G2 phase and M phase (mitosis). Each phase can be identified based on the relative amount of DNA present in the cell, with G0/G1 cells having the least and G2/M cells having the most amount of DNA (Figure 7). These relative differences in DNA content are easily detected by fluorescence intensity and can be utilized to establish quantitative cell cycle profiles. As the cell progresses through the cell cycle it will increase its DNA content to prepare for cell division. At the end of the cell cycle, each daughter cell will receive identical amounts of DNA. Theoretically, cells in the G2/M phase should have double the quantity of DNA relative to cells in G0/G1 phase. Practically however, most methods detect a relative ratio closer to 1.7 or 1.8 for these two phases.

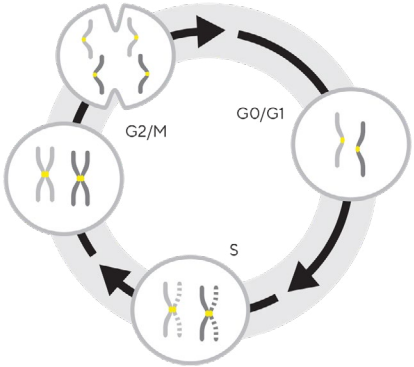


Figure 7
Determining Cell Cycle Stage by DNA Content

Note. The iQue® Cell Cycle Kit uses a fluorescent dye that intercalates into DNA, reporting content with enough sensitivity to distinguish between the G0 | G1, G2 | M and S phases. Unlike traditional methods, the kit requires no wash steps, and the live-cell stain can be added without the need to permeabilize, fix or perform an RNase treatment, requiring only a single, 1-hour incubation.

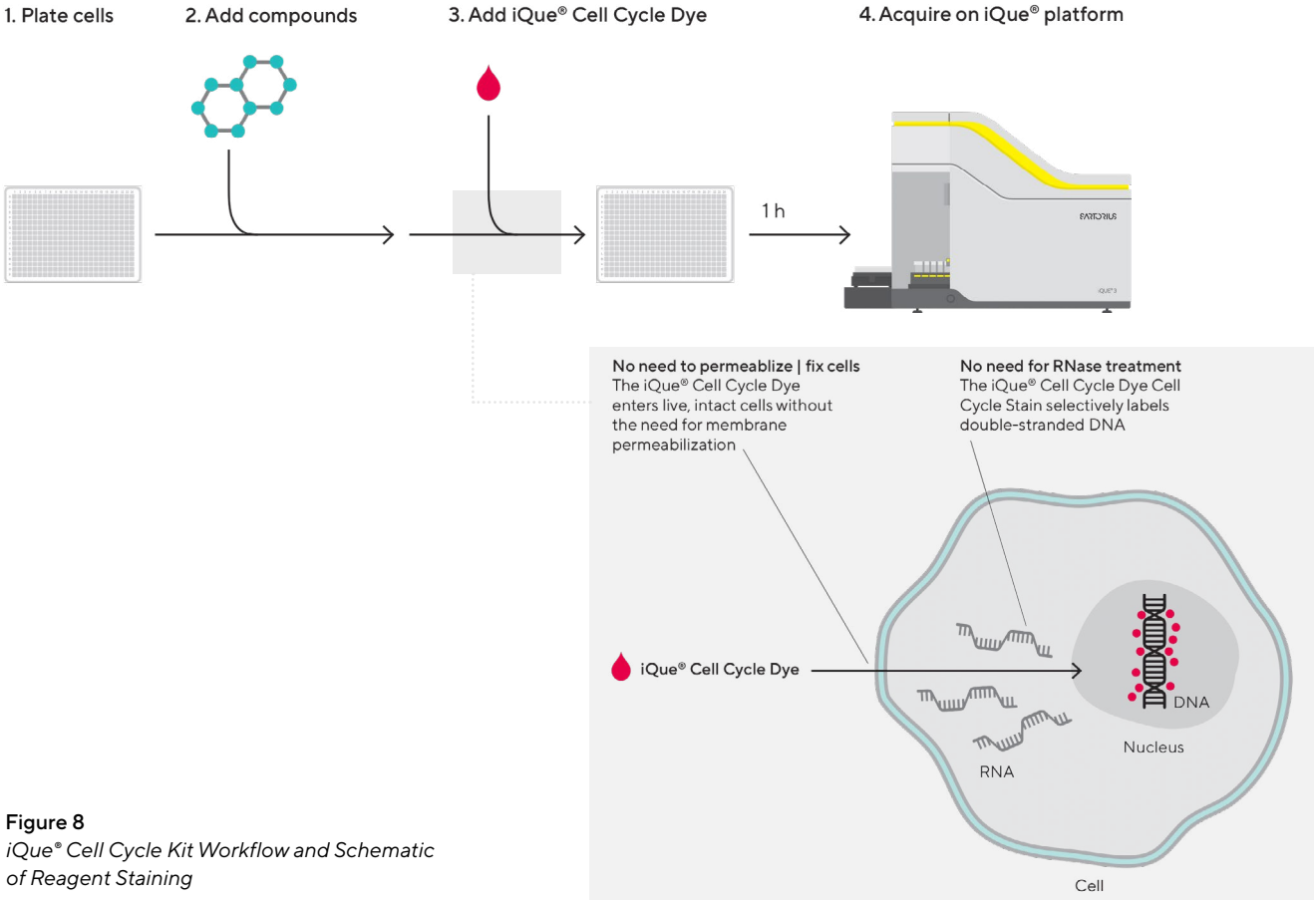


Figure 8
iQue® Cell Cycle Kit Workflow and Schematic of Reagent Staining

Example Results

Cell cycle inhibitors, some of which are used as chemotherapeutics, can disrupt the cell cycle and have distinct mechanisms of action that can be leveraged to investigate cytotoxic effects. The ability to screen for cell cycle perturbations is an important aspect of cytotoxic compound profiling. To detect cell cycle perturbations, eight different cytotoxic compounds were applied to Jurkat cells in a 384-well screening assay. Each compound was run in triplicate with top concentrations of 10 µM and 1:2 serial titrations for a total of 12 concentrations, along with a negative control. The dose-dependent cell cycle arrest in G2|M can be seen for nocodazole in Figure 9.

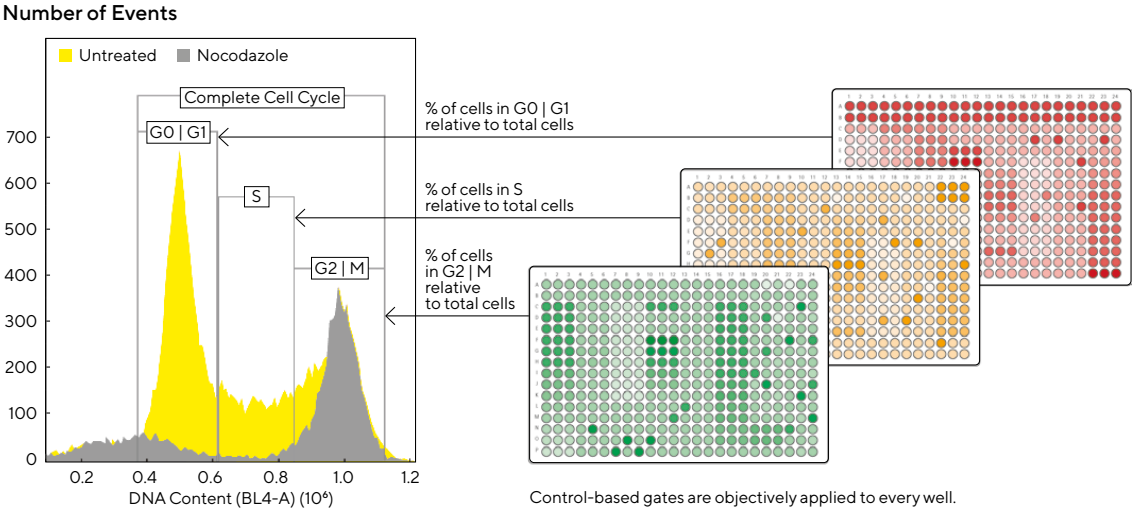


Figure 9

The Effects of the Cell Cycle Inhibitor

Note. Nocodazole (gray histogram), can be compared to untreated cells (yellow histogram) using the iQue® Cell Cycle Kit. The percentage of cells in each cycle (G0 | G1, G2 | M, and S) can be quickly compared across multiple plates using heat maps feature in the iQue Forecyt® Software.

Apoptosis and Cytotoxicity Assays

Apoptosis, the biological process by which cells undergo programmed cell death, is required for normal tissue maintenance and development. However, aberrations in apoptotic signaling networks are implicated in numerous human diseases including neurodegeneration and cancer.[2] Drugs that modulate levels of apoptosis have long been a target for drug discovery in a multitude of therapeutic areas. For example, many chemotherapy drugs kill cancer cells by upregulating apoptosis.

Numerous factors can induce apoptosis, such as: infection; severe DNA damage; hypoxia and disrupted cell cycle signaling.[3,4] These cellular stresses begin a molecular cascade through one of two main apoptotic pathways: extrinsic or intrinsic. The early phases of the intrinsic pathway involve depolarization across the mitochondrial membrane whilst the extrinsic pathway relies on signaling by 'death ligands'.[5] Both pathways join to follow a final execution pathway, beginning with activation of Caspase 3: a key enzyme for molecular cleavage in downstream events. The execution pathway involves mass biochemical and morphological changes, including protein degradation, DNA fragmentation, membrane blebbing, and extrinsic phosphatidylserine expression to promote phagocytosis.[5]

Flow cytometry assays offer significant benefits for studying the hallmarks of apoptosis—with the ability to quantify multiple cellular parameters at once. These assays also interrogate apoptosis at the single-cell level, rather than as an estimate from the bulk population, unlike some of the more traditional colorimetric assays.

iQue® Advanced Flow Cytometry Apoptosis and Cytotoxicity Assays at a Glance

The no-wash iQue® Human 4-Plex Apoptosis Kit allows the simultaneous detection of Caspase 3/7 activation, Annexin V binding, cell viability, and mitochondrial depolarization from a single sample (Figure 10), in addition to total cell count to identify overly toxic treatments.

The iQue® Apoptosis Kits are comprised of four spectrally distinct and mechanistically unique reagents that can either be used

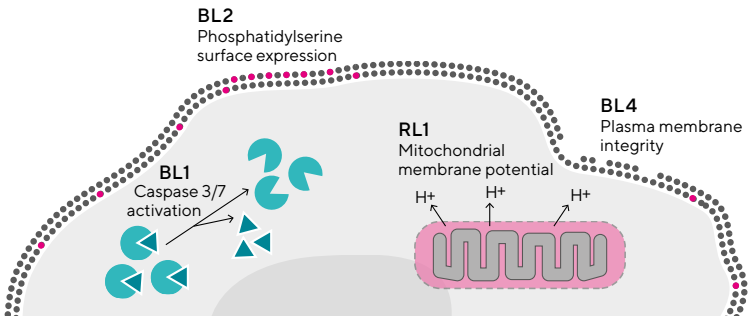
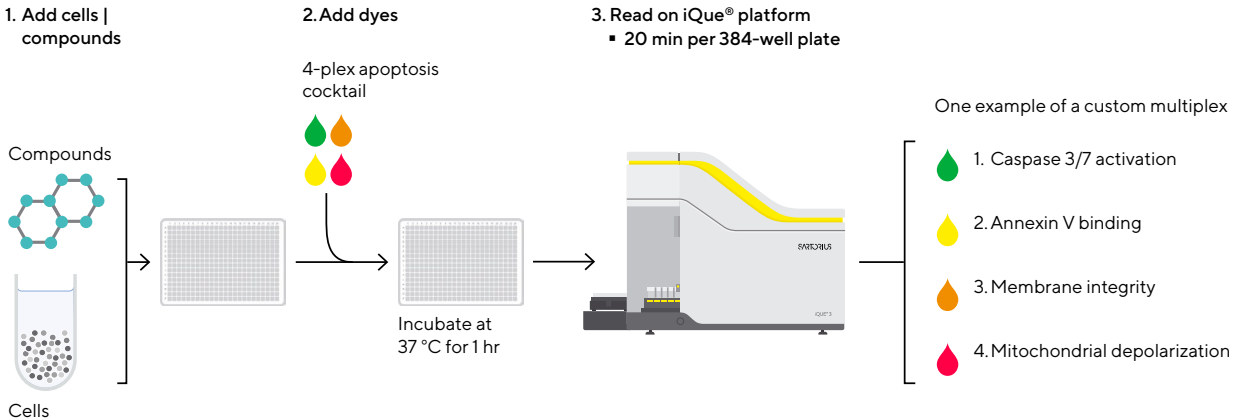


Figure 10
Principle of the iQue® Human 4-Plex Apoptosis Kit

individually or in multiplex. Each proprietary reagent has been carefully developed and optimized to match the detection capabilities of iQue® screening platforms.

- Activation of Caspase 3 and 7 is detected by the iQue® Caspase-3/7 Reagent substrate, which upon cleavage by activated enzyme, results in a fluorescent signal (BL1).
- Surface expression of phosphatidylserine is detected by the binding of Annexin V to the cell surface (BL2).
- Cell viability, as measured by membrane integrity, is determined by the inability to exclude a DNA binding dye due to compromised (porous) membranes (BL4).
- Mitochondrial membrane potential is determined by sequestration of a small fluorescent molecule inside the lumen of intact mitochondria with an active membrane potential. Upon mitochondrial depolarization the dye leaks into the cytoplasm and the cell exhibits a decrease in fluorescence (RL1).

The kit can be used as part of a drug discovery compound screening workflow as suitable for both 96- and 384-well plate assays (Figure 11). This has also been shown to work well for characterizing differential mechanisms, for example cytotoxic, verses cytostatic compounds.



*Apoptosis reagents can be ordered separately for custom multiplexing.

Figure 11
Schematic of the Simple, Mix-and-Read iQue® Human 4-Plex Apoptosis Kit Workflow

Example Results

To quantify the apoptotic profile, the percentage of cells that are negative or positive for each endpoint was determined. In the case of Caspase 3/7 activation, Annexin V binding, and cell membrane integrity, stained cells show an increase in fluorescence and are scored as positive for an apoptotic response. In cells with depolarized mitochondria, the loss of fluorescence is indicative of an apoptotic response. All four reagents can be used simultaneously (Figures 12A and B), or in different combinations according to experimental objectives.

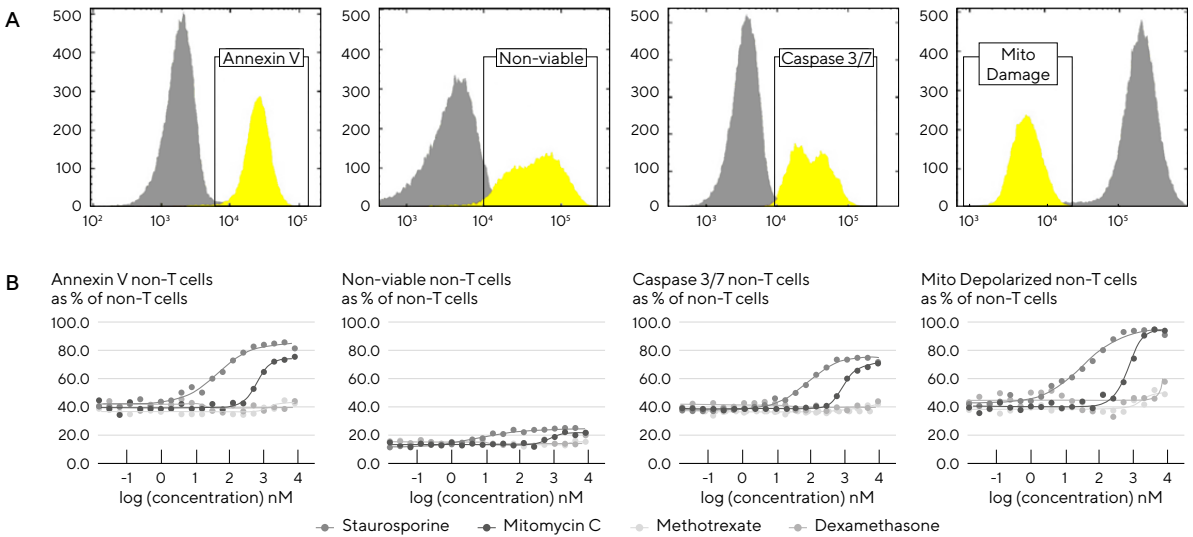


Figure 12

Note. (A) Four distinct hallmarks of cell death enable identification of apoptosis pathways. (B) Compound dose response curves to the addition of Staurosporine, Mitomycin C, Methotrexate, and Dexamethasone. Histograms and dose response curves generated on the iQue Forecyt® Software.

Apoptotic Markers Can Be Quantified in High-Throughput Using a 384-Well Plate Format

Drug discovery is reliant on high-throughput methods to provide fast profiling of large compound libraries. To illustrate the use of the iQue® Advanced Flow Cytometry Platform as a high-throughput tool for quantification of apoptosis, we screened the response of Jurkat cells to eight cytotoxic compounds. These compounds are common in scientific literature and induce apoptosis through well-defined mechanisms of action (Table 2). Jurkats were added to serial dilutions of each compound in a 384-well plate. Concentrations of each compound required to produce curves with defined maximal and minimal levels of apoptosis (over 24 hours) had been determined by previous experiments. Results in Figure 13 show the damaging effect (%) of each compound on the cells' mitochondria. Each compound displayed a unique pharmacological profile for induction of apoptosis. The iQue Forecyt® Panorama feature was used to rank the compounds from the most to the least potent based on depolarization of mitochondria. The ordered list of compounds and their corresponding EC_{50} values were exported directly from iQue Forecyt® and inserted into Table 2.

Drug	EC_{50} (nM)*	Mechanism of Action from Literature
Vinblastine	2.2	Chemotherapeutic; induces cell cycle arrest through disruption of microtubules[6]
Nocodazole	46.6	Induces microtubule depolymerization[7]
Staurosporine	126.7	Pan-inhibitor of protein kinase activity[8]
Mitomycin C	971.0	Chemotherapeutic; alkylating agent the cross-links DNA[9]
Etoposide	1582.1	Anti-cancer drug that induces DNA damage by binding to topoisomerase II[10]
Thioguanine	4114.9	Chemotherapeutic; incorporates into DNA as a nucleotide to exert toxicity[11]
Terfenadine	9533.9	H1 histamine receptor agonist; can induce DNA damage and activate caspases[12]
Thioridazine	30093.1	Inhibits the PI3K/Akt/mTOR/p70S6K signalling pathways[13]

* EC_{50} values exported from iQue Forecyt®

Table 2
Cytotoxic Drugs Ranked from Most to Least Potent Based on Mitochondrial Depolarization Effect

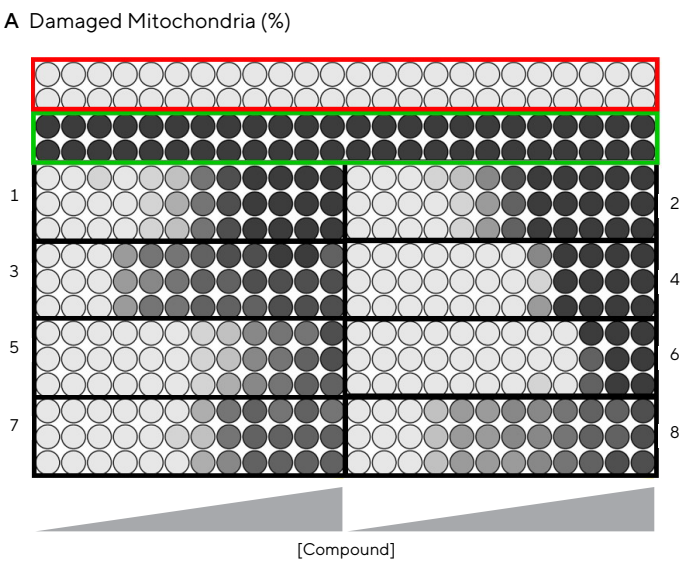


Figure 13

Screening Cytotoxic Compounds Reveals Varied Pharmacological Profiles

Note. Jurkat cells (1E6/mL) were incubated in a 384-well plate for 24 hours with a range of concentrations of eight cytotoxic compounds (n=3). (A) Heat map from iQue Forecyt® Software compares the proportion of cells with mitochondrial damage in each well. Red and green boxes show the negative and positive controls, respectively. Black boxes separate wells containing each compound (1=Staurosporine, 2=Etoposide, 3=Nocodazole, 4=Terfenadine, 5=Mitomycin C, 6=Thioridazine, 7=Vinblastine, and 8=Thioguanine). (B and C) Concentration-response curves for mitochondrial damage response (%). These were used to calculate EC_{50} values for each compound.

Summary
The iQue® Human 4-Plex Apoptosis Kit used in conjunction with the iQue® advanced flow cytometry platform provides a robust assay for measuring cellular progression through apoptosis via multiple pathways. This delivers valuable insight into the mechanism of action and pharmacological profile of cytotoxic drugs. Utilizing the speed of the iQue® and the inbuilt iQue Forecyt® software enables rapid profiling of compound libraries.

The experiments described have highlighted:

- Assays can be run in 96- or 384-well plate formats. The high throughput of the iQue® advanced flow cytometry platform facilitates gathering of pharmacological information, such as EC_{50} values, about multiple drugs in a short space of time.
- Readouts can reveal mechanistic differences between drugs.
- The iQue® Human 4-Plex Apoptosis Kit in combination with the iQue Forecyt® software can be used to easily study the effects of varying cytotoxic compound concentration on the apoptotic response.

Combined, these benefits make this technique a powerful tool with potential applications in drug discovery and cell biology.

For further supporting information (videos and literature), see Appendix.

References

1. Kepp, O., Galluzzi, L., Lipinski, M., Uan, J. and Kroemer, G. Cell death assays for drug discovery. *Nature Reviews* 2011 10:221.
2. Cotter TG. Apoptosis and Cancer: The Genesis of a Research Field. Nat Rev Cancer 2009, 9(7):501-507.
3. Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: cytometry in studies of programmed cell death. Methods Cell Biol, 103;55-98 (2011) Papaliagkas V, Anogianaki A, Anogianakis G, Ilonidis G. The proteins and the mechanisms of apoptosis: a minireview of the fundamentals. Hippokratia, 11(3);108-113 (2007)
4. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol, 35(4);495-516 (2007)
5. Anttila JV, et al. Contrasting the impact of cytotoxic and cytostatic drug therapies on tumour progression. PLoS Comput Biol, 15(11);e1007493 (2019)
6. Moudi M, Go R, Yien CY, Nazre M. Vinca alkaloids. Int J Prev Med, 4(11);1231-1235 (2013)
7. Beswick RW, Ambrose HE, Wagner SD. Nocodazole, a microtubule de-polymerising agent, induces apoptosis of chronic lymphocytic leukaemia cells associated with changes in Bcl-2 phosphorylation and expression. Leuk Res, 30(4);427-436 (2006)
8. Karaman MW, et al. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol. 26(1);127-132 (2008)
9. Tomasz M. Mitomycin C: small, fast and deadly (but very selective). Chem Biol, 2(9);575-579 (1995)
10. Azarova AM, et al. Roles of DNA topoisomerase II isozymes in chemotherapy and secondary malignancies. Proc Natl Acad Sci USA, 104(26);11014- 11019 (2007)
11. Nelson JA, Carpenter JW, Rose LM, Adamson DJ. Mechanisms of Action of 6-Thioguanine, 6-Mercaptopurine, and 8-Azaguanine. Cancer Res, October 1; 35(10);2872-2878 (1975)
12. Jangi SM, et al. H1 histamine receptor antagonists induce genotoxic and caspase-2-dependent apoptosis in human melanoma cells. Carcinogenesis, 27(9);1787- 1796 (2006)
13. Kang S, et al. Thioridazine induces apoptosis by targeting the PI3K/Akt/mTOR pathway in cervical and endometrial cancer cells. Apoptosis, 17(9);989-997 (2012)

Chapter 3

Advanced Flow Cytometry for Secreted Protein Quantification

Cellular signaling pathways encompass a broad range of secreted proteins and cytokines. Quantification of these molecules is critical in basic research and drug development, as it can facilitate increased understanding of the pathways of immune cell activation and cellular signaling cascades in inflammation and oncology. Aberrant production and/or secretion of proteins have long-reaching effects for physiological processes, and dysfunctional signaling is implicated in virtually all diseases. The detection and quantification of soluble proteins in solution provides key information for researchers in therapeutic areas ranging from cancer and immunology to aging and regeneration. Characterizing the complex signaling pathways between cells is an important target across the drug discovery process, from primary screening to toxicity profiling.

Studies often revolve around the ability to detect, quantify, and discriminate specific or multiple cytokines from a multitude of biomolecules present in any given sample. Researchers currently employ a variety of established immunoassay techniques, such as enzyme-linked immunosorbent assay (ELISA), multiplexed assays, and flow cytometry-based assays to analyze the level of secreted proteins in various disease models and exploratory research areas. However, traditional techniques for measuring protein secretion have some drawbacks:

- Low-throughput, laborious and time-consuming, requiring steps such as labeling, protocol optimization, long incubations, and repetitive washes
- Require centrifugation steps to separate supernatants from cells
- Generally limited to analyzing one analyte per sample, increasing data variability, and consuming large volumes of precious sample and reagents
- Complicated, manual analysis of datasets

iQue Qbeads® are a family of reagents that allow researchers to analyze secreted proteins through their capture on distinct bead types, enabling multiplexed quantitation of biological parameters on the iQue® Advanced Flow Cytometry Platform. iQue Qbeads® are available in two general classifications: iQue Qbeads® Plexscreen and iQue Qbeads® Devscreen.

The iQue Qbeads® Plexscreen class are ready-to-use kits complete with buffers, detection reagents and standard analytes. In total, there are over 50 analytes available to quantify human-, mouse- or rat-secreted proteins. The iQue Qbeads®

Devscreen class, including streptavidin-coated beads (SAv beads for conjugation with biotinylated proteins) and SH-derivatized beads (for conjugation with any protein), are building block reagents used to build custom beads.

iQue Qbeads® are highly reproducible, significantly reduce sample volume requirements (as low as 10 µL) and decrease the time to results with less hands-on time and a no wash assay. The iQue Qbeads® assay can be multiplexed with other iQue® kits, for high-throughput measurement of protein secretion in addition to viability, cell surface marker expression and proliferation through a simplified, streamlined workflow. Following sample acquisition, pharmacological analysis can be performed quickly and easily using the integrated iQue Forecyt® software, with secreted protein concentrations automatically calculated through comparison to a standard curve.

Assay Principles

iQue Qbeads® function on the same principles as a sandwich ELISA. Using a single analyte as an example (Figure 1), beads coated with capture antibodies targeting an analyte of interest are combined with the sample. Samples can be from cell culture supernatant, serum, or plasma. Once the analyte is bound to the capture beads, a fluorescent detection antibody is added which binds to the analyte and forms a "sandwich." The fluorescence signal intensity of the bead complex directly correlates to the quantity of bound analyte.

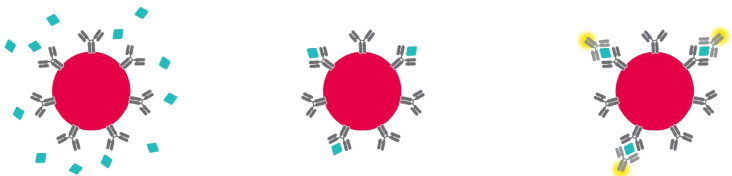


Figure 1

Schematic of a Bead-Based Sandwich Assay Showing a Single Capture Bead Detecting a Single Analyte Type

Note. Once the complex is fully formed, the fluorescence signal is directly associated with the bead, and the signal intensity is proportional to the bound analyte concentration.

iQue Qbeads® uniquely enable a screening-friendly, no-wash protocol (Figure 2A). If desired, greater low-end sensitivity can be achieved for some analytes by performing a single wash step to reduce background just prior to reading on the iQue® Platform. Quantitative readouts from this assay can be measured as fluorescence intensity or interpolated to a concentration (pg/mL) in solution via the use of a standard curve. Specific beads designed to capture different analytes are also fluorescently tagged with a unique signature, allowing for up to 30 beads to be combined in a single sample (Figure 2B). The assay builder provides a simple system to help design your custom Qbeads® Plexscreen by guiding you through the choice of 50 available analytes. The iQue® Platform then discriminates the various bead sub-populations and the associated analytes during analysis. On the iQue® Platform, cells and Qbeads® in the same sample are easily distinguished based on their scatter characteristics (Figure 2C). This separation of cells and beads and the ability to discriminate between them is maintained when other cell types or even complex cell mixtures such as PBMCs are used.

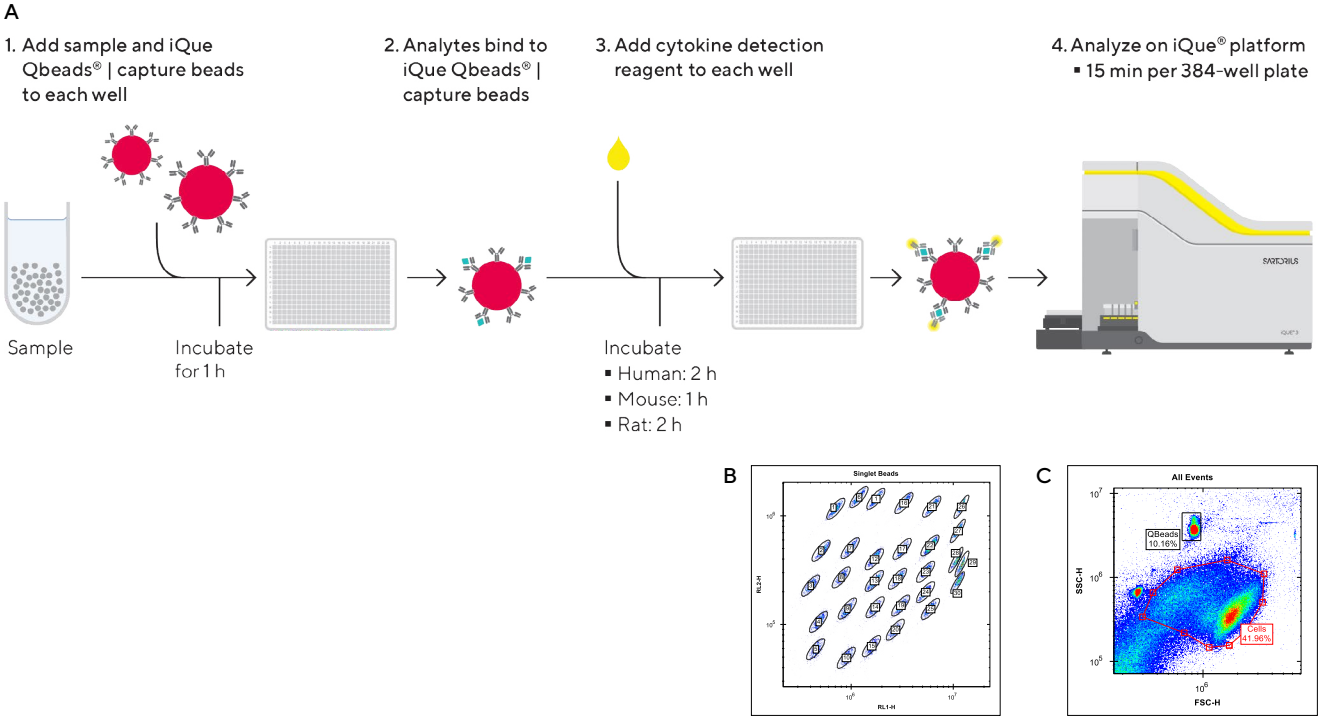


Figure 2

A Schematic of the iQue Qbeads® No-Wash Workflow on the iQue® Platform

Note. (A) Combine 10 µL of mixed beads and 10 µL of sample, then incubate for 1 hour. Add 10 µL of detection reagent and incubate for 2 hours for human and rat analytes, or 1 hour for mouse analytes. Finally, directly read the plate on an iQue® Platform. (B) Distinct identification of iQue Qbeads® and cells characterized by iQue Forecyt® Software population gating. (C) The iQue Qbeads® Assay Builder provides a flexible assay format that facilitates analysis of up to 30 secreted protein concentrations in a single well.

Example Results

Pembrolizumab Induces Inflammatory Cytokine Release in a Mixed Lymphocyte Reaction (MLR)

Common side effects of checkpoint inhibitors relate to the increase in T-cell responses causing an attack on healthy body cells, resulting in adverse inflammatory responses.[1] Signals that propagate these adverse responses include pro-inflammatory cytokines. One such checkpoint inhibitor currently in clinical use is Pembrolizumab, which is a monoclonal antibody against the programmed cell death protein 1 (PD-1). To examine the secreted protein release profile in response to Pembrolizumab in a mixed lymphocyte reaction (MLR), the iQue Qbeads® Human Inflammation Panel Kit was used to measure the concentrations of seven inflammatory cytokines and chemokines in a multiplex assay (Figure 3). This kit is a pre-defined panel covering a range of inflammatory markers that are implicated in inflammatory responses to disease states, including autoimmune diseases, chronic inflammation, and infections. Analytes offered in the iQue Qbeads® Human Inflammation Panel Kit include Human Interferon gamma (IFNγ), Interleukin-2 (IL-2), Interleukin-6 (IL-6), CCL2 (MCP-1), CCL3 (MIP-1α), CXCL9 (MIG), and CXCL10 (IP-10). The kit is supplied with a pre-gated template for easy use.

Pre-activated dendritic cells (DCs) were seeded (40K/well) with CD4+ T cells from a different donor (3:1 T cell-to-DC ratio) and treated with Pembrolizumab. 10 µL samples were analyzed on Days 2 and 6 using the iQue Qbeads® Human Inflammation Panel Kit. Pembrolizumab induced a concentration dependent increase in 6 out of the 7 secreted proteins measured (representative data for IL-2, IL-6, CCL2 and CCL3 shown in Figure 4). The only signaling molecule for which this effect was not observed was CXCL9 (data not shown), however by Day 6, release of this chemokine had exceeded the maximum range of this assay across the entire Pembrolizumab concentration range used, so the results for drug concentration-dependent effects were inconclusive. IL-2 provided an indication of early-stage inflammatory response, with an EC_{50} of 0.53 µg/mL for IL-2 release in response to Pembrolizumab on Day 2. By Day 6 all IL-2 production had stopped. IL-6, CCL2 and CCL3 levels all increased from Day 2 to 6, with very similar EC_{50} values on Day 6 for CCL2 and CCL3 release at 0.98 and 1.01 µg/mL, respectively.

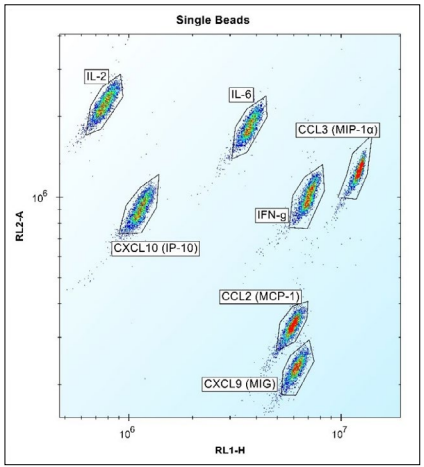


Figure 3
iQue Qbeads® Human Inflammation Panel Kit Pre-Gating

Note. Showing the seven distinct bead populations.

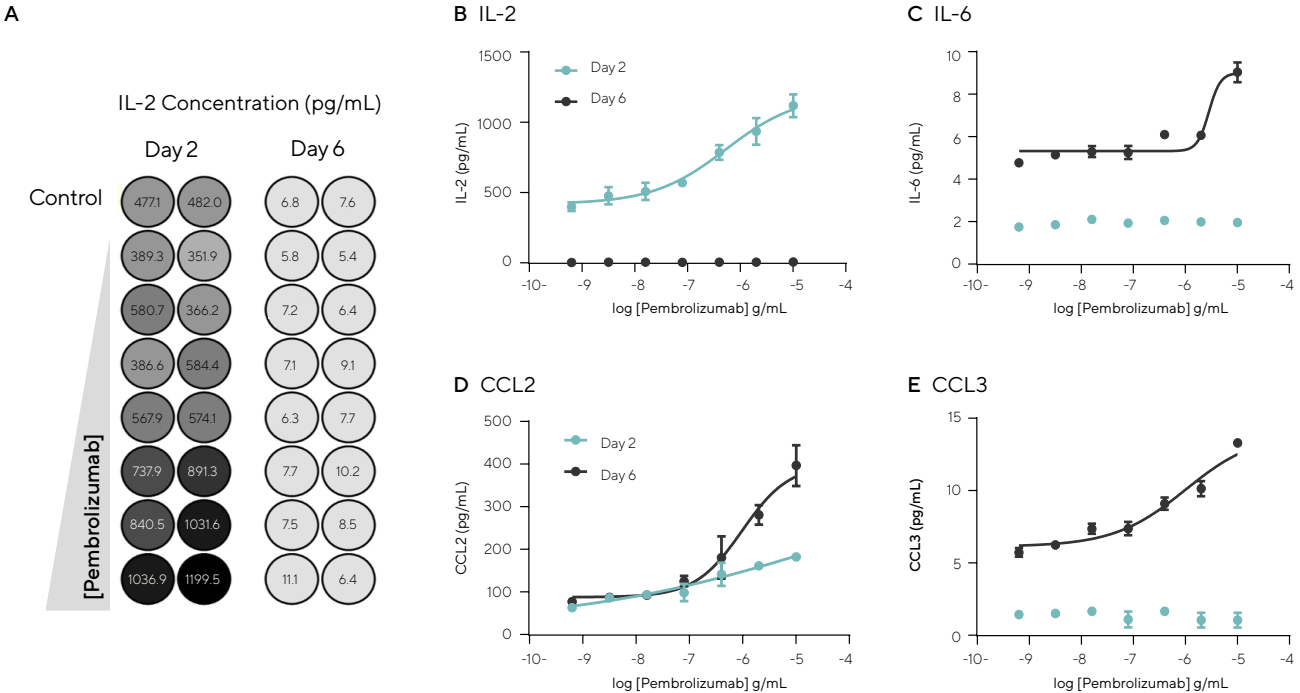


Figure 4

Release of Inflammatory Cytokines Fluctuates Temporally and Increases in the Presence of Pembrolizumab

Note. (A) Heat map showing IL-2 (pg/mL) release per well. Control wells contained T cells and DCs in the absence of drug. (B-E) curves highlight temporal and Pembrolizumab concentration-dependent release of inflammatory cytokines and chemokines: IL-2, IL-6, CCL2, and CCL3.

Summary

The iQue® used in conjunction with a validated suite of Qbeads® provides a simple and flexible workflow for measuring secreted protein levels in media samples and directly from assay plates. Combining fast sample acquisition by the iQue® Platform with pre-set gating and analysis using the built-in iQue Forecyt® software means multiple samples can be profiled for their activity in minimal time. This chapter has highlighted the advantages of this workflow:

- Small sample volume (10 µL) requirement means a single assay plate can feed multiple readouts and time points, allowing the user to generate a wide range of assay outputs with minimal usage of precious sample.
- Pharmacological readouts, such as EC_{50} values, generated using the Forecyt software, can be used to profile drug effects.
- High-throughput data acquisition by the iQue® Platform means that a 96-well assay can be read in 9 minutes or a 384-well in 20 minutes. This facilitates rapid screening of multiple conditions.
- It is possible for cell markers and cytokines to be measured in multiplex, speeding up time to actionable results and without having to correlate data from multiple platforms. This improves data coherence with all readouts for each treatment provided by the same population of cells at a single time point.

Together, these benefits make this technique a powerful tool with potential applications in research and drug discovery. The combination of Qbeads® and cell marker readouts will be explored in greater depth in later chapters (see Chapter 5 Advanced Flow Cytometry Assays for Antibody Discovery and Chapter 6 Advanced Flow Cytometry for Immune Cell Profiling).

References:

1. Burla J., Bluemel S., Biedermann L. et al. Retrospective Analysis of Treatment and Complications of Immune Checkpoint Inhibitor-Associated Colitis: Histological Ulcerations as Potential Predictor for a Steroid-Refractory Disease Course. Inflamm Intest Dis; 5:109-116 (2020)

For further supporting information (videos and literature), see Appendix.

Chapter 4

Advanced Flow Cytometry Assays to Support Cell Line Development

Biologics, also known as biopharmaceuticals, are manufactured using biotechnological methods. These products originate from biological sources usually involving live organisms or their active cellular components.[1] Biologics account for almost 50% of recent new drug approvals and include products such as vaccines, gene therapies, and recombinant proteins. Over the past four years, 84% of the biologics approved for therapeutic use were produced using mammalian cells.[2,3]

In 2017, 10 therapeutic monoclonal antibodies were approved for clinical use in the EU or US with nine additional candidates under regulatory review and 60 in phase III clinical trials.[4] Monoclonal antibodies (mAbs) are the backbone of many treatment modalities including unconjugated therapeutic antibodies, antibody drug conjugates and bispecific antibodies. Many of the approved antibody drugs engage the same target or target multiple members of a single signalling pathway. For example, multiple approved antibody drugs target the human epidermal growth factor receptor 2 (HER2), the B-cell antigen CD20 and the programmed cell death 1 ligand (PDL1), although the mechanism of action, antigenic epitope and antibody format differs. This highly competitive landscape incentivizes drug developers to produce next generation antibody drugs against new targets with improved mechanism of actions, pharmacokinetics, and delivery systems.[5]

High-performing, stable and robust cell lines form the foundation of biologics production. However, cell line development is logistically and technically demanding. Costly errors can occur and impede the process of drug development.

The goal of cell line development is to produce a monoculture expressing desired characteristics, such as the production of a specific antibody. For biologics, these characteristics, known as critical quality attributes (CQAs), must fall within a specific range to ensure the efficacy and safety of the product.[6] CQAs should be defined and validated early in the cell line development process to reduce time lost pursuing poorly performing clones. This also gives confidence that an observed effect is reproducible in later stages. Often, the methods for identifying optimal clones are time consuming, single endpoint assays, such as enzyme-linked immunosorbent assays (ELISAs). The process required to examine CQAs and measure protein output is therefore a significant bottleneck with multiple, lengthy assays.

Viable Cell Count

The use of clinical biologics is underpinned by the efficient development of cell lines to support the manufacture of these therapeutics. From gene cloning and initial clone selection through to final cell evaluation, the continual assessment of cell count and viability is important for determining the best growing and highest producing clones. This information can be used for monitoring proliferation rates, optimizing growth conditions, and normalizing cell data for further studies. Traditional methods for measuring cell count and viability are often low throughput, time-consuming and lacking in linearity. Advanced flow cytometry provides a fast, accurate and reproducible solution for cell viability and density quantification, which is essential for efficient biologics development processes.

Here we document the use of the iQue® Cell Count and Viability Kit in combination with the iQue® Advanced Flow Cytometry Platform to accurately quantify both cell count and viability in 96- or 384-well plates. The data presented demonstrates that the iQue® Cell Count and Viability Kit alongside the integrated iQue Forecyt® analysis software can be effectively used with various non-adherent cell types for rapid and accurate quantification, requiring small cell volumes. The data also demonstrates how this can be easily, rapidly, and successfully utilized for a cell line development workflow.

Assay Principles

The iQue® Cell Count and Viability Kit is designed for reproducible, quantitative analysis of cell count and viability. The kit provides fast quantification of a variety of non-adherent cell lines (not optimized for adherent cell types) across a large linear range. The streamlined workflow (Figure 1) guides the user from cell labeling, using a simple no-wash protocol, through to analysis within the iQue Forecyt® software. The kit includes validated reagents and preset gating templates to enable simple, automated identification of live cells in both 96- and 384-well plates. Samples can be either cultured cells or freshly-thawed cells.

To validate the accuracy of the kit, it was tested across a range of non-adherent cell types to assess the linear range of quantification compared to an alternative quantification method. Jurkat, Raji or Ramos cells were grown in culture and mixed with heat-killed cells at various ratios to provide specific cell viability percentages. For heat killing, cells were heated to > 60 °C in a water bath for a minimum of 10 minutes before cooling. Mixed samples were taken, and cell viability was assessed using the iQue® Cell Count and Viability Kit and an alternative technique based on trypan blue exclusion counting (Figure 2A). The data clearly demonstrates a linear correlation between the two counting techniques (R^2 > 0.99) for all cell types across 0 to > 90% viability range. A similar assessment was performed using Jurkat cells at various cell densities (Figure 2B) showing a linear correlation (R^2 > 0.97) across cells densities from 8×10^4 to 2×10^7 cells/mL.

Example Results

Assessment of viable cell concentration (VCC) is an important quantification during a cell line development strategy. This is of key interest during the phases of pool evaluation, single cell cloning and then

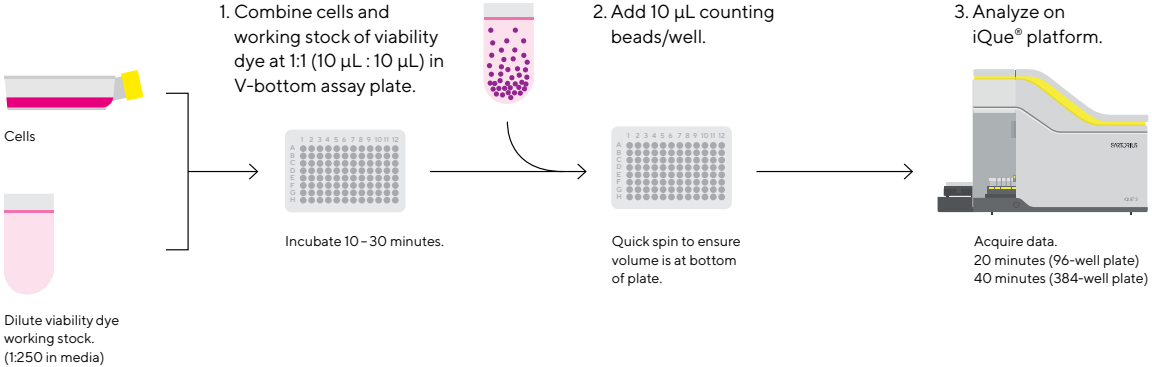


Figure 1: *iQue® Cell Count and Viability Kit Workflow*

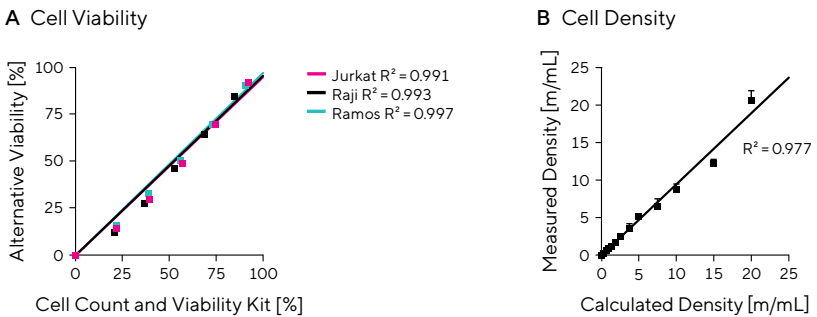


Figure 2: *Linear Assessment of Quantification*

Note. Comparison of the iQue® Cell Count and Viability Kit to an alternative counting method to assess the viability of various ratios of live and heat-killed cells (A) or Jurkat cell density (B). Data shown as mean of (A) 12 or (B) 4 samples ± SD, with linear regression analysis.

clone evaluation. During these stages, there can be high numbers of samples (> 100 pools and clones) that need assessment at regular intervals. The use of a high throughput, reproducible, fast analysis system is advantageous to ensure the identification of the best clones. This selection process has traditionally been performed using methods such as comparison of titer measurements as the assessment of viable cell count has been too laborious and time consuming.

The iQue® Cell Count and Viability Kit was evaluated in a simulated clonal expansion phase used during a standard cell line development process to analyze the reliability of the iQue® workflow. The acceptance criteria values were defined to be within ± 20% deviation from an alternative count method. Ten clones of the cell line CHO-DG44 were seeded in duplicate to a VCC of 2×10^5 cell/mL in a 25 mL culture volume. Cells were measured and split based on VCC determination by the iQue® and an alternative counting method covering the period of six splits over 25 days in culture.

When the ten clones were assessed across splits (Figure 3A), 63.3% of values were within the ± 20% deviation criteria. In general, the better growing clones were identified by both methods (data not shown). Example data is shown for two of the ten clones (Figure 3B and 3C) showing how the VCC compares over the various splits for the two counting methods. This data confirms that the iQue® Cell Count and Viability Kit in combination with the iQue® Advanced Flow Cytometer can be used as part of a clonal expansion phase. In addition, the user sample preparation time was reduced using the iQue® Platform method, meaning multiple replicates could be run for each sample, increasing the accuracy and robustness of the data generated.

Summary

The data demonstrates the use of the iQue® Cell Count and Viability Kit for both cell counting and viable cell density quantification of non-adherent cells. The kit components in combination with a simplified sample handling workflow and fully integrated, pre-defined data analysis provides the user with an accurate, high-throughput and rapid cell quantification method. Further validation demonstrates how this workflow can be integrated into a cell line development process to aid the rapid identification of healthy growing clones.

Conclusions from the data collected during the clonal expansion experiments demonstrate comparable quantification of VCC compared to alternative accepted methods. Further significant advantages in time required for experiment and analysis were recorded using the iQue® workflow:

- Significant increase in capacity to quantify VCC samples enabled through use of 96- or 384-well plates, in combination with rapid analysis.
- User-friendly templates to enable plug-and-play measurement and analysis within the iQue Forecyt® software.
- Access to both total cell count and viable cell count data within the template, which saves on analysis time.

The workflow using the iQue® Cell Count and Viability Kit in combination with the iQue® Advanced Flow Cytometry Platform along with the iQue Forecyt® software offers the user a high-throughput method for viable cell count determination for use throughout the cell line development process.

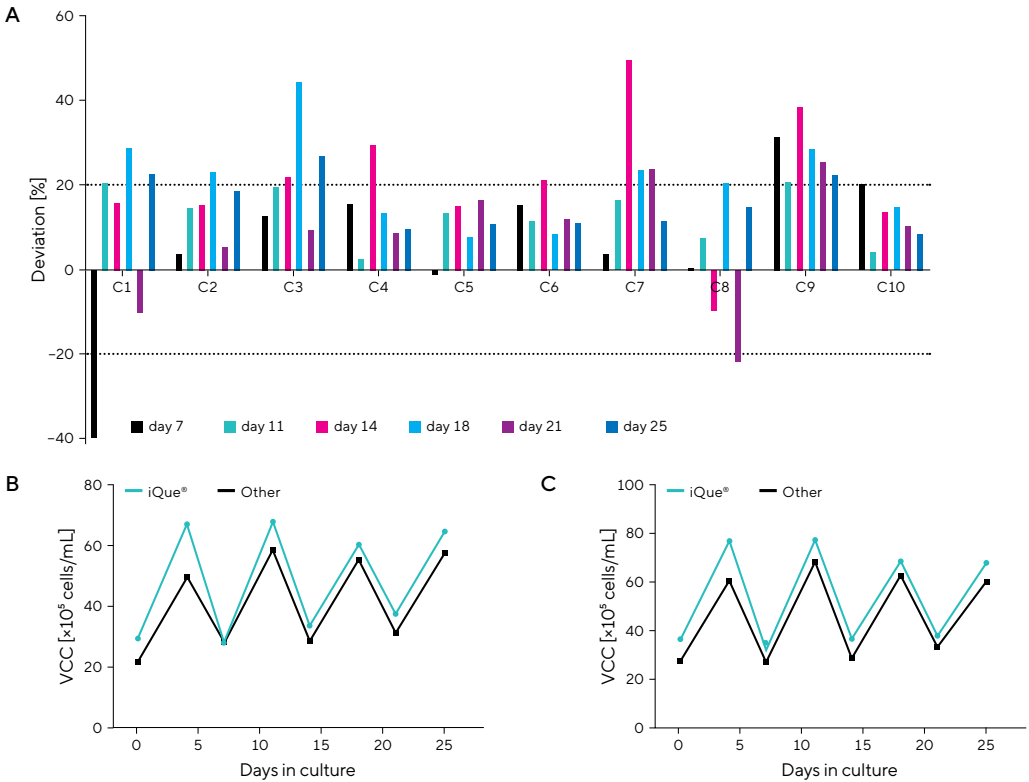


Figure 3

Use of VCC as Part of Clonal Expansion Protocol

Note. The deviation (%) between iQue® Workflow and an alternative counting method was compared across ten clones (C1–C10) over 25 days of culture (A), dotted lines represent the acceptance criteria of ± 20%. Absolute VCC data for two clones are shown in detail (B and C) over the various splits for the iQue® Platform (teal circles) and alternative counting method (black squares).

Titer and Viability Determination

A key component of therapeutic monoclonal antibody development is identifying a cell line that secretes high levels of antibody into the culture supernatant. Early identification of high producing cell lines significantly increases the probability of success in downstream cell line manufacturing.

The iQue® Advanced Flow Cytometry Platform, combined with the iQue® Human IgG Titer and Viability Kit or Mouse IgG Type and Titer Kit provides a high-throughput analysis platform that uses low sample volumes to simultaneously report on antibody output and cell viability. The no-wash, no-dilution workflow reduces variability of results, while the walkaway automation with rapid protocols reduces time cost. Automated high-throughput solutions can relieve bottlenecks and give more in-depth insights for clone ranking to obtain rapid, actionable results early in the cell line development process.

Both kits are designed for ease of use in multiplexing, enabling a straightforward workflow without sacrificing assay performance. Compared to other IgG quantitation methods, the iQue® solution offers several unique advantages:

- Simultaneous quantification of secreted IgG and viable cell count—enables the precise quantitation of IgG per cell, IgG per viable cell, and cell viability and growth.
- Wide dynamic range—enables transfer of cell culture samples directly into assay plates, without the need for dilution steps.
- No wash assay—mix and read format minimizes screen time, cost, and variability.
- Mouse kit also enables simultaneous isotyping measurement using multiplexed isotype-capture beads.

Assay Principles

The iQue® Human IgG Titer & Viability Kit (Figures 4 and 5) is a competition assay in which fluorescently labeled human IgG (human FITC-IgG) is added to samples containing secreted IgG and CHO production cells. The human FITC-IgG and non-labeled sample IgG compete for binding to IgG capture beads. The amount of IgG present in the sample is inversely proportional to the bead-associated FITC fluorescence. Cell viability is measured simultaneously in each well using a cell membrane integrity dye. Healthy cells with intact cell membranes exclude the dye and are not fluorescent. Unhealthy cells with compromised membranes will allow entry of the dye into the intracellular space, where it then localizes to the nucleus and binds to DNA by intercalation.

Quantitative readouts from this assay can be measured as fluorescence intensity or extrapolated to a concentration in solution via the use of a standard curve. For a screening workflow, distribute IgG secreting cells grown for the appropriate time into culture plates by standard methods including limiting dilution or

single cell cloning. After growth, samples are transferred (20 µL) from each well (including both cells and supernatants) to assay plates. Human FITC-IgG and iQue® Cell Membrane Integrity (R/Red) Dye and IgG Capture Beads are added to the wells and mixed. After incubation at room temperature for 60 minutes, plates are read directly on the iQue® Platform. The assay is suitable for both 96- and 384-well screening. Readouts captured include IgG concentration, cell number, cell viability, IgG per cell and IgG per viable cell.

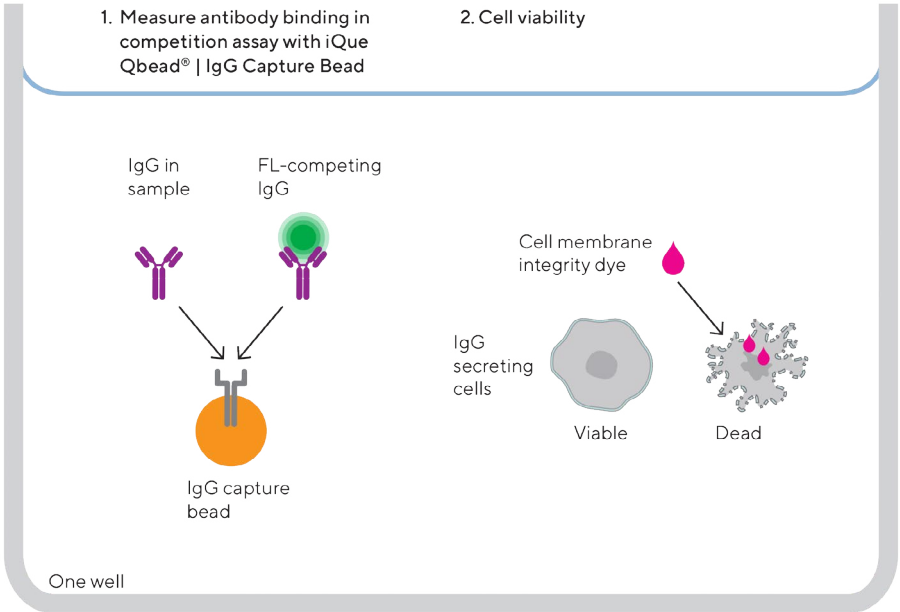


Figure 4
Principle of the iQue® Human IgG Titer and Viability Kit

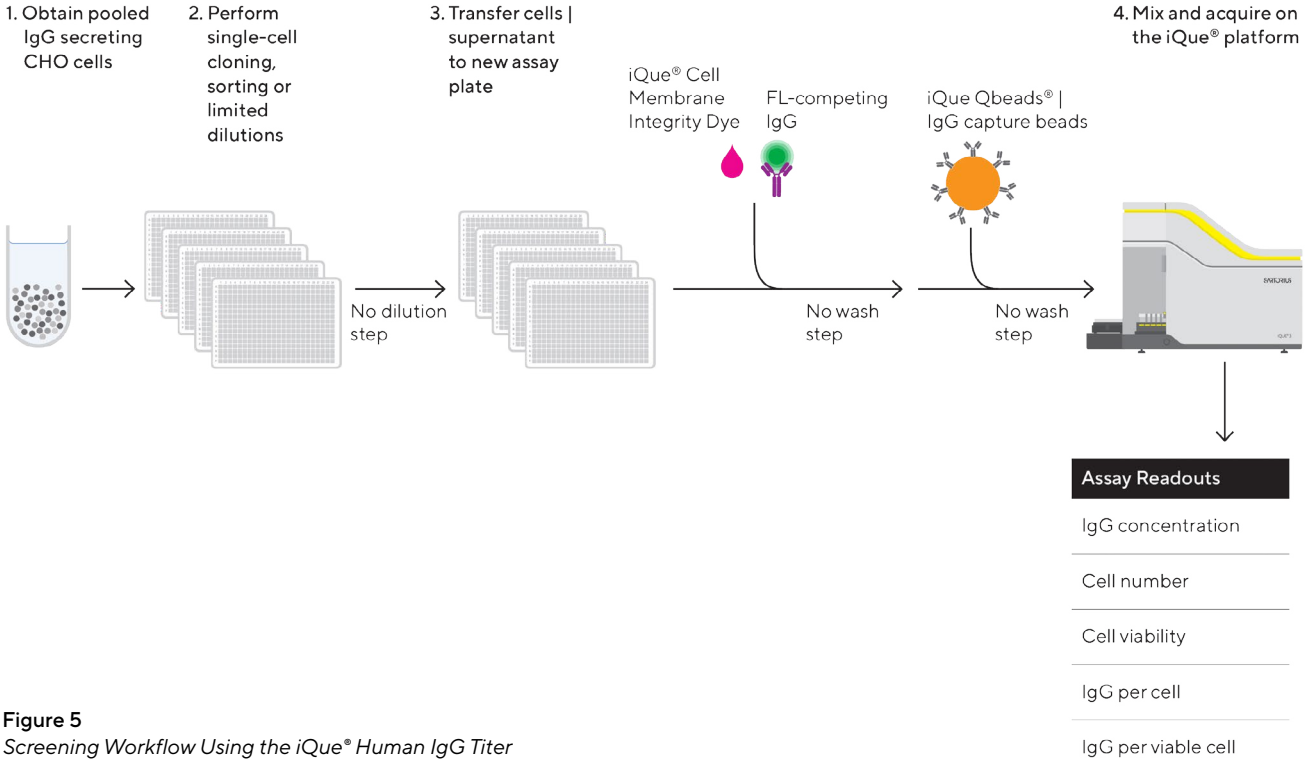


Figure 5
Screening Workflow Using the iQue® Human IgG Titer and Viability Kit

The iQue® Mouse IgG Type and Titer Kit (Figures 6 and 7) enables the simultaneous measurement of mouse IgG isotypes, the mouse IgG quantity for each isotype, cell number and cell viability from each well of the screening plates. As with the previously described kit, this is a competition assay in which fluorescently labeled mouse IgG (mouse FITC-IgG) is added to mouse samples containing secreted IgG from hybridomas or B-cell cultures. The mouse FITC-IgG and non-labeled mouse sample IgG compete for binding to IgG capture beads in an isotype-specific manner. The amount of IgG isotype present in the sample is inversely proportional to the isotype-specific bead-associated fluorescence. Signals across four different isotype-specific beads determine the IgG isotype in the assay well. Cell viability is measured simultaneously as before using a cell membrane integrity dye. Quantitative readouts from this assay are again measured as fluorescence intensity or extrapolated to a concentration (µg/mL) in solution via the use of isotype-specific standard curve.

For a screening workflow, distribute mouse hybridoma fusion cells (grown for the appropriate time) into culture plates by either limiting dilution or single cell cloning or single cell sorting. After growth, samples are transferred (20 µL) from each well (supernatant only or supernatant plus cells) to assay plates. Mouse FITC-IgG and iQue® Cell Membrane Integrity (R/Red) Dye are added to the samples, and then mixed with mouse IgG capture beads. After incubation at room temperature for 60 minutes, plates are read directly on the iQue® Platform. Readouts captured include: IgG isotypes (1, 2a, 2b, and 3), IgG isotype concentration, total IgG concentration, cell number, and cell viability.

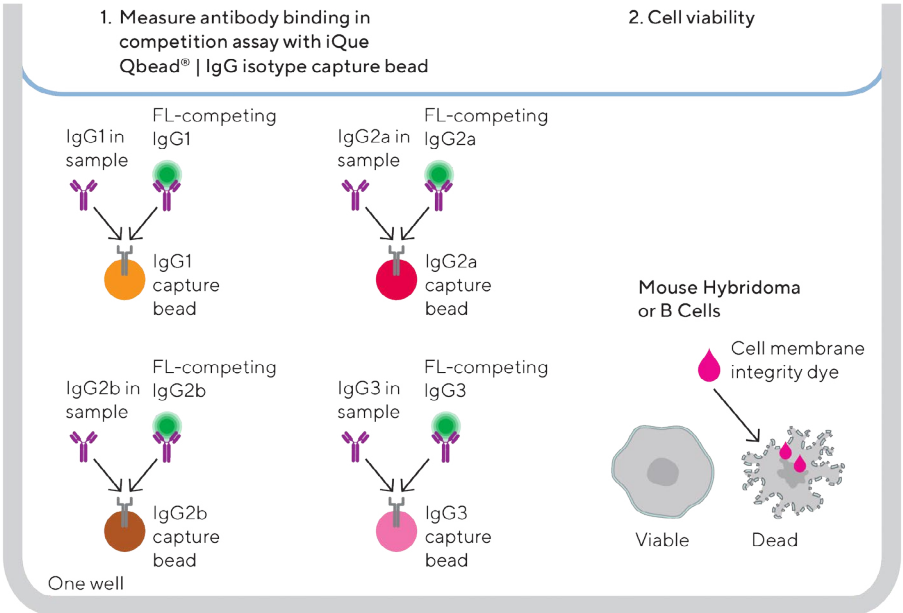
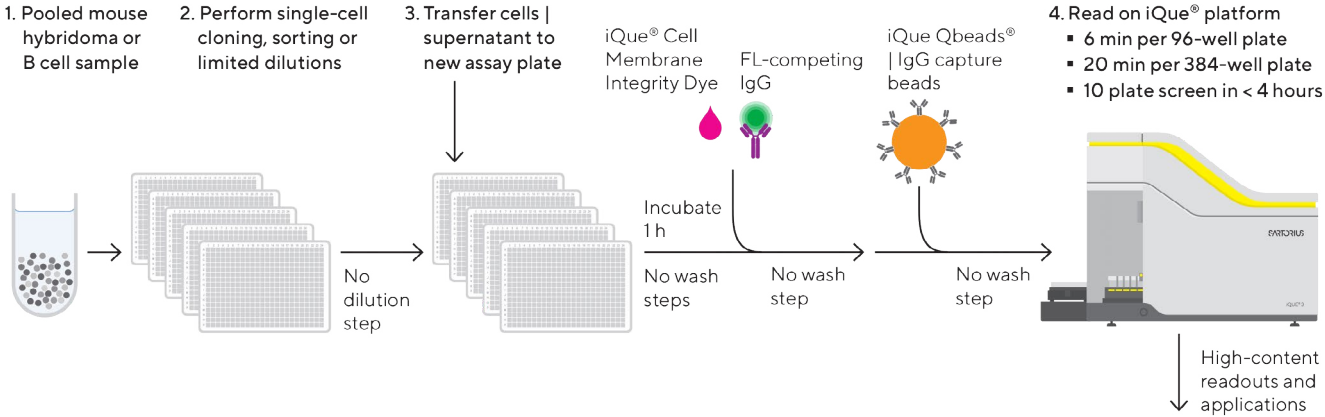


Figure 6
Principle of the iQue® Mouse IgG Type and Titer Kit



Readouts	Applications
IgG isotypes	▪ Isotyping: 1, 2a, 2b, 3 or mixture ▪ Identify pure monoclonal ▪ Determine DNA primers for gene cloning
IgG isotype concentration	▪ Normalize concentrations for downstream functional assays
Total IgG concentration	▪ Confirm hybridoma | B cell stability with antibody secretion
Cell count and health	▪ Monitor hybridoma | B cell proliferation and health in original cell cloning plates

Figure 7
Screening Workflow Using the iQue® Mouse IgG Type and Titer Kit

Example Results

Quantification of antibody concentration from cell culture supernatant is traditionally assessed using an ELISA assay. ELISA is a time consuming, single-endpoint assay, often requiring sample dilution and multiple washes. Additionally, separate IgG isotyping and cell count/health assays are performed to provide the scientific insight for downstream antibody cloning. Using the iQue® Platform, researchers can streamline their antibody discovery workflows. The following example focuses on data acquired using the iQue® Mouse IgG Type and Titer Kit.

The sample analyzed was a mixture of mouse hybridoma cells and supernatant assessed in a single 96-well plate. The iQue® Mouse IgG Type and Titer Kit was used to analyze the mouse IgG isotype and quantity and as well as cell count/cell health.

Using one 96-well plate assay with mouse hybridoma samples, analysis of hybridoma hits at plate level was performed (Figure 8). The template included in the kit automatically generated four isotype-specific standard curves after sample acquisition for quantification of concentrations. All IgG information was automatically populated in the heat maps and dot plots. Using the Panorama function in the iQue Forecyt® software, an overlay line graph was generated with all IgG isotype in the sample wells. The mouse hybridoma samples with IgG isotypes and quantity information were displayed in the line graph for easy, rapid monitoring and quality-control of the screening results at the single plate level.

Summary

The iQue® Mouse IgG Type and Titer and iQue® Human Titer and Viability Kits provide a novel assay platform for antibody discovery that disrupts the traditional time-consuming workflow. The iQue® Platform provides high throughput and rapid sampling for a screening campaign. iQue Forecyt® quickly transforms large raw data sets into rich visual context by applying multi-plate Panorama analysis algorithms. This allows researchers to quickly identify the critical antibody clone attributes required for actionable decisions that ultimately result in robust antibody discovery.

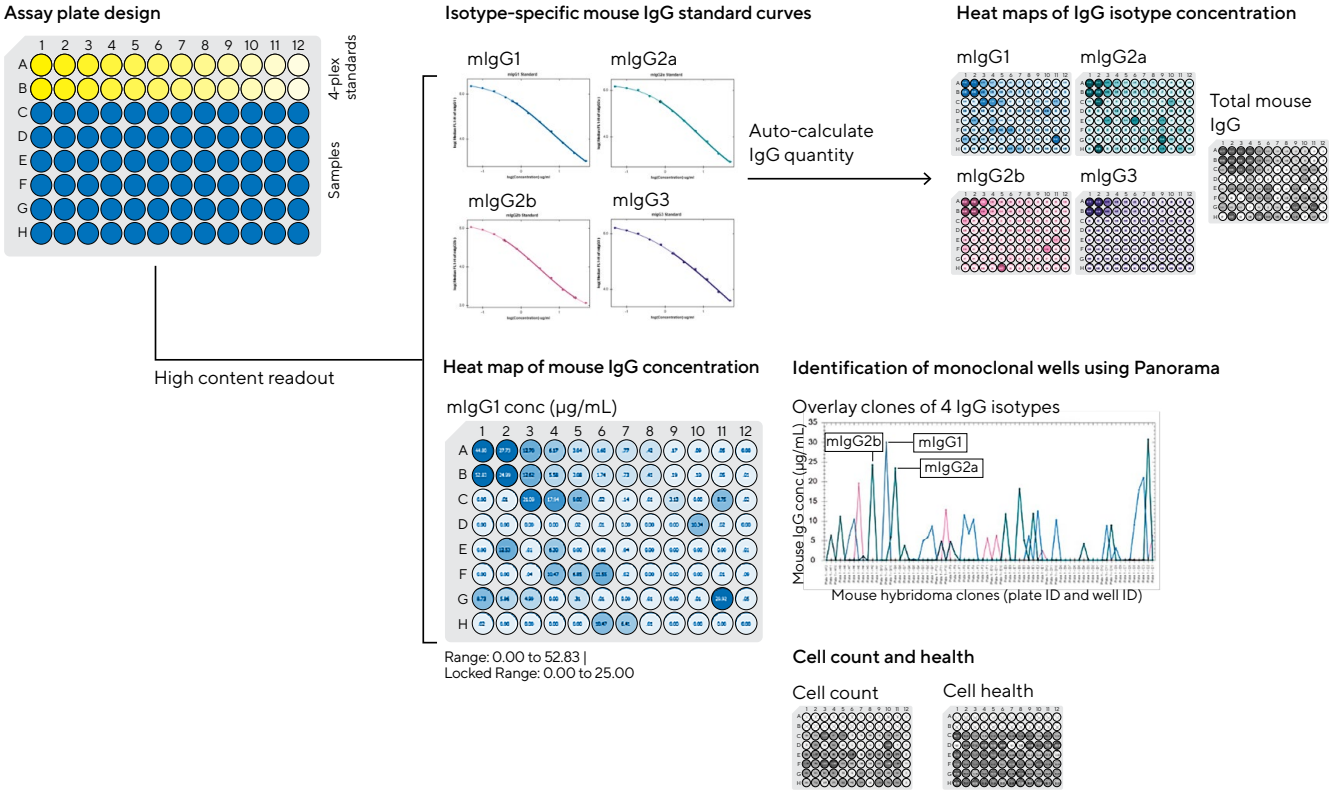


Figure 8

Data Analysis of High Content Readouts in iQue® Mouse IgG Type and Titer Kit with Mouse Hybridoma Samples

Hybridoma Screening

Next generation antibody drugs require the development of antibodies with specific characterizations and are against difficult targets such as multi-pass membrane proteins. These efforts require high-throughput methods to perform large screening campaigns and software capable of easily analyzing large, multiplexed data sets. The iQue® Advanced Flow Cytometry Platform with its combined high-throughput sampling and on-board analysis with plate-level analytics can deliver rich content with rapid sampling times. Eliminating the dead volumes of traditional flow cytometry, assay volumes can be reduced to microliters saving precious cells and reagent costs. Data acquisition, analysis and visualization happen on the fly for each plate using user defined templates, dramatically reducing the time and effort to generate useful results.

The first stage in this process is often to use multiple hybridoma clones to increase the chance of finding the right constructs and producing clones. To exemplify this process ImmunoPrecise Antibodies Ltd. applied the iQue® Platform in their antibody discovery process. The ImmunoPrecise Antibodies Ltd antibody discovery workflow is divided into three stages:

- Target cell line engineering to generate cell lines that express high surface levels of the target antigens.
- Immunizing mice with multiple types of antigens, followed by testing of the immune sera for binding to target antigens.
- Large-scale screening of hybridoma clones for antibody binding and target specificity.

Example Results

Developing antibodies against these challenging epitopes requires presenting the antigens in a variety of ways to the immune system in order to find antibodies with the desired characteristics. In this study, BALB/c mice were immunized with either purified protein, a DNA plasmid expressing antigen, antigen expressing cells, or liposome embedded antigen. Antibody binding was determined using a fluorescent detection antibody and antibody titers were measured using a duplicate eight point, 2-fold dilution series of either pre-immune or immune sera. To ensure antibody specificity, a multiplexed approach was used where cells expressing the antigen, a related antigen or the parental cell line was encoded with different intensities of a fluorescent dye and mixed into the same well. Multiplexing target and control cells in the same well allowed the combination of three separate assays into a single binding assay expediting the time to actionable data.

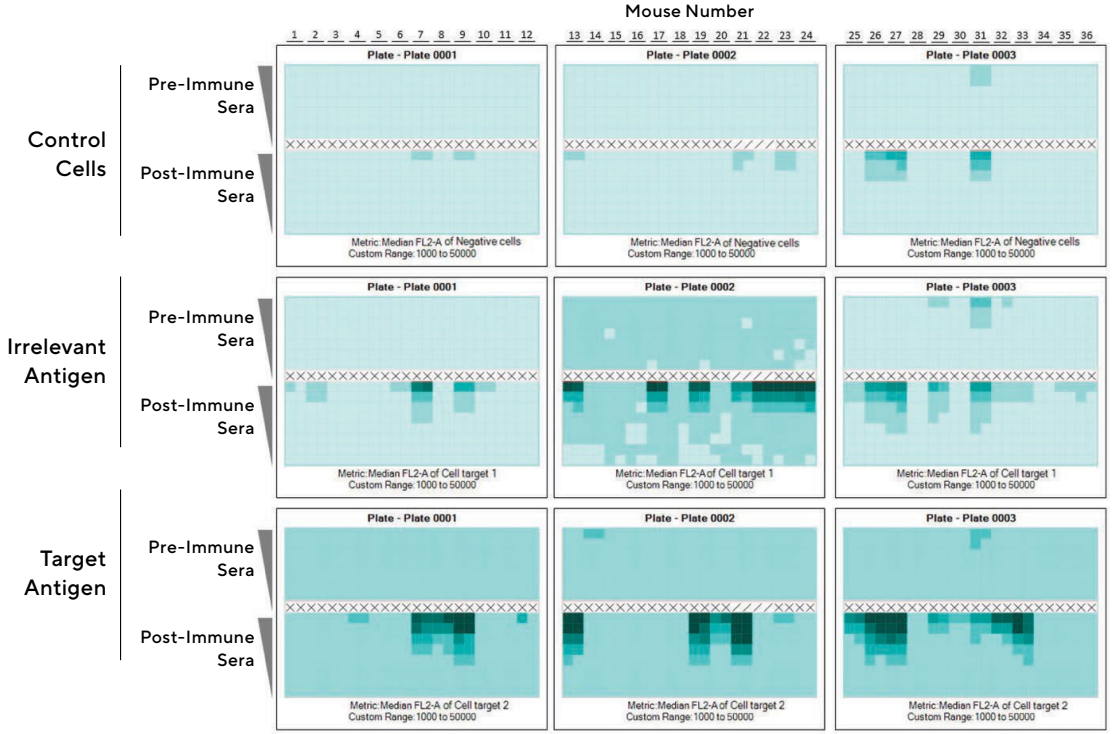


Figure 9

Multiplexed Assay for Mouse Sera Evaluation

Note. Data show results from the testing visualized using the heat maps feature in iQue Forecyt® Software. Three cell lines were color coded with the fluorescent Cell Encoding Dye, mixed together and dispensed into wells of 384-well plates. Serum samples were added to each well, followed by a fluorescent detection antibody and plates were analyzed using the iQue® Advanced Flow Cytometry Platform. Sera from 36 mice pre-immunization and post-immunization with the target antigen were tested in duplicate in 8-fold dilutions. Heat maps show results for antibody binding to control cells, cells expressing target antigen and cells expressing a related but irrelevant antigen.

Each cell population was resolved based on fluorescence and the amount of antibody binding from pre-immune, or immune sera from each cell population is shown on the heat maps in Figure 9. Little antibody binding was observed with either pre-immune or immune sera in the parental control cells. In contrast, immune sera from many animals showed high binding to the antigen-specific expressing cells whereas no binding was observed in the pre immune sera. A moderate amount of binding was measured in the related antibody expressing cells and these overlapped with antigen specific cells. This suggests the generation of polyclonal antibody responses that can bind to related proteins. From this data, two mice were chosen for hybridoma generation.

Summary

ImmunoPrecise Antibodies Ltd uses the iQue® Platform at multiple steps of the antibody discovery workflow to accelerate the time to lead generation. The value of the iQue® Advanced Flow Cytometry Platform and integrated iQue Forecyt® software to ImmunoPrecise Antibodies Ltd and their customers lie in the rapid, high-throughput sampling and the real-time and multi-plate data analysis. Multiplexed cell analysis combines multiple assays into a single study, which simplifies and speeds up the antibody screening workflow. Assay miniaturization reduces costs and saves precious antibody supernatant that can be used for additional confirmatory or functional studies.

References

1. What are "biologics" questions and answers. U.S. Food and Drug Administration. What Are "Biologics" Questions and Answers | FDA
2. A perfect fit, from start to finish. Sartorius. Cell Line Development | Sartorius
3. Fischer S, Handrick R, Otte K. The art of CHO cell engineering: a comprehensive retrospect and future perspectives. Biotech Adv. 2015;33(8):1878-1896. doi:10.1016/j.biotechadv.2015.10.015
4. Kaplon, H., & Reichert, J. M. Antibodies to Watch in 2018. mAbs (2018), 1-21.
5. Carter, P. J. & Laazar, G. A. Next Generation antibody Drugs: Pursuit of the "High-Hanging Fruit." Nature Reviews, Drug Discovery (2018), 17, 197-223.
6. Witcher MF. Why controlling CQAs isn't good enough for gene and cell therapies. Cell and Gene. https://www.cellandgene.com/doc/why-controlling-cqas-isn-t-goodenough-for-gene-cell-therapies-0001. Published March 2020. Accessed September 13, 2022.

For further supporting information (videos and literature), see Appendix.

Chapter 5

Advanced Flow Cytometry Assays for Antibody Discovery

Multiplexed, multiparameter screening assays are foundational to the drug discovery process. By collecting more information more rapidly, and earlier in the process, researchers have more confidence in therapeutic candidates as they progress towards the clinical pipeline.

The iQue® Advanced Flow Cytometry Platform represents a powerful and versatile tool for multiplexed, high-throughput measurement of cell-specific parameters, protein analysis, immunophenotyping, functional assessments and profiling, including antibody screening and immune cell activation.

In this chapter we will explore a range of live-cell assays that can be performed using the iQue® Platform during antibody characterization. These span a multitude of key features of antibodies that must be measured during antibody discovery, from assessment of antibody binding and internalization to analysis of *in vitro* functional activity. A walkthrough of the assays for profiling antibody function will begin by measuring the three key Fc mediated functions of antibodies: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). This chapter will conclude with a look at how the iQue® Platform can be used to quantify antibody cytotoxicity, using antibody drug conjugates (ADCs), in 2D and 3D models, as an example.

Antibody Binding

The success of monoclonal antibodies (mAbs) as a class of therapeutics is largely due to their high specificity and affinity for the target antigen. Evaluation of mAb target binding is a critical part of the antibody development process and robust, high-throughput techniques are needed to facilitate rapid screening of antibody libraries.

Antibody binding can be measured using a range of techniques, from protein-protein interaction-based assessment using techniques such as biolayer interferometry (BLI) or surface plasmon resonance (SPR) to live-cell methods like flow cytometry. Each method comes with its own advantages. For example, BLI and SPR can be used to measure the kinetics of antibody to antigen interactions, yielding values such as affinity,

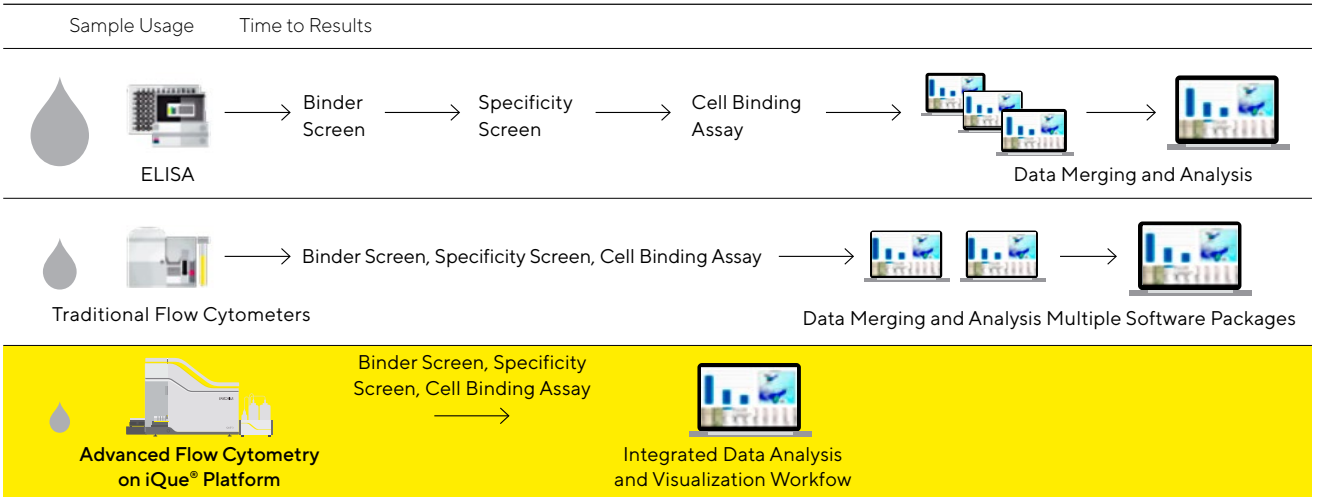


Figure 1
Comparison of Sample Usage and Time to Results in a Typical Biological Workflow

on rate, and off rate. On the other hand, flow cytometry measures the binding of the antibody to the target antigen as it is found on live cells, rather than a solubilised version of the protein as is required for BLI and SPR assays, which means it can more closely reflect binding *in vivo*.

iQue® Assay Principles

Here we describe two simple assays that utilize the iQue® Advanced Flow Cytometry Platform and validated reagents to measure binding of unlabeled therapeutic mAbs to targets on live cells. The first is a simple, direct antibody binding assay (Figure 2) which enables mAbs to be ranked based on binding to live cells, with the ability to analyze binding to multiple cell types in a single well. Target cells are optionally labeled with the iQue® Cell Proliferation and Encoding (V/Blue) Dye and incubated with test mAbs for 30 minutes on ice to prevent internalization. Plates are then washed and labeled with the iQue® Cell Membrane Integrity (R/Red) Dye. After 30 minutes incubation, plates are washed and run on the iQue® Platform.

The second is a competitive binding assay (Figure 3), which can assess mAbs that target different epitopes. A competing antibody is pre-labeled in a tube with a fluorophore-conjugated secondary antibody, prior to combination with the

Direct binding assay

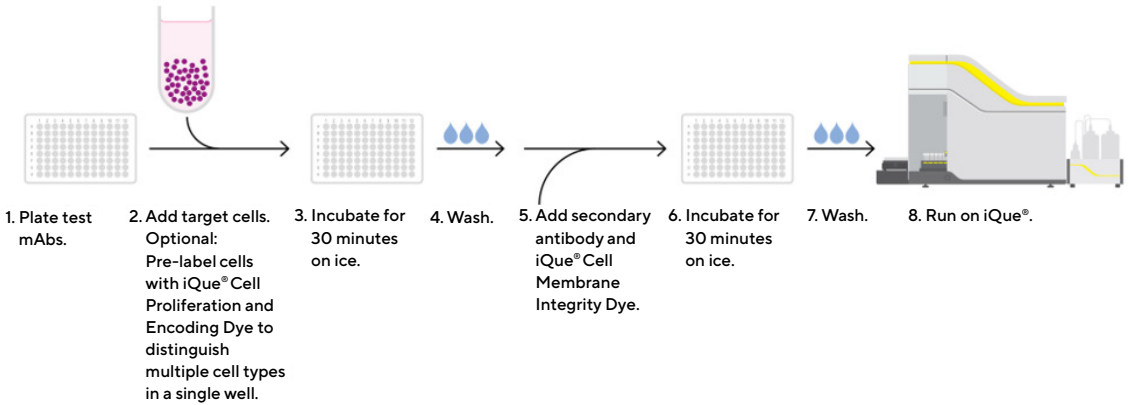


Figure 2
Direct Antibody Binding iQue® Assay Workflow

unlabeled test antibody of interest in the assay plate. The target cells are then added to this antibody mixture and the competing labeled mAb and the test mAb will compete for binding to the target antigen, provided they both target the same epitope. Therefore, the greater the concentration of the test antibody, the less of the competing labeled antibody will bind, resulting in a lower Mean fluorescence intensity (MFI) value for the secondary antibody fluorophore. If the two antibodies do not compete for the same epitope, the MFI should be unaffected by test mAb concentration.

Competitive binding assay

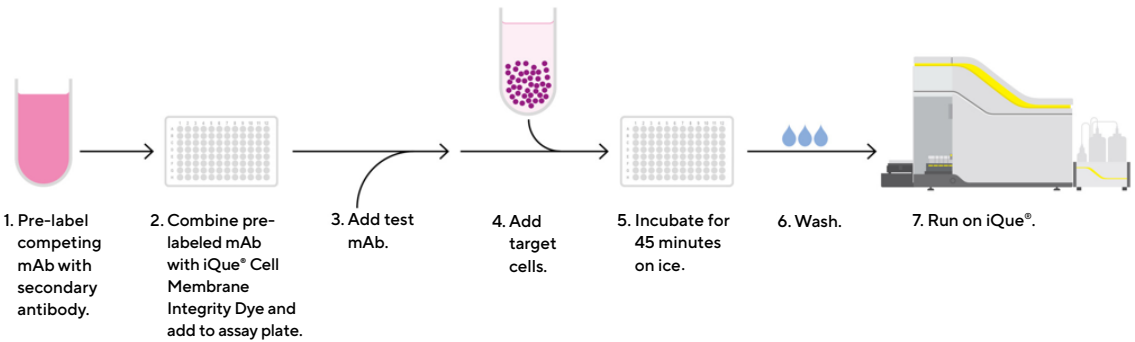


Figure 3
iQue® Competition Binding Assay Workflow

The iQue® antibody binding workflows provide key advantages over traditional methods.

- Faster data acquisition facilitates high-throughput screening and enhanced replication leading to more robust data.
- Easy-to-follow protocols facilitate assessment of binding to both adherent and suspension cell targets.
- Low volume requirements allow for conservation of precious antibody samples.
- The broad range of detection channels gives the flexibility to mix and match dyes, such as encoder or membrane integrity dyes, in a panel with your chosen secondary antibody fluorophore.

Example Results

Assessment of mAb Binding and Specificity in Both Adherent and Suspension Cell Models

To accommodate the assessment of antibody binding to a broad range of tumor targets, the iQue® antibody binding assay was validated for use with both suspension and adherent cell types. For the suspension cell model, binding of anti-CD20 antibody Rituximab to CD20-antigen positive Ramos cells, from a B lymphocyte cell line, and CD20-negative Jurkat cells, from a T lymphocyte line was quantified. For the adherent cell model, binding of anti-HER2 antibody Trastuzumab to HER2-positive AU565 cells and HER2-negative MDA-MB-468 cells, both from breast cancer cell lines, was measured. In both models, the antigen-negative cell type was labeled using the iQue® Cell Proliferation and Encoder (V/Blue) Dye to distinguish them from the antigen-positive cells, as displayed in the dot plots in Figure 4.

In both models, there was an antigen-positive cell type-specific increase in binding with increasing antibody concentration. There was no binding observed with the antigen negative cell types, or with the IgG control antibody. It is crucial to assess whether there is any off-target binding of a novel antibody candidate in the early stages of drug discovery using *in vitro* assays, as this can present issues, such as reduced efficacy and increased toxicity *in vivo*.[1]

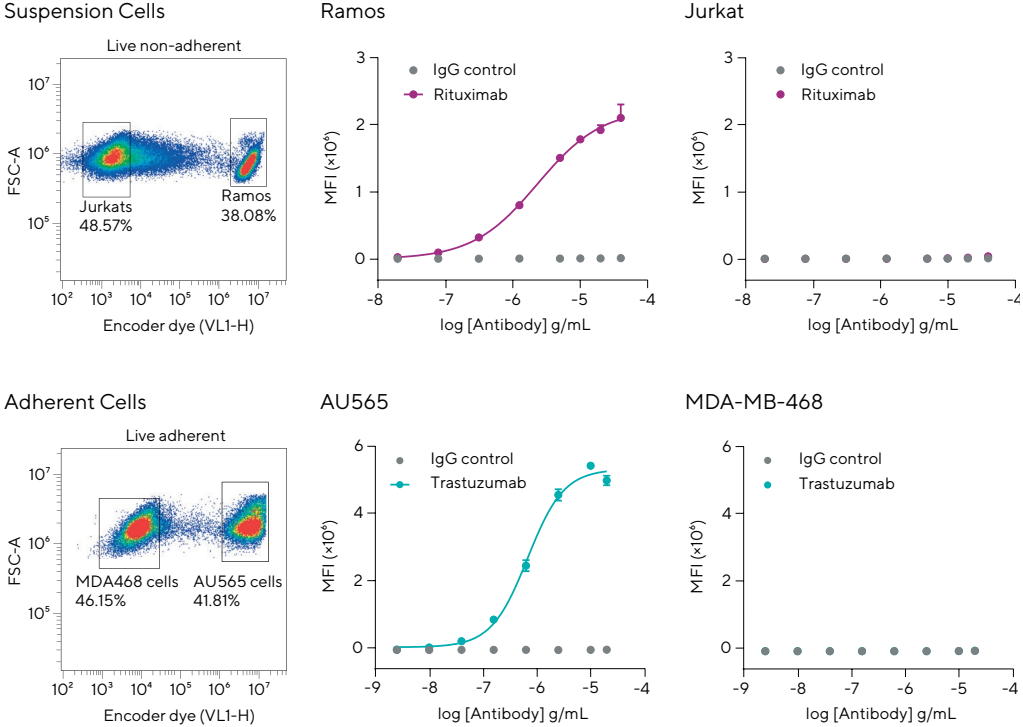


Figure 4

Assess Specificity of Rituximab and a Trastuzumab Biosimilar Binding to Target Antigens on Both Suspension and Adherent Cells

Note. CD20-positive Ramos cells and HER2-positive AU565 cells were labeled with iQue® Cell Proliferation and Encoding (V/Blue) Dye to distinguish them from antigen-negative Jurkat and MDA-MB-468 cells. Cells were incubated with a range of concentrations of unlabeled test mAb or an IgG control followed by a single concentration of RPE-conjugated secondary antibody (5 µg/mL).

Competition Binding Assay Reveals Antibodies That Bind to the Same Epitope

During antibody discovery, it is important to determine which epitope a novel drug candidate binds to on the target antigen. If it is a well characterized target for which antibodies are available with known epitope binding, this can be achieved by evaluating whether the novel antibody competes with those known binders. This is exemplified by the data in Figure 5 which shows the results from an iQue® competition assay used to assess competition between anti-HER2 antibodies. In these experiments, a single concentration of each antibody was labeled with fluorophore-conjugated secondary antibody, and the presence or absence of competition with unlabeled antibody was assessed, in a pairwise manner.

Trastuzumab binds HER2 at the C-terminal portion of domain IV, meaning Kadycla, with the same variable region should also bind domain IV.[2] Pertuzumab binds HER2 in a central region of domain II, which is thought to inhibit HER2 dimerization.[2] The data in Figure 5 support the expected epitope binding, with competition between Trastuzumab and Kadcyla indicated by the drop in MFI (left shift of the histogram

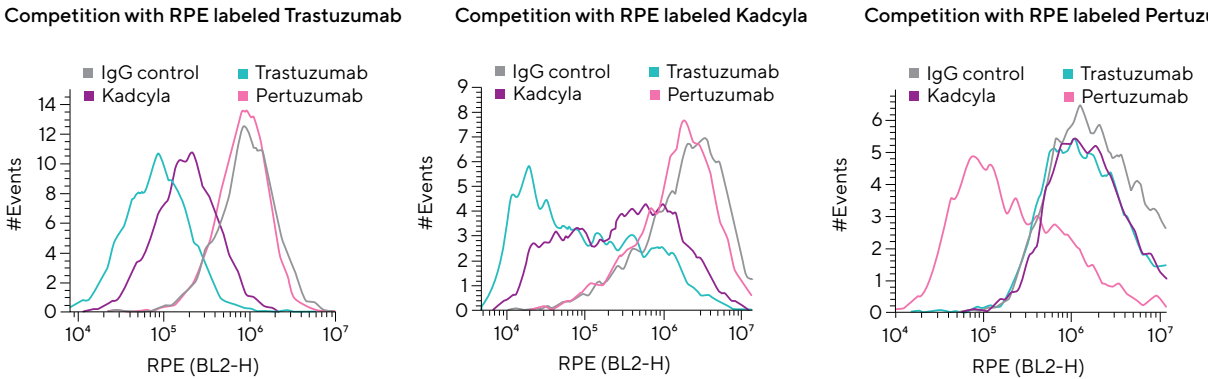


Figure 5

Competition Binding Assay Reveals Antibodies that Bind the Same Epitope on the Target Antigen

Note. A single concentration (1.5 µg/mL) of three mAbs (a Trastuzumab biosimilar, Kadcyla® [an ADC based on Trastuzumab] and a Pertuzumab biosimilar) were labeled with RPE-secondary antibody. Labeled mAbs were paired with unlabeled mAbs (a βGal-IgG1 control, Trastuzumab, Kadcyla, and Pertuzumab, 20 µg/mL) and added to HER2-positive AU565 cells. A left shift in the histogram relative to the IgG control shows a decrease in intensity of RPE, indicating competition for binding to HER2.

relative to the IgG control) when these two antibodies were combined. There was no competition between the Trastuzumab-based antibodies and Pertuzumab as they bind different epitopes. This illustrates why these antibodies can be given as a combination therapy, which has been shown to increase median overall survival in treatment of HER2-positive breast cancers.[3]

Summary
The iQue® Advanced Flow Cytometer with optimized assays and validated reagents enables high-throughput measurement of antibody binding activity. The direct binding workflow provides robust quantification of binding to native protein on live cells, with simple generation of pharmacological readouts, such as EC_{50} values using the iQue Forecyt® software. The competition binding assay provides a streamlined workflow to assess antibodies that bind the same epitope on the target antigen. These assays have the potential to enhance the speed and quality of hits generated during antibody discovery.

Antibody Internalization (ABI)

Antibodies specific to cell surface antigens induce endocytosis, which leads to the cellular internalization of those antibodies along with any molecules conjugated to them. The process of antibody internalization induces a multitude of effects inside cells, which can be harnessed by therapeutic agents to fight disease. For example, through delivery of highly toxic drugs to specific cancer cells via antibody drug conjugates (ADCs), removal or degradation of surface receptors from cancer cells (i.e. EGFR), and antibody immunotherapies used to identify tumor cells for immune cell killing (i.e. ADCC or ADCP).

Many fluorescence-based methods are available to assess internalization, utilizing analytical methods such as traditional flow cytometry or confocal microscopy. The downside to these techniques is that they can involve lengthy, complicated workflows, large sample sizes, multiple wash steps and low-throughput instrumentation.

iQue® Assay Principles
The iQue® Antibody Internalization Reagent is designed to measure internalization of a large number of antibodies in 96- and 384-well formats. The reagent includes a novel pH-sensitive fluorescent probe that enables one-step, no-wash labeling of isotype matched antibodies. A fluorescent signal is generated as internalized antibody is processed into the acidic endosome and lysosome pathway (Figure 6). Antibody internalization is quantified as the percentage of live cells positive for the fluorescent probe.

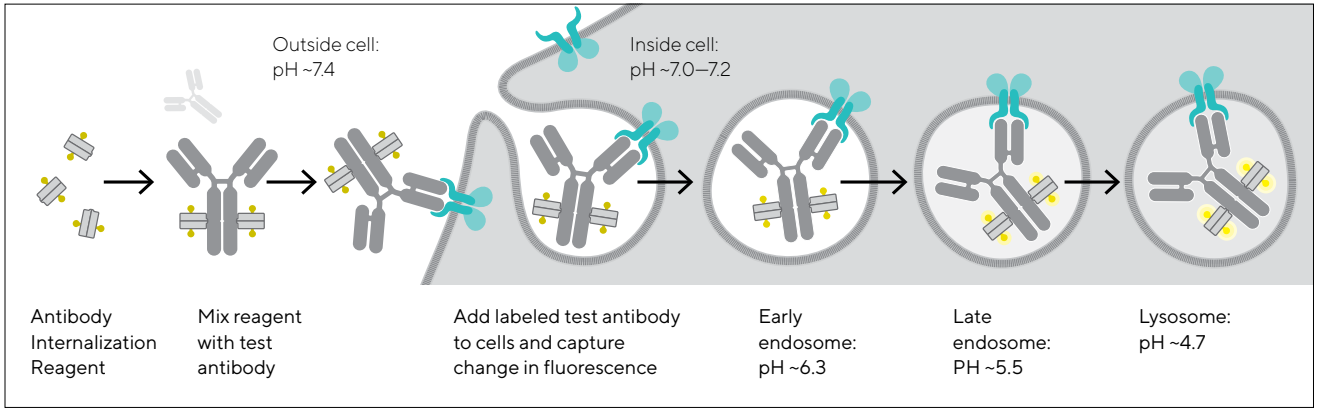


Figure 6
Antibody Internalization pH-Sensitive Fluorescent Probe Principle

The internalization reagent can also be used as part of the iQue® Multiplex Antibody Internalization Kit, which is a high throughput, multiplex, no-wash assay for simultaneous quantification of antibody internalization, cell specificity, and cell health using 10 µL sample of cells and 10 µL antibody. The iQue® Cell Proliferation and Encoding (V/Blue) Dye can be used at various concentrations to differentially-label cell types with different intensities of fluorophore to facilitate multiplexed assessment of ABI into multiple cell types per well. Cell viability is measured using the included iQue® Cell Membrane Integrity (B/Green) Dye. These reagents are combined into a simple workflow, suitable for use with both adherent and suspension cell types (Figure 7). The easy antibody labeling step can be performed in full media suitable for early-stage hybridoma screening. It requires a low quantity of antibody and can be tested on multiple cell types for multiplex screening of internalization and cell health readout.

Example Results

To demonstrate the multiplexing capabilities of this novel pH-sensitive dye on the iQue® Platform, Ramos and Raji cells were stained with two intensities of violet encoding dye, combined with unstained Jurkat cells. Cells were incubated with a serial dilution of dye-conjugated specificity antibodies: isotype-matched anti-CD3 (T cell marker), anti-CD19, anti-CD20, anti-CD22, or anti-CD79b (B-cell markers), anti-CD71 (positive control), and IgG (negative control). The cell membrane integrity dye

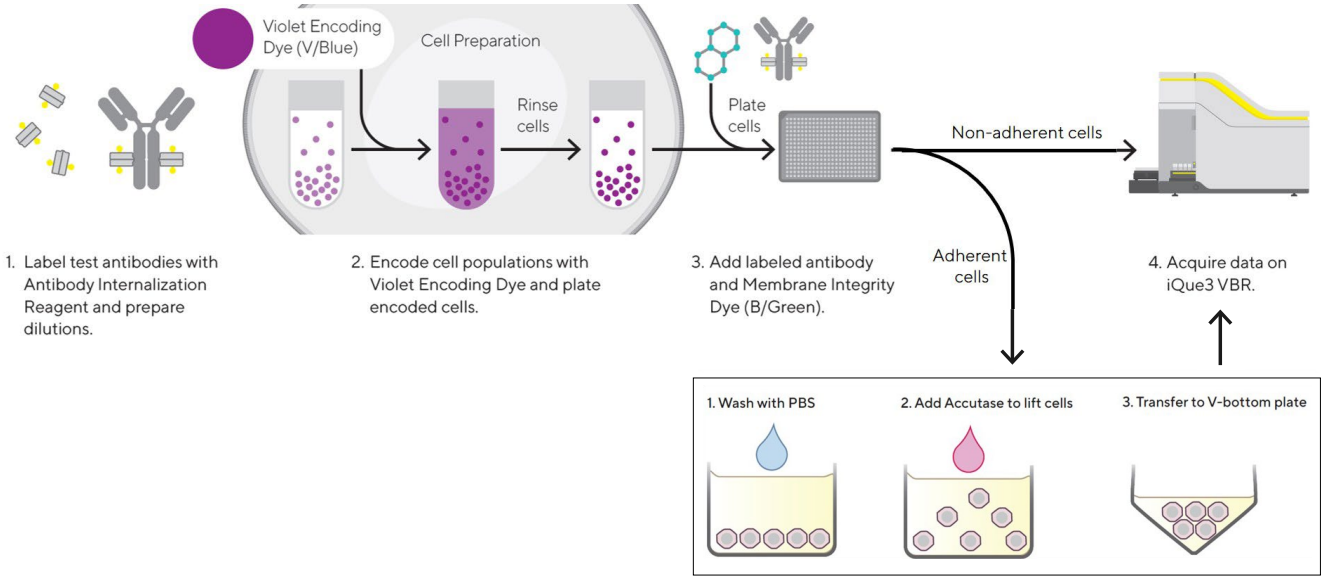


Figure 7
Schematic of the iQue® Antibody Internalization Kit Workflow

was also added to enable identification of viable cells. The three cell types were then spectrally separated based on encoder dye labeling; this means internalization of each antibody into each cell line could be distinguished (Figure 8).

Jurkats, from a T cell line, showed internalization of anti-CD3, but not anti-CD19 or anti-CD22, whereas the Rajis, from a B cell line, internalized anti-CD19 and anti-CD22, but not anti-CD3. Only Ramos cells, also from a B cell line, showed internalization of anti-CD79b, an ADC drug target for non-Hodgkin's lymphoma. In the three-hour assay time frame, we did not observe anti-CD20 internalization, but this was evident in the two B cell lines by 24 hours (data not shown).

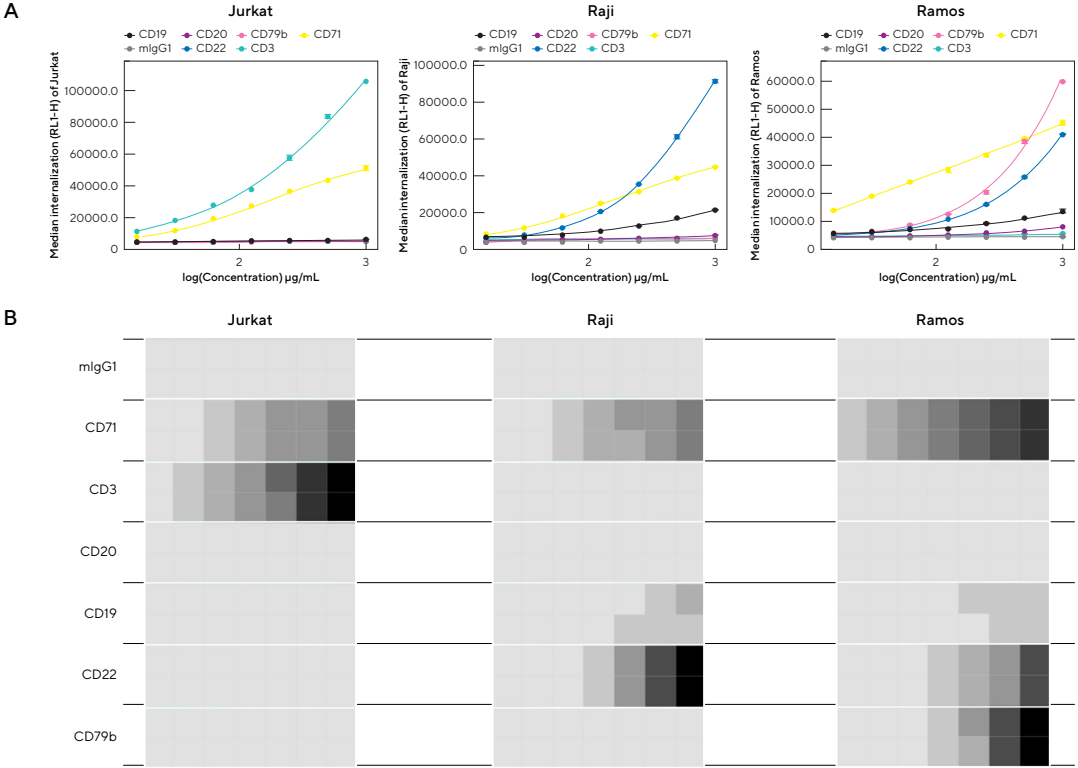


Figure 8

Multiplexed Assessment of Antibody Internalization into Several Cell Types Reveals Differential Internalization Specificities

Note. (A) Serial dilution curves for internalization-labeled antibodies. A serial dilution of each antibody with a top concentration of 1 mg/mL was prepared and incubated with encoded Jurkat, Raji, and Ramos cells in the same well. (B) Heat maps show median fluorescent intensity (MFI) for internalization in (A).

Summary
The iQue® Platform and integrated Forecyt® software provide an integrated solution that rapidly profiles antibody internalization and other critical characteristics using data analysis and visualization with plate-level analytics to accelerate antibody discovery, development, and screening of antibody drug candidates for potential drug efficacy and toxicity. Compared to performing a series of single-plex assays, a multiplexed assay approach enables you to analyze multiple readouts (internalization, viability, cell type) from a single well, decreasing the number of tests needed to perform a comprehensive functional characterization of the antibody candidate.

Antibody-Dependent Cellular Cytotoxicity (ADCC)

Natural Killer (NK) cells are an essential part of the innate immune system and play a crucial role in immune surveillance and anti-tumor responses. Currently, several types of NK cell related immunotherapeutics are being developed for the treatment of cancers. Tumor-specific monoclonal antibodies (mAb) that are able to induce NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) have proven to be successful against several types of cancer. Multiple bispecific and trispecific NK cell engaging antibodies (BiKES, and TRiKES) are also currently in preclinical and clinical development.[4-7]

A decisive factor in evaluating the potency of any new tumor-specific mAb is its ability to induce NK cell-mediated ADCC against the targeted tumor cells. Complexity arises from donor cell variability, which can modulate the degree of NK cell mediated ADCC. Thus, in addition to assessing cytolytic potential, it is also important to characterize the number and activation state of the donor NK cells to provide insight into the potential treatment efficacy. Traditional cytotoxicity assays, such as Chromium-51 (Cr-51) release or use of fluorescent DNA binding vital dyes with standard flow cytometry methods, are time intensive and require additional downstream assays for characterization of donor effector cells. The iQue® Human NK Cell Killing Kit provides a multiplexed approach that is faster and provides more insights than traditional methods.

iQue® Assay Principles

- NK cells mediate ADCC through engagement of their FcγRIIIa (CD16a) with the constant (Fc) region of Abs bound to a target cell. This process triggers strong NK cell activation including the release of cytolytic granules containing perforin and proteases known as granzymes, up-regulation of Fas ligand expression, and production of cytokines, such as interferon gamma (IFNγ). The iQue® Human NK Cell Killing Kit is a multiplexed, high-throughput assay that simultaneously measures target cell killing, expression of NK cell phenotypic and activation markers, and quantification of secreted effector proteins and cytokines in a single well of a 96- or 384-well plate (Figure 9).

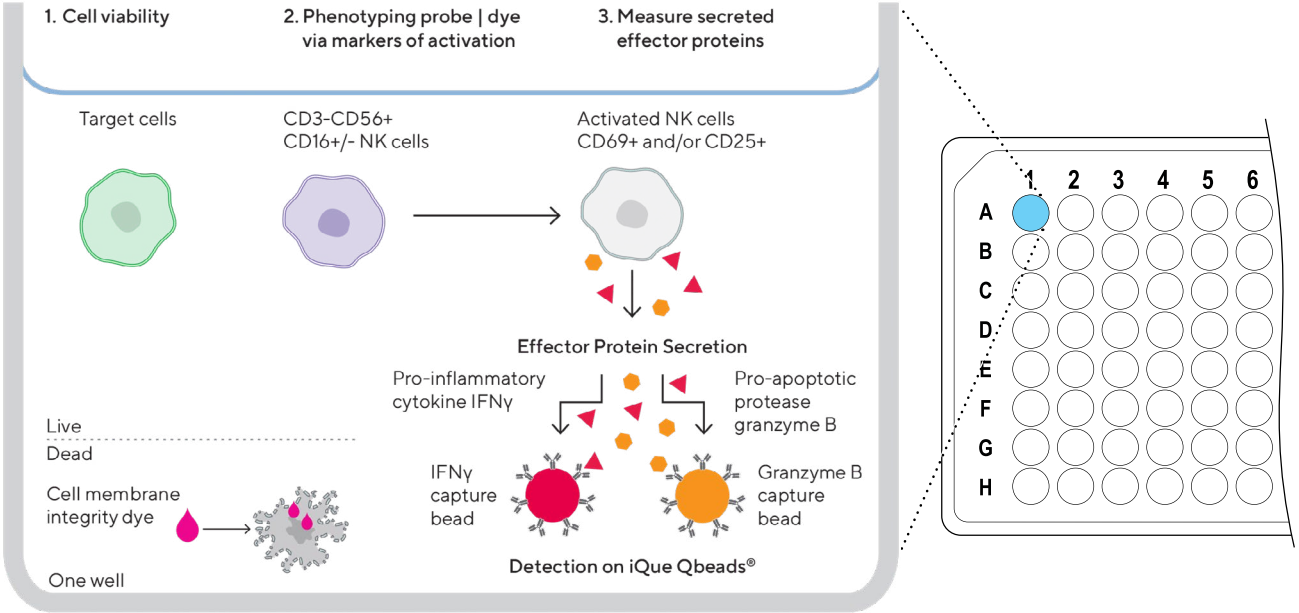


Figure 9
Illustration of iQue® Human NK Cell Killing Kit Assay Principles

Target cells are distinguished from effector cells by staining with a fluorescent encoder dye, whilst live and dead populations are separated by staining with a fluorescent membrane integrity dye. ADCC activity is determined through quantification of the number of dead target cells/well. NK cells are immunophenotyped by staining with a fluorescent antibody panel to identify CD3- CD56+ NK cells, and then expression of CD16 can be assessed. This is important since differences in CD16 expression levels have been associated with differences in ADCC activity.[8] The phenotyping panel also includes two NK cell functional activation markers; CD69 and CD25. Release of IFNγ and pro-apoptotic protease, Granzyme B is quantified using 2-plex iQue Qbeads® in a sandwich with immunoassay format. Combining the iQue® Human NK Cell Killing Kit with the iQue® Human NK Cell

Companion Kits provides the flexibility to quantitate additional effector proteins, including Granzyme A, CD178 (Fas Ligand), TNF, GM-CSF, MIP-1a (CCL3), and RANTES (CCL5), all in the same well.

IC/EC_{50} curves and color compensation matrices are auto-generated using the iQue® Human NK Cell Killing Kit template and iQue Forecyt® software. Templated analysis workflows reduce time to actionable results due to the inclusion of pre-set color compensation matrices, circumventing the need for single-color compensation controls and subsequent color compensation analysis. Templated gating allows individual cytokine populations to be identified from the singlet iQue Qbeads® population. Separate effector and target cell populations are identified from the singlet cell population based on an encoding dye. Live/dead cell populations can then be further identified for both the effector and target cell populations using a cell membrane integrity dye (Figure 10A). Further gating strategy was used to identify CD3-CD56+ NK cells, followed by analysis of CD16 expression and the activation markers, CD69 and/or CD25 (Figure 10B).

Example Results

To test the ADCC activity of three anti-hCD20 mAbs, we utilized the iQue® Human NK Cell Killing Kit plus iQue® Human NK Companion Kits. The mAbs included Rtx-G1, a human IgG1 with Rituximab variable region and Ob-G1, a human IgG1 with Obinutuzumab variable region. The Ob-A2 antibody was used as a negative control. It also possessed the variable region of Obinutuzumab but had a human IgA2 constant region and did not bind CD16a. PBMCs were added at a 10:1 E:T or direct antibody-mediated killing of tumor cells was assessed by culturing Raji cells with mAbs alone in the absence of effector cells. Following co-culture for 4 hours, 10 µL samples were labeled and analyzed using the iQue® Platform.

The Ob-G1 anti-hCD20 mAb demonstrated greater ADCC potency as compared to Rtx-G1 for all donors tested (Figure 11). No increase in ADCC above baseline levels was seen when the negative control anti-hCD20 mAb, Ob-A2, was included in the co-cultures, and only very low levels of direct killing of Raji tumor cells was observed using the anti-hCD20 mAbs alone. Differences in the level of tumor cell killing was also observed between donors, with PBMCs from Donor 1 with a FcγRIIIa-158 V/V genotype, exhibiting greater ADCC than Donor 2 (FcγRIIIa-158 V/F) or Donor 3 (FcγRIIIa-158 F/F) using either the Ob-G1 or Rtx-G1 anti-hCD20 mAbs. In addition, the secretion levels of several effector proteins and cytokines reflected the level of ADCC induced by the different mAbs tested, as exemplified by the data for Donor 1 in Figure 12.

Summary

The iQue® Human NK Cell Killing Kit provides a multiplexed approach to simultaneously measure ADCC, assess the NK cell activation state, and quantitate effector proteins (IFNγ and Granzyme B) secreted in a single well of a 96- or 384-well plate. The iQue® Human NK Cell Companion Kits add the flexibility to quantitate up to six additional effector proteins and cytokines within the same wells. Samples are acquired using the iQue® and analyzed using the integrated iQue Forecyt® software with pre-set compensation matrices. This collapses traditional workflows into a single assay platform which allows for streamlined and rapid data acquisition and can be used to rapidly screen NK cell effector functions and new, potential NK cell-based therapeutics.

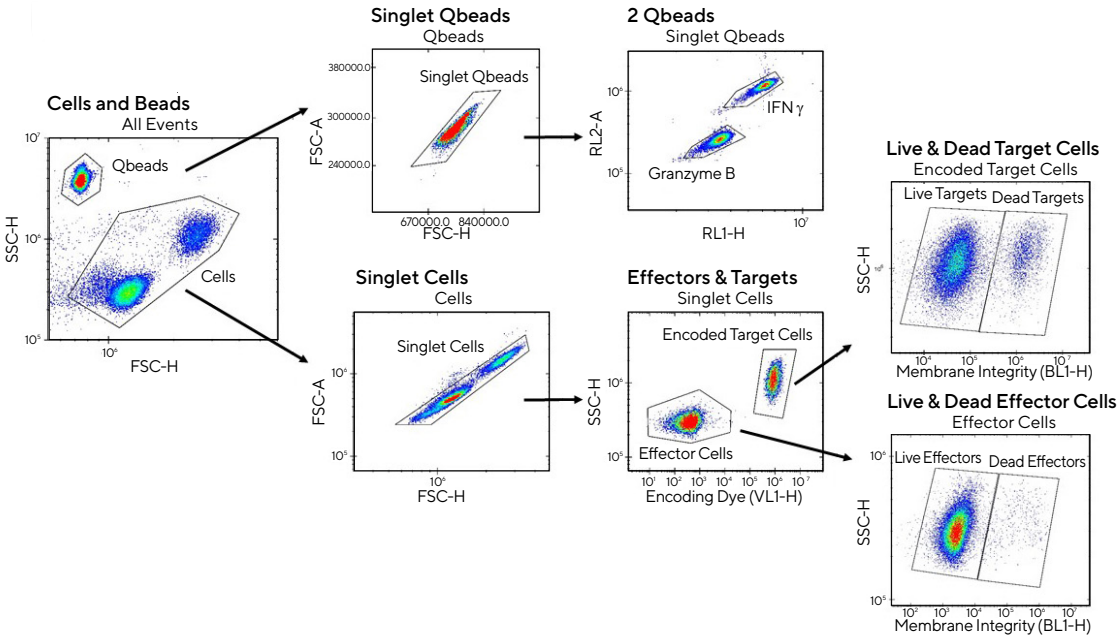


Figure 10A

Simultaneous Assessment of NK Cell Cytolytic Activity along with NK Cell Phenotypic and Activation Markers

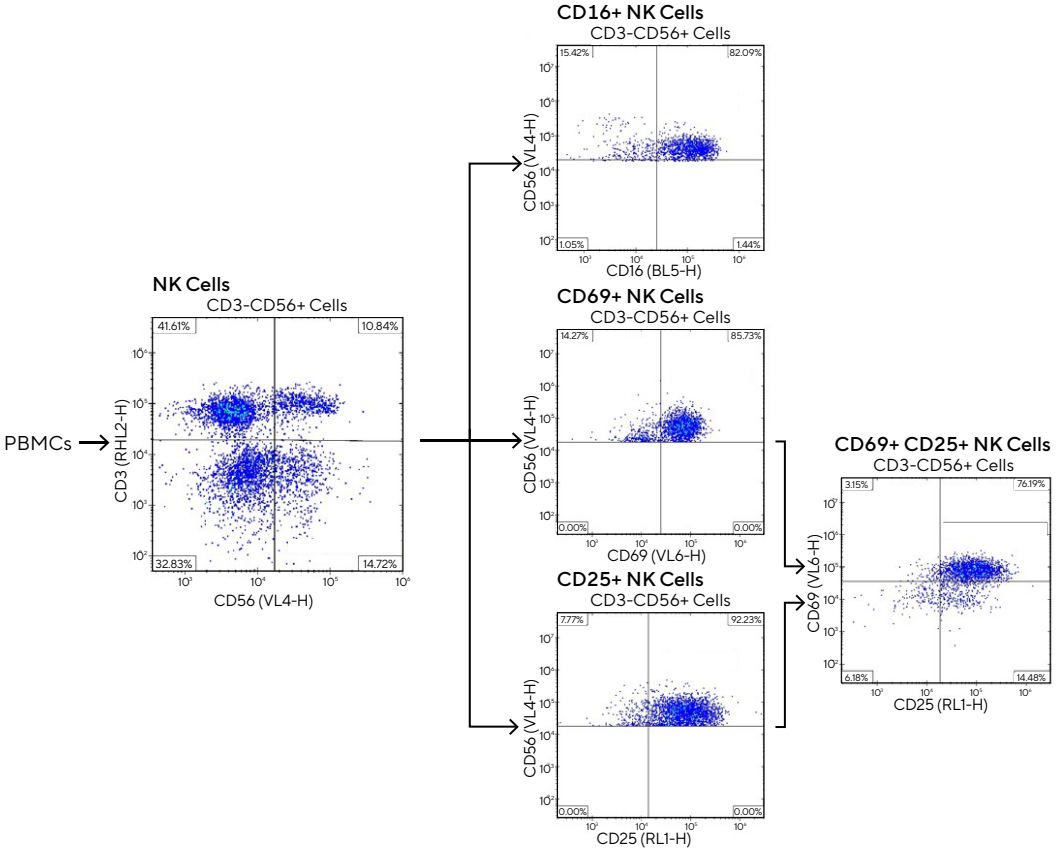


Figure 10B

Simultaneous Assessment of NK Cell Cytolytic Activity along with NK Cell Phenotypic and Activation Markers

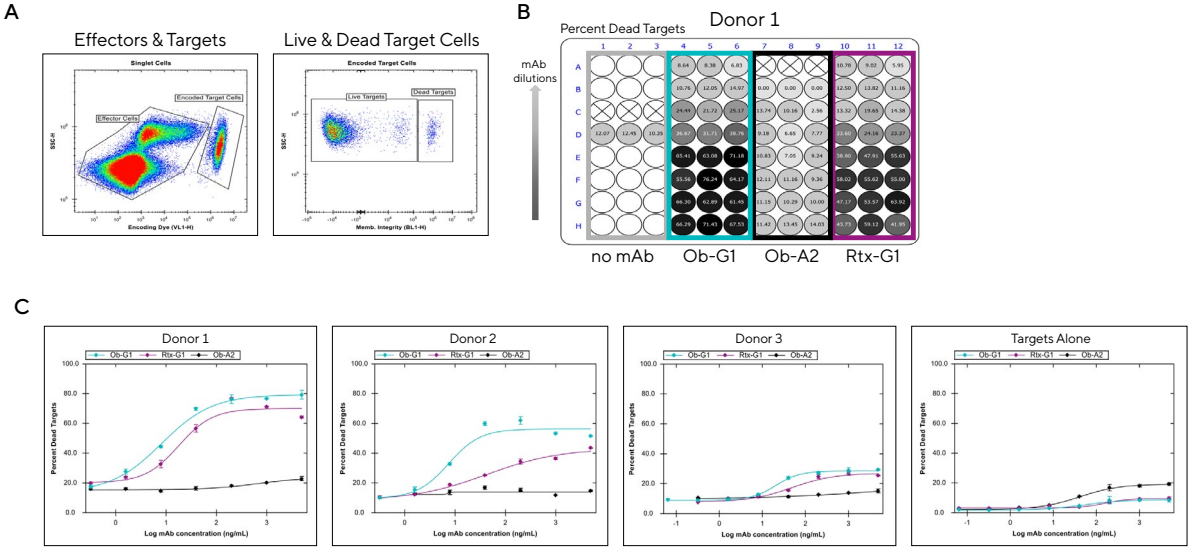


Figure 11

ADCC Activity Assessment

Note. The level of ADCC is mAb and PBMC donor dependent. PBMCs (200K/well) from three donors were co-cultured with Raji tumor cells (20K/well) in the presence of anti-hCD20 mAbs (Ob-G1, Rtx-G1, or Ob-A2, as a negative control). After 4 hours, 10 µL samples were analyzed using the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits. (A) Target cells were distinguished from effector cells with the use of a fluorescent encoder dye. Target cell killing was determined using a cell membrane integrity dye. (B) Percent dead targets displayed as a plate heat-map for Donor 1 over serial dilutions (from 10 µg/mL). (C) Curve showing tumor cell killing by effector cells at five-fold serial dilutions (from 5 µg/mL). Rajis were incubated with the mAbs alone to evaluate only direct Ab-mediated cytotoxicity targets.

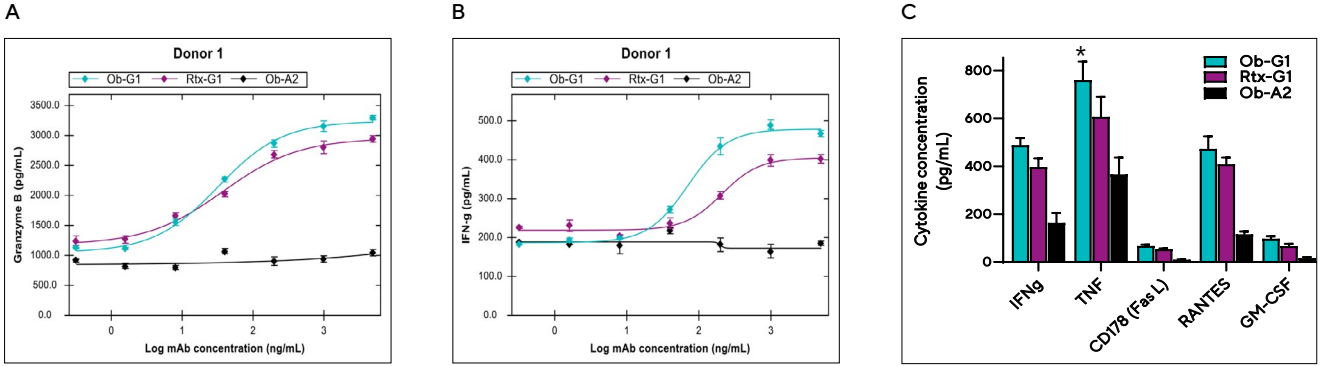


Figure 12

Effector Protein and Cytokine Secretion Levels Correspond with mAb Potency

Note. Secretion levels of (A) Granzyme B and (B) IFNγ by PBMCs from Donor 1 cultured with Raji tumor cells in the presence of anti-hCD20 mAbs at concentrations ranging between 0–5 µg/mL. (C) Cytokines produced by Donor 1 PBMCs co-cultured with Raji cells and three different mAbs at 1 µg/mL dose. Bar graph data presented as Mean +/-1 SD, 3 replicates/mAb dose. (*) Significantly higher levels of cytokines produced in co-culture using Ob-G1 compared to Rtx-G1 anti-CD20 mAb, $p < 0.05$.

Antibody-Dependent Cellular Phagocytosis (ADCP)

ADCP is the immunological process of antibody-stimulated engulfment of tumor cells by phagocytic immune cells, such as monocytes and macrophages. This results in specific internalization and degradation of tumor cells by the immune cells. ADCP, alongside ADCC and CDC, is one of three key mechanisms of action (MoAs) adopted by mAbs to induce clearance of cancerous cells from the body. Powerful, high-throughput techniques are therefore crucial to facilitate discovery of novel therapeutics by screening large libraries of antibodies for their involvement in these MoAs in minimal time.

Conventional techniques for measuring ADCP, such as traditional flow cytometry and confocal microscopy are limited due to the following reasons:

- Assay workflows are complex and time-consuming resulting in low throughput.
- They require lengthy fixation protocols, repetitive wash steps and complicated data analysis.
- These methods often involve multiple rounds of protocol optimization and necessitate large volumes of precious sample.
- Other techniques such as reporter assays are reliant on engineered reporter cell lines. True ADCP is challenging to quantify, meaning traditional assays may either provide only qualitative readouts, or are heavily prone to measuring artifacts.

iQue® Assay Principles

We describe a high throughput, multiplex assay that measures ADCP via co-localization of encoding dye-labeled target cells with CD14 positive effector cells using a simplified, streamlined workflow (Figure 13). Validated mix and read reagents from the iQue® Human Antibody Dependent Cellular Phagocytosis Kit are added to co-cultures of adherent or suspension target cells alongside primary effector cells. Target cells labeled with iQue® Proliferation and Encoding (B/Green) Dye are incubated with test antibody in 96- or 384-well plates prior to the addition of unlabeled effector cells (either PBMCs or an enriched population of monocytes or macrophages). Live and dead cells are separated using the iQue® Cell Membrane Integrity (R/Red) Dye. ADCP is quantified as the percentage of live, CD14+ effector cells that are positive for the target cell encoder. The combination of fluorophores used in this kit means it is compatible with both the iQue® BR and VBR configurations.

The pre-set gating template provided with the iQue® Human Antibody Dependent Cellular Phagocytosis Kit (Figure 14) combined with automated data analysis by iQue Forecyt® software provides instant identification of co-localized live, encoded target cells with CD14+ effectors and generation of pharmacological readouts, such as EC_{50} values.

Example Results

The iQue® Human Antibody Dependent Cellular Phagocytosis Kit workflow was used to measure ADCP response to Truxima (anti-CD20-IgG1), a Rituximab biosimilar treatment for CD20 positive B cell cancers. The mAb was added to Ramos cells labeled with encoder dye in a 384-well plate, then PBMCs from two different donors were then added at various effector-to-target ratios (E:T) (Figure 15). After 1 hour, all cells were labeled with viability dye and the Human ADCP Antibody Detection Reagent so that co-localization between live encoded targets and CD14+ monocytes could be quantified using the iQue® Platform.

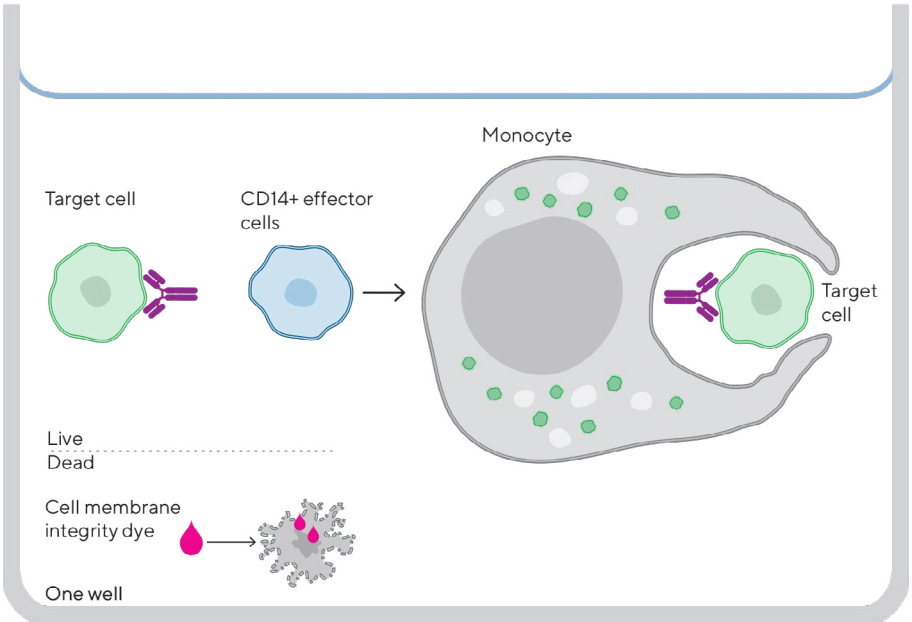


Figure 13
Illustration of the iQue® Human Antibody-Dependent Cellular Phagocytosis Kit Assay Principles

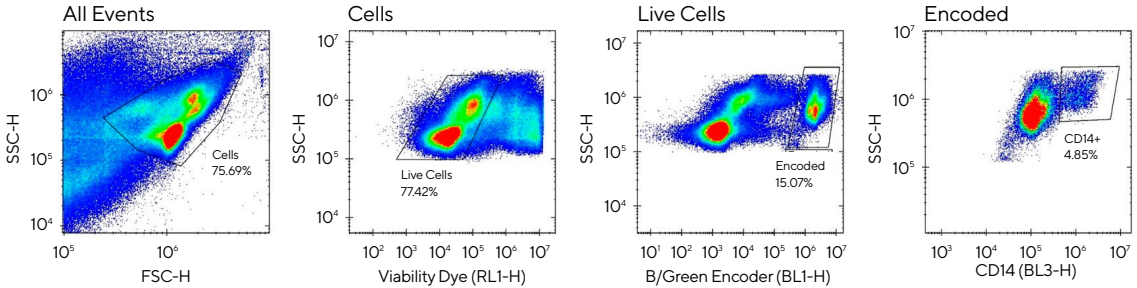


Figure 14

iQue® Human Antibody-Dependent Cellular Phagocytosis Kit Pre-Set Gating Template

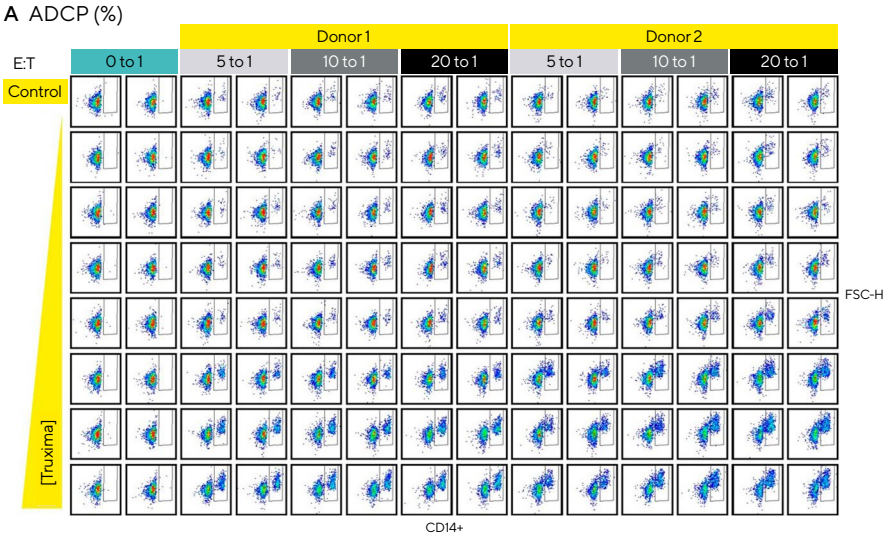
Note. Omission of a single cell gate combined with a simple 3-step gating strategy ensures measurement of a true ADCP signal.

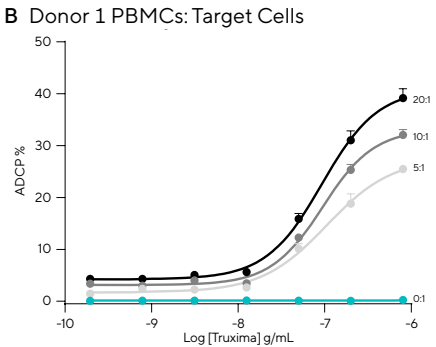
Truxima induced a concentration-dependent increase in ADCP of target cells mediated by the effector cells. This increase was observed at 5:1, 10:1, and 20:1 E:T, but not in the absence of PBMCs (0:1). At the highest E:T (20:1), maximal ADCP (%) was very similar between donor 1 and donor 2, at 41 ± 5% and 43 ± 2% respectively. However, as the E:T decreased, the difference in response between the two donors became more pronounced, with a maximal response at 5:1 E:T of 28 ± 8% with donor 1 compared to 19 ± 6 % with donor 2. Donor-to-donor variability in the level of mAb stimulated clearance of tumor cells may relate to differences in the immune cells' Fc receptor density, polymorphisms in the Fc receptor affecting IgG binding or variability in the number of phagocytic immune cells within each PBMC population.[9,10]

Summary

The iQue® Human Antibody Dependent Cellular Phagocytosis Kit workflow provides a simple, robust assay for measuring ADCP. The pre-set gating template provided with the kit enables instantaneous quantification of co-localized targets and effectors which can be used to generate pharmacological readouts for ADCP response to mAbs.

The flexibility to use either adherent or suspension cells in this assay means it is suitable for modeling mAb effects on both blood cancers and solid tumors. The high-throughput power of the iQue® Platform combined with inbuilt, automated data analysis by iQue Forecyt® software can profile libraries of mAbs for their effects on ADCP in minimal time. This technique is a powerful tool with potential to reduce the time for MoA characterization in drug discovery applications.





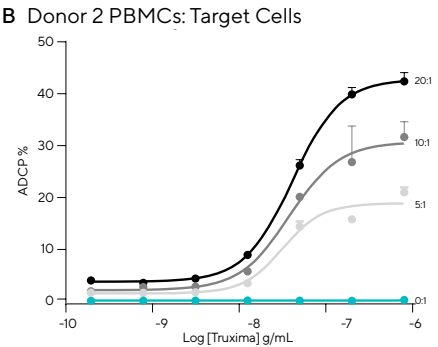


Figure 15

ADCP Response to Truxima Increases with Concentration and Effector-to-Target Ratio

Note. Ramos cells (2.5K/well) were incubated with varying concentrations of Truxima (anti-CD20-IgG1 mAb; Rituximab biosimilar; 0.05 ng/mL to 200 ng/mL). PBMCs were added at 5:1, 10:1, and 20:1 effector-to-target ratios. Control wells contained no antibody. ADCP was analyzed using the iQue® Human Antibody Dependent Cellular Phagocytosis Kit and the iQue® Platform. ADCP (%) is defined as the % CD14 and B/Green Encoder+ cells in the live population. (A) Plate view with gate for CD14+ cells within the B/Green encoded live population in each well. (B, C) Concentration-response curves with ADCP (%) by donor 1 and 2 PBMCs, respectively.

Complement-Dependent Cytotoxicity (CDC)

There are several mechanisms of action (MoAs) through which therapeutic mAbs are cytotoxic towards target cells, such as cancer cells. These MoAs often harness the power of the body's own immune system to exert anti-tumor effects. Included in this are the three key Fc-mediated functions: ADCC, ADCP and CDC. ADCC and ADCP rely on a mAb simultaneously engaging with Fc receptors on immune cells and the antigen on target cells. This brings the target cells into proximity with cytotoxic cells (e.g. natural killer cells) or phagocytic cells (e.g., monocytes or macrophages), leading to enhanced immune clearance. Contrastingly, in CDC, the antigen-bound mAb recruits proteins present in the blood to induce lysis. This process begins with binding of the C1q protein to the mAb Fc region, which triggers activation of the complement pathway. A complex molecular cascade follows, which culminates in the formation of the membrane attack complex (MAC).[11] The MAC creates a pore in the target cell membrane, resulting in cell death.

During early-stage mAb development, *in vitro* assays are often used to profile activity towards several MoAs, including CDC, to ensure mAbs with desirable characteristics are selected for clinical evaluation. Conventional techniques for measuring CDC activity often:

- Require large volumes of precious antibody and serum samples
- Use instrumentation with low-throughput acquisition (e.g. traditional flow cytometry)
- Are laborious and time-consuming, requiring steps such as protocol optimization, fixation, and multiple washes

iQue® Assay Principles

The iQue® CDC assay is a simple, streamlined assay for measuring CDC activity of monoclonal antibodies against live target cells using the iQue® Advanced Flow Cytometry Platform (Figure 16). Target cells are cultured with mAbs of interest in the presence of human serum, which contains the proteins required to initiate the complement cascade and for subsequent formation of the membrane attack complex on cells. Target cells can be labeled using the iQue® Cell Proliferation and Encoding (V/Blue) Dye so that multiple cell types can be distinguished in a single well (for example a bright, mid-bright, dim, and unstained population). This assay is appropriate for use with both suspension and adherent target cells. Cells are labeled using iQue® Cell Membrane Integrity (R/Red) Dye to enable quantification of cell death as an indicator of CDC activity. Fluorophore-conjugated antibodies may be included alongside the integrity dye to reveal changes in surface marker expression associated with CDC activity, for example surface expression of complement proteins. Following a final wash step, cells are resuspended and run on the iQue® Advanced Flow Cytometry Platform. In the integrated iQue Forecyt® software, a simple gating strategy is applied to determine the proportion of dead cells per well (Figure 17).

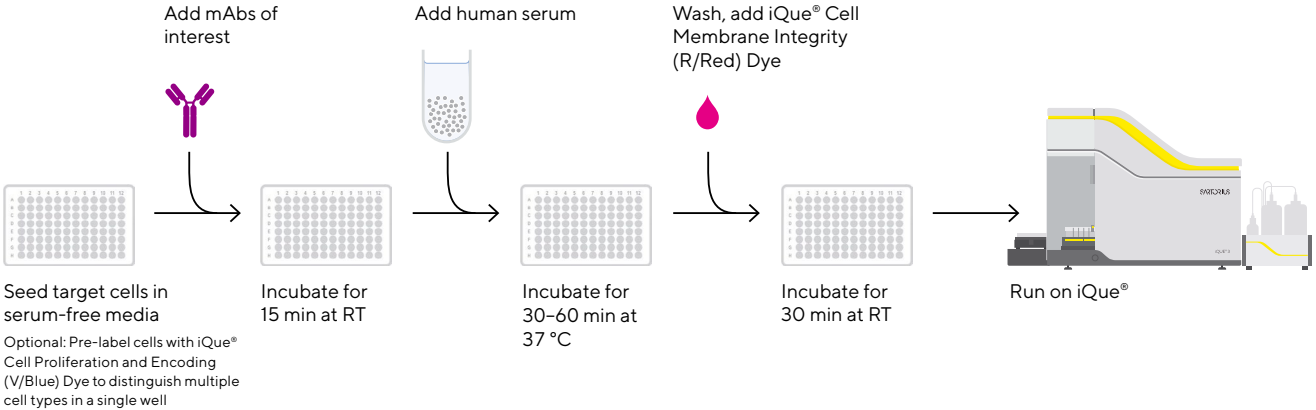


Figure 16

Workflow Schematic for CDC Assay Using High-Throughput Flow Cytometry

Note. Combine target cells and mAbs of interest (10 µL of each per well, in serum-free media) in a 96- or 384-well plate and incubate for 15 minutes at room temperature (RT) to promote antibody binding to targets. Add human serum (15%, 10 µL/well) and incubate for a further 30 mins (37 °C). Wash then label with iQue® Cell Membrane Integrity Dye (choice of V/Blue, B/Green, B/Red, R/Red) for 30 minutes (RT). Wash then collect data using the iQue® Advanced Flow Cytometer.

Example Results

Levels of CD20 Expression on Target Cells Correlated with CDC Activity

Several studies have shown links between CD20 expression on cancerous target cells and the level of CDC activity exerted on them by anti-CD20 antibodies.[12,13] Our experiments aimed to explore differences in CDC between suspension cell types using the iQue® assay. The histogram in Figure 18A shows the relative cell surface CD20 expression on three cancer cell lines: Ramos, Rajis (another B-lymphocyte cell line) and Jurkats (a T-lymphocyte cell line). These data show that CD20 expression is highest on Ramos cells, with a median fluorescence intensity (MFI) x 10^6 of 3.08 ± 0.1, compared to 1.51 ± 0.03 on Raji cells. Expression of CD20 on Jurkat cells was similar to the IgG background control, with an MFI x 10^6 of 0.06 ± 0.0005. To conserve time and reagents, iQue® Cell Proliferation and Encoding (V/Blue) Dye was used to differentially label cells so they can be distinguished within a single well, allowing comparison of CDC across multiple

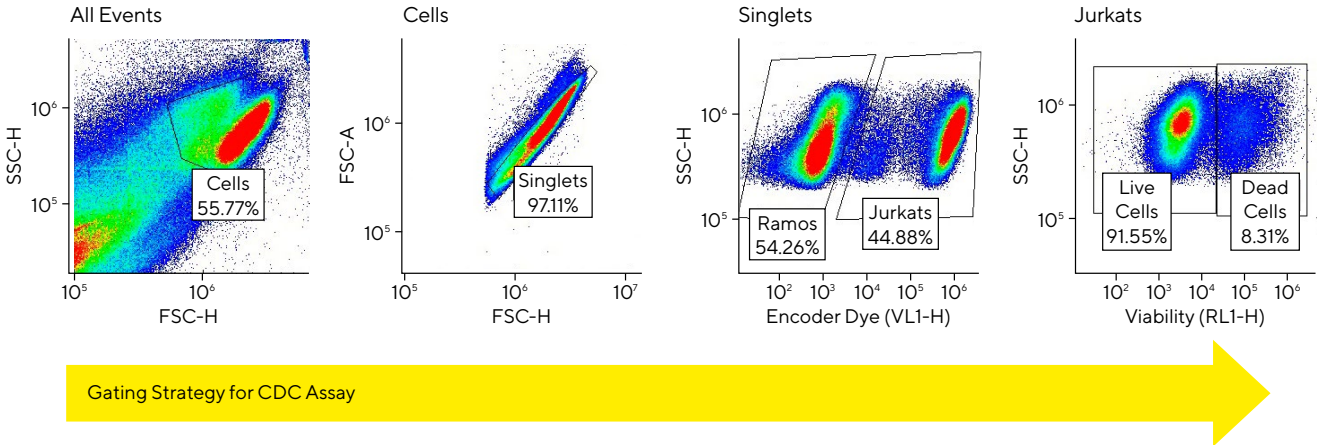


Figure 17

Gating Strategy for Quantifying Cell Death in the iQue® CDC Assay

Note. First, cells and single cells are gated. If multiple cell types were included in the well, they can then be separated based on fluorescence of the iQue® Cell Proliferation and Encoding (V/Blue) Dye. Cell viability can then be determined based on iQue® Cell Membrane Integrity (R/Red) Dye staining.

antibodies, concentrations, and cell types using a single 384-well plate. The histogram in Figure 18B shows how the cell types were separated, with the Rajis brightly-labeled, Ramos more dimly-labeled, and Jurkats unlabeled.

Figures 18C and 18D reveal how CDC induction by both Rituximab and Truxima® differed depending on the target cell type. The high-CD20-expressing Ramos cells saw the greatest percentage cell death at the highest concentrations of both mAbs, with a 68 and 70% reduction in live cells compared to the IgG control for Rituximab and Truxima®, respectively. The mid-CD20-expressing Raji cells saw considerably less CD20 mAb induction of CDC compared to the Ramos, at 34% (Rituximab) and 24% (Truxima®). As expected, no CDC was induced in the presence of CD20-negative Jurkat cells.

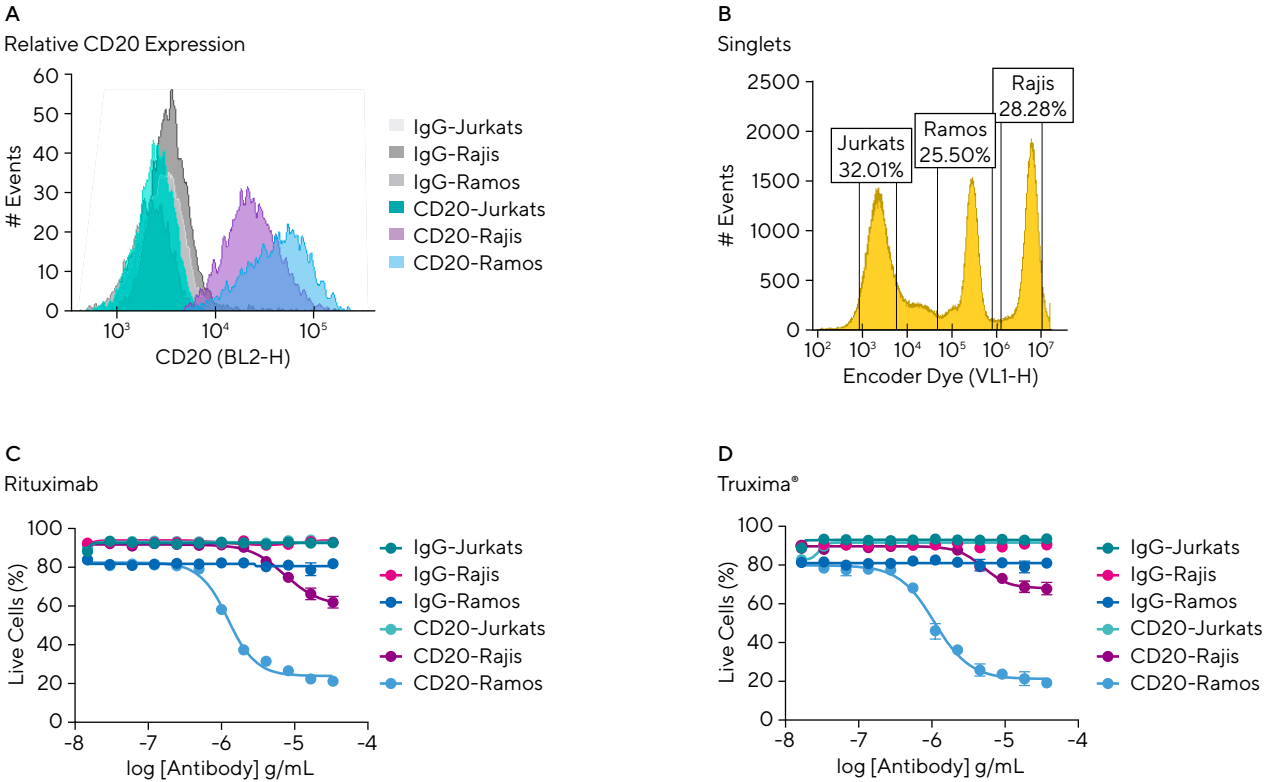


Figure 18: *Higher CD20 Expression Elicits Greater CDC Induction by Anti-CD20 mAbs*

Note. (A) Relative CD20 expression on Jurkat, Raji, and Ramos cells compared to an IgG background control. (B) 5K/well of each cell type were seeded in a 384-well plate for a CDC assay and separated using differential labeling with iQue® Cell Proliferation and Encoding (V/Blue) Dye. CDC activity was measured with a range of concentrations of (C) Rituximab and (D) Truxima® (a Rituximab biosimilar).

Protein Binding and CDC Activity

To delve deeper into the CDC mechanism, it can be useful to combine quantification of CDC activity with measurement of expression of cell surface markers or binding of proteins, for example those involved in the formation of the complement complex. The data in Figure 19 show the results from a CDC assay in which a FITC-labeled antibody was included during the labeling step to look at the binding of complement proteins C4c and C4b. C4b binds cells during the complement cascade and is processed to C4c, which remains bound to the cell surface.[14] The cell surface location and lack of occlusion of these complement proteins makes them an ideal target for binding antibodies. This method did not work for measuring binding of the C1q protein (data not shown). This is likely due to recruitment of other proteins to C1q in the complex obstructing antibody binding. Instead, its binding should be measured using purified C1q protein, a method which has been utilized in other studies.[15] The results in Figure 19 show that both CDC and C4c and C4b binding increased in response to increasing Rituximab concentrations on the high-CD20-expressing Ramos cells. The EC_{50} value for CDC activity was 0.17 µg/mL while the EC_{50} for C4c and C4b binding was 0.64 µg/mL. No CDC or C4c and C4b binding was observed with either the IgG control antibody or with the antigen negative Jurkat cell line.

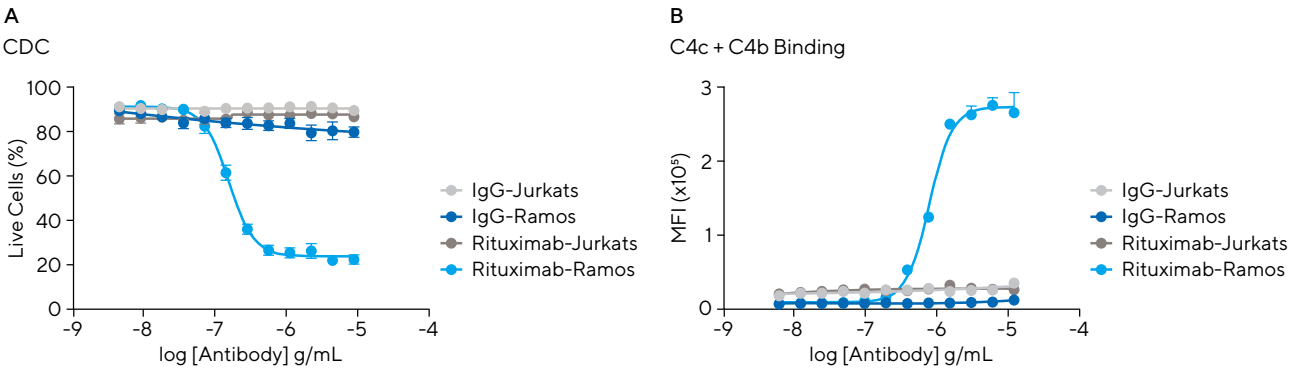


Figure 19

Binding of Complement Proteins C4c and C4b to the Cell Surface Increased with CDC.

Note. 10K/well each of Ramos and Jurkat cells were seeded with a range of concentrations of Rituximab or an IgG control antibody. Human serum was added for 30 minutes to induce CDC. (A) Quantification of the percentage of live cells to give an indication of cell death due to CDC activity. (B) MFI on cells for binding of an anti-C4c + C4b antibody (part of the complement protein cascade).

Summary
This is an easy-to-follow workflow for quantifying CDC activity using the iQue® Advanced Flow Cytometry Platform. Fast sample acquisition by the iQue® combined with streamlined data analysis using the iQue Forecyt® software enables rapid comparisons between antibody function to be drawn. The CDC readout can be multiplexed with other markers and dyes, allowing much more information to be gathered from a single assay and negating the need to combine data from multiple sources. Combining CDC data with Fc function analysis using the iQue® Human ADCP Kit and iQue® Natural Killer Cell Killing Kit can provide full profiling of the three key Fc receptor-mediated functions of antibodies using a single instrument. This workflow can improve antibody drug discovery processes, both through enhanced speed and quality of hits generated.

Antibody Cytotoxicity

Antibody-drug conjugates (ADCs) are one of several adaptations made to monoclonal antibodies (mAbs) to improve their anti-tumor activity. They unite both immunotherapeutic and chemotherapeutic interventions to create an efficacious and targeted cancer treatment, leveraging the highly specific nature of therapeutic mAbs as a backbone for delivery of potent cytotoxic drugs. These mAbs have been adapted to include a linker in their constant region, onto which a toxic payload is attached. ADCs can be characterized based on whether the linkers are cleavable (released by proteases) or non-cleavable (degraded in acidic lysosomes).[16] Both types rely on internalization of the ADC into tumor cells to trigger the cytotoxic action of the payload drug, and therefore work to prevent release of the drug in the blood stream or healthy tissues. The payloads typically used are chemotherapeutic drugs, with mechanisms of action (MoAs) that interfere with processes such as microtubule polymerization or that induce DNA damage.[17] Drugs with these MoAs preferentially kill rapidly proliferating cells, such as cancer cells, adding an extra layer of specificity to ADC cytotoxicity.[18,19]

Traditionally, assays for quantification of antibody cytotoxicity have been limited due to:

- Low-throughput instrumentation (e.g. traditional flow cytometry)
- Laborious, time-consuming processes involving protocol optimization, fixation, and repetitive washes
- Requirements for large volumes of precious sample and antibody
- Complex and lengthy data analysis

Assay Principles
Here we present an iQue® Advanced Flow Cytometry assay for quantifying antibody cytotoxicity towards adherent, solid tumor target cells. Unlabeled target cells or cells labeled with Incucyte® Nuclight Green Lentivirus are seeded overnight in a 2D monolayer format in 96-well flat bottom plates (5K/well) before test antibodies are added at a range of concentrations. After 3–6 days, cells are lifted using Accutase and transferred to V-bottom plates for labeling with iQue® Cell Membrane Integrity (R/Red) Dye. The appropriate time for transfer of the plate onto the iQue® Platform could be selected by

monitoring the cell counts over time using the Incucyte® Live-Cell Analysis Platform. After 30 minutes of incubation plates are washed and run on the iQue® Platform. The percentage of live target cells is quantified using the iQue Forecyt® software.

This assay could be translated into a 3D single spheroid model by instead seeding the target cells (unlabeled or labeled with Incucyte® Nuclight Green Lentivirus) in an ultra-low attachment (ULA plate) for 72 hours to promote formation of spheroids. If required for tight spheroid formation, Corning® Matrigel® Matrix can be added to a final concentration of 1.5%. At assay endpoint, spheroids are dissociated using the method described in the Sartorius T Cell Killing in Single Spheroids Protocol, following the steps for spheroids formed with or without the inclusion of Matrigel.[20] Cells are then labeled following the same steps as in the 2D monolayer.

The 2D cell monolayer has long been used due to its simplicity, cost-effectiveness and scalability, however it can lack some critical features of the 3D tumor microenvironment (TME). For this reason, many researchers now use 3D models, such as spheroids or organoids, to assess drug effects *in vitro*. These models provide a much closer reflection of the TME, with more complex cell-cell interactions and the option to include extracellular matrix proteins. The tumor cells also grow in a more layered structure, with the outer layer undergoing fast proliferation and interaction with the TME, followed by a middle quiescent layer and an inner necrotic core.[21,22] This facilitates more representative modeling of how a drug may act *in vivo*, for example by allowing the comparison of tumor penetration of candidate drugs.

Example Results

These experiments used the iQue® Advanced Flow Cytometry Platform to profile the cytotoxicity of three anti-HER2 antibodies: Trastuzumab, Kadcyla® and Enhertu®. Trastuzumab is an anti-HER2 mAb therapeutic, whilst Kadcyla® and Enhertu® are ADCs which have been based on Trastuzumab. Incucyte® Nuclight Green Lentivirus labeled cells (high HER2-expressing AU565s or HER2-negative MDA-MB-468s) were seeded with a range of concentrations of antibody. After 96 hours, cells were lifted, labeled with viability dye and analyzed using the iQue® Platform. Figure 20 shows ADC induced death of high HER- expressing AU565 cells after 96 hours, with a 2-fold lower EC_{50} for Kadcyla® activity (0.075 µg/mL) compared to Enhertu® (0.16 µg/mL). Trastuzumab induced minimal effect on the growth of AU565 cells, highlighting that without the addition of the cytotoxic payload, Trastuzumab activity is reliant on the presence of immune cells to act via the FcγRs. Death of the HER2-negative MDA-MB-468 cells was minimal, again reinforcing the high specificity of the ADCs.

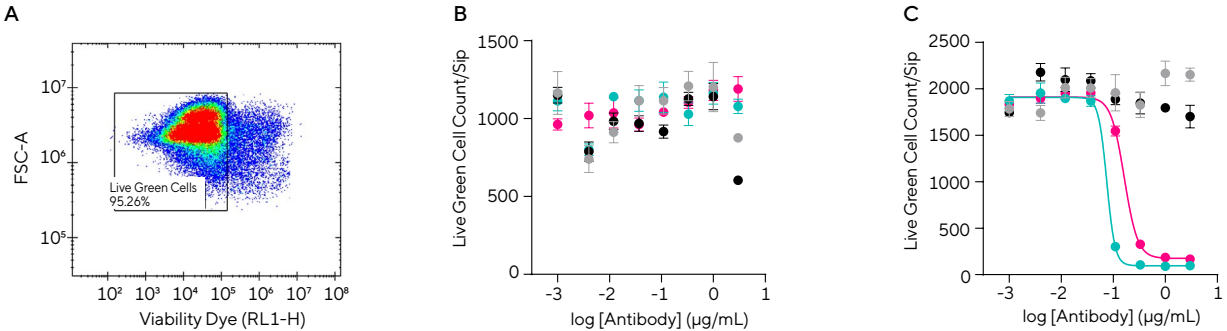


Figure 20

Anti-HER2 ADCs Induce Death of High HER2-Expressing Cells in Monoculture

Note. Incucyte® Nuclight Green Lentivirus labeled cells (high HER2-expressing AU565s or HER2-negative MDA-MB-468s) were seeded with a range of concentrations of antibody: IgG control, Trastuzumab, Kadcyla®, or Enhertu®. After 96 hours, cells were lifted, labeled with viability dye, and analyzed using the iQue® Platform. (A) Dot plot showing gating of live green target cell population (B) and (C) Live green cell count curves for MDA-MB-468 and AU565 target cells.

To assess the activity of the anti-HER2 antibodies in a 3D single spheroid model, BT474 cells were seeded in ULA plates to promote spheroid formation, then incubated with the antibodies for 10 days. Spheroid dissociation and quantification of the live cell population using the iQue® Platform revealed much more potent activity of Enhertu across the concentration range tested, when compared to Kadcyla® (Figure 21). This contrasted the 2D monolayer model (Figure 20) which displayed 2-fold greater potency of Kadcyla® than Enhertu®. We hypothesize the reason for this difference may be due to the high permeability of the Enhertu® cytotoxic payload, which has been shown to be responsible for its 'bystander activity', where once the payload has been released from the antibody backbone following antibody internalization, it can then diffuse into and kill neighboring cells.[23] It is possible that the proximity of the cells in the tight 3D spheroid structure may increase the diffusion of the payload into surrounding cells and account for this large difference in potency.

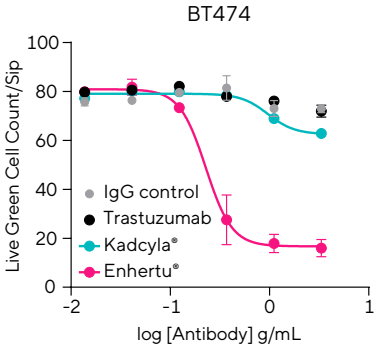


Figure 21

Induction of Cell Death by Enhertu® Was Much More Potent Than Kadcyla® in 3D Single Spheroids

Note. Incucyte® Nuclight Green Lentivirus labeled high HER2 expressing BT474 cells were seeded in ULA plates for 72 hours to promote formation of single spheroids. IgG control, Trastuzumab, Kadcyla®, or Enhertu® were added at a range of concentrations for 10 days. Spheroids were then dissociated using Accutase®, labeled with viability dye, and analyzed using the iQue® Platform.

Summary

This shows how the iQue® Advanced Flow Cytometry Platform can be used for *in vitro* quantification of antibody cytotoxicity, using the example of ADCs. Advantages of this workflow include:

- Simple, pre-optimized workflows allow for easy cytotoxicity assessment
- The ability to translate to 3D models to increase relevance of measured *in vitro* drug response
- High-throughput instrumentation and streamlined data processing speeds the time to actionable results

Together, these advantages create a powerful workflow for ADC characterization that can uncover differences in antibody functional cytotoxicity, which can inform and enhance the antibody discovery processes.

For further supporting information (videos and literature), see Appendix.

References

1. Bumbaca, D., et al., 2011. Highly specific off-target binding identified and eliminated during the humanization of an antibody against FGF receptor 4. mAbs, 3(4), pp.376-386.
2. Rockberg, J., Schwenk, J. and Uhlén, M., 2009. Discovery of epitopes for targeting the human epidermal growth factor receptor 2 (HER2) with antibodies. Molecular Oncology, 3(3), pp.238-247.
3. Jagosky, M. and Tan, A., 2021. Combination of Pertuzumab and Trastuzumab in the Treatment of HER2-Positive Early Breast Cancer: A Review of the Emerging Clinical Data. Breast Cancer: Targets and Therapy, Volume 13, pp.393-407.
4. Rothe A, Sasse S, Topp MS, Eichenauer DA, Hummel H, Reiners KS, Dietlein M, Kuhnert G, Kessler J, Buerkle C, Ravic M, Knackmuss S, Marschner JP, Pogge von Strandmann E, Borchmann P, Engert A. A phase 1 study of the bispecific anti-CD30/CD16A antibody construct AFM13 in patients with relapsed or refractory Hodgkin lymphoma. Blood. 2015;125(26):4024-31.
5. Felices M, Kodal B, Hinderlie P, Kaminski MF, Cooley S, Weisdorf DJ, Vallera DA, Miller JS, Bachanova V. Novel CD19-targeted TriKE restores NK cell function and proliferative capacity in CLL. Blood Adv. 2019;3(6):897-907.
6. Vallera DA, Felices M, McElmurry R, McCullar V, Zhou X, Schmohl JU, Zhang B, Lenvik AJ, Panoskaltsis-Mortari A, Verneris MR, Tolar J, Cooley S, Weisdorf DJ, Blazar BR, Miller JS. IL15 Trispecific Killer Engagers (TriKE) Make Natural Killer Cells Specific to CD33+ Targets While Also Inducing Persistence, In Vivo Expansion, and Enhanced Function. Clin Cancer Res. 2016;22(14):3440-50.
7. Nayyar G, Chu Y, Cairo MS. Overcoming Resistance to Natural Killer Cell Based Immunotherapies for Solid Tumors. Front Oncol. 2019;9:51.
8. Hatjiharissi E, Xu L, Santos DD, Hunter ZR, Ciccarelli BT, Verselis S, Modica M, Cao Y, Manning RJ, Leleu X, Dimmock EA, Kortsaris A, Mitsiades C, Anderson KC, Fox EA, Treon SP. Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the Fc{gamma} RIIIa-158 V/V and V/F polymorphism. Blood. 2007;110(7):2561-4.
9. Parekh BS, Berger E, Sibley S, et al. Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay. MAbs. 2012;4(3):310-318. doi:10.4161/mabs.19873
10. Matlawska-Wasowska K, Ward E, Stevens S, Wang Y, Herbst R, Winter SS, Wilson BS. Macrophage and NK mediated killing of precursor-B acute lymphoblastic leukemia cells targeted with a-fucosylated anti-CD19 humanized antibodies. Leukemia. 2013 Jun;27(6):1263-74. doi: 10.1038/leu.2013.5. Epub 2013 Jan 11. PMID: 23307031; PMCID: PMC4249624.
11. Wang B, Yang C, Jin X, et al. Regulation of antibody mediated complement-dependent cytotoxicity by modulating the intrinsic affinity and binding valency of IgG for target antigen. mAbs. 2020;12(1):1690959. doi:10.1080/19420862.2019.1690959
12. Bondza S, Marosan A, Kara S, et al. Complement dependent activity of CD20-specific IgG correlates with bivalent antigen binding and C1q binding strength. Front Immunol. 2021;11:609941. doi:10.3389/fimmu.2020.609941
13. Loeff FC, van Egmond HME, Nijmeijer BA, Falkenburg JHF, Halkes CJ, Jedema I. Complement-dependent cytotoxicity induced by therapeutic antibodies in B-cell acute lymphoblastic leukemia is dictated by target antigen expression levels and augmented by loss of membrane-bound complement inhibitors. Leuk Lymphoma. 2017;58(9):1-14. doi:10.1080/10428194.2017.1281411
14. Ramsey-Goldman R, Li J, Dervieux T, Alexander RV. Cell-bound complement activation products in SLE. Lupus Sci Med. 2017;4(1):e000236. doi:10.1136/lupus-2017-000236
15. Pawluczkowycz AW, Beurskens FJ, Beum PV, et al. Binding of submaximal C1q promotes complement dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or Rituximab (RTX): considerably higher levels of CDC areinduced by OFA than by RTX. J Immunol.2009;183(1):749-758. doi:10.4049/jimmunol.0900632
16. Hafeez, U. et al. (2020) "Antibody–drug conjugates for cancer therapy," Molecules, 25(20), p. 4764.
17. Ponziani, S. et al. (2020) "Antibody-drug conjugates: The New Frontier of chemotherapy," International Journal of Molecular Sciences, 21(15), p. 5510.
18. Dumontet, C. and Jordan, M.A. (2010) "Microtubule-binding agents: A dynamic field of cancer therapeutics," Nature Reviews Drug Discovery, 9(10), p. 790–803.
19. Reuvers, T.G., Kanaar, R. and Nonnekens, J. (2020) "DNA damage-inducing anticancer therapies: From global to precision damage," Cancers, 12(8), p. 2098.
20. Sartorius. T Cell Killing in Single Spheroids Protocol
21. 3D Cell Culture Systems: Tumor Application, Advantages, and Disadvantages. Ola Habanjar,1
22. Zanoni M, Piccinini F, Arienti C, et al. 3D tumor spheroid models for in vitro therapeutic screening: A systematic approach to enhance the biological relevance of data obtained. Sci Rep. 2016;6(1).
23. Ogitani, Y. et al. (2016) "Bystander killing effect of ds-8201A, a novel anti-human epidermal growth factor receptor 2 antibody–drug conjugate, in tumors with human epidermal growth factor receptor 2 heterogeneity," Cancer Science, 107(7), p. 1039–1046.

Chapter 6

Advanced Flow Cytometry Assays for Immune Cell Profiling

The human immune system is made up of a complex network of cells that provide continuous surveillance for anything that is non-self, such as bacteria or viruses. There are two main branches of the immune system, innate and adaptive immunity, which serve different functions but work together to overcome the different challenges the body may face.

Innate immune cells are responsible for the initial response, rapidly responding to foreign invaders, and generating a pro-inflammatory response to quickly and efficiently contain it. This includes the recruitment of macrophages, neutrophils, monocytes, and dendritic cells, which in turn stimulate cells in the adaptive immunity pathway to multiply and differentiate to overcome the specific infection. The adaptive branch of the immune system specifically recognizes foreign antigens, is mainly made up of the thymus-derived lymphocytes (T lymphocytes) and bone-marrow-derived lymphocytes (B lymphocytes), and its roles include the elimination of foreign species, the formation of immunological memory, and ensuring tolerance to self-antigens.[1] Natural killer cells (NK cells) lie in between the two branches, as they possess some qualities of both.

In the past, cancer treatment was limited to surgery, chemotherapy, and radiotherapy, but recent advances in the field of immunotherapy have changed the paradigm by recruiting the body's immune system to fight tumors. Immune profiling is the process of analyzing the immune system to identify the specific immune cells and molecules that are involved in a particular disease or condition. This information is important in the development of immunotherapies because it can help identify potential targets for therapy and predict how a patient will respond to treatment.

Immune profiling can also help identify the specific immune cells that are involved in attacking the cancer cells. This information can be used to develop antibody-based therapies like bispecific T cell engager (BiTE) therapies, which work by binding to both the cancer cell and an immune cell, bringing them into close proximity and triggering an immune response against the cancer cell. Another type of immunotherapy is checkpoint inhibitors, which work by blocking proteins that inhibit the immune response against cancer cells. Knowing which checkpoint proteins are overexpressed in a patient's cancer cells, is important in the development of targeted checkpoint inhibitors. Adoptive cell therapies like chimeric antigen receptor (CAR) T cells and

CAR-NK cells involve engineering a patient's own immune cells to recognize and attack cancer cells. This will be investigated further in Chapter 7 where we will expand on assays to support cell therapy.

Traditional methods, only allow for the measurement of one parameter or analyte per sample. In contrast, advanced flow cytometry can be used to study a wide range of immune cell parameters, from assessing cytokine production, cellular proliferation and cell viability, to analyzing the cell cycle, rare events, and immunophenotyping. In addition to analyzing intact cells, this approach can be used to measure extracellular analytes of interest, such as cytokines. On the iQue® Platform, this is performed using a "sandwich" immune assay format by combining fluorescent analyte-specific capture beads (iQue Qbeads® as discussed in Chapter 3) with fluorescent detection antibodies, thus allowing multiple parameters to be assessed in a single experiment. High-throughput, multiparametric assays allow scientists to derive the maximum amount of data from a small volume of sample, helping to limit resource use and speed up time to results.

T Cells

T cells are pivotal in adaptive immune responses. Specialized T cells are critical for both the induction of humoral (antibody) responses and cell-mediated immune responses, in which certain types of T cells orchestrate the influx and activation of innate immune cells to the site of infection, while other types of T cells function as direct cytotoxic effector cells. Understanding the processes controlling the activation, function, memory and exhaustion of T cells at the molecular level is fundamental to the identification and validation of novel immunotherapies, creating a need for assays to accurately profile T cell function and health.

T Cell Activation

T cells emerge from the thymus in a resting, naïve state (T_N cells), unable to produce any kind of immune response.[2] Activation from the naïve state is therefore critical for their proliferation and programming to the correct subtype. This is achieved through a complex series of interactions between the T cell and an antigen presenting cell (APC).

Naïve T cells are first activated upon engagement of the T cell receptor (TCR)-CD3 complex on the T cells with a foreign peptide bound to a major histocompatibility complex (MHC) on an APC. There are two types of MHC: class I molecules that interact with the CD8 co-receptor on cytotoxic T (T_C) cells, while MHC class II interacts with the CD4 co-receptor on T helper (Th) cells.[2] Binding of the peptide + MHC complex by the TCR, as well as CD4 or CD8 co-receptors binding to the MHC, triggers CD3 to transduce an activation signal into the T cell cytosol. There is also co-stimulation through several other membrane protein complexes. This combined signaling induces IL-2 production, secretion and full T cell activation.[1]

Upon activation, T cells are induced to proliferate and differentiate into effector cells and generate long-lived memory T cells. CD4+ T cells can differentiate into specialized T helper (Th) subsets or regulatory T cells (Tregs) that serve different functions and differ in the types of cytokines they produce. T cells may also differentiate into T_C that can directly kill infected cells and tumor cells. T_Cs may be CD4+ or CD8+, but are predominantly CD8+ T cells, and are critical for protection against viruses and other intracellular pathogens, as well as in cancer prevention.

T_Cs primarily kill their targets by programming them to undergo apoptosis. The main killing mechanism is by the release of lytic granules that contain several cytotoxic effector proteins. One is perforin, which polymerizes to form transmembrane pores in target cell membranes. Another is a class of cytotoxic serine proteases called granzymes.

In addition to direct target cell killing, cytotoxic CD8 T cells also release the cytokines, IFNγ and TNFα, which contribute to host defences. IFNγ can induce inhibition of viral replication in cells, as well as increased expression of MHC class I, which increases the chance that infected cells will be recognized as targets for cytotoxic attack. IFNγ also activates other immune cells, recruiting them to sites of infection. TNFα can synergize with IFNγ in macrophage activation and may also induce apoptosis in target cells that express the TNF Receptor-1 (TNFR-I).

Modulating TCR engagement and signaling pathway using biologics, small molecules or genetic engineering is highly relevant to many therapeutic areas including cancer immunotherapy, adoptive cell therapy, and vaccine development. Below, we present some examples of multiplex analysis using the iQue® Platform and iQue® kits to streamline characterization of T cell phenotype and function.

Assay Principles

The iQue® Human T cell Activation Kit (TCA kit) was developed to rapidly analyze the phenotype, health, and activation status of T cells, along with measuring secreted cytokines in a multiplexed, high-throughput format (Figure 1). The TCA kit detection antibody cocktail includes antibodies directed against human CD3, CD4, CD8, CD25, CD69 and HLA-DR. The CD3, CD4 and CD8 markers are used to identify helper and cytotoxic T cell subsets, while CD25, CD69 and HLA-DR are known markers of early-, mid- and late-stages of T cell activation, respectively. Two effector cytokines, IFNγ and TNFα are also quantified using 2-plex Qbeads® in a sandwich immunoassay format in the same well. The kit also contains an optional proliferation and encoding dye that can be used either to examine proliferation of the effector T cells, or to label and distinguish target cells (e.g. tumor cells), allowing assessment of target cell killing.

The iQue® TCA kit allows you to gain additional biological insight into T cell activation status in physiologically relevant models, with advantages including:

- Simultaneous quantification of surface proteins and cytokine expression
- Easy to use and analyze data with a predefined gating strategy
- Minimal sample manipulation required, with low volume requirements (10 µl) and a single-wash, no dilution, mix-and-read format

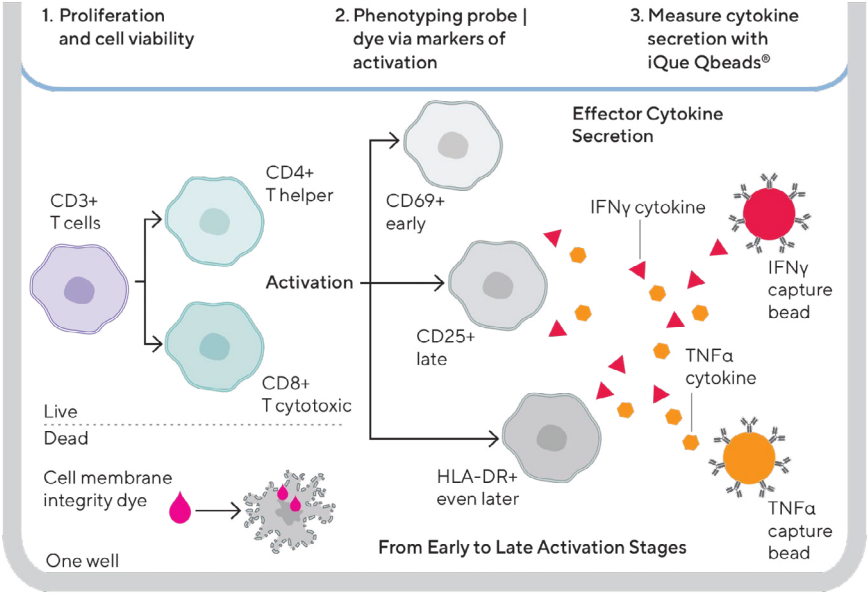


Figure 1
Illustration of iQue® Human T Cell Activation Kit Assay Principles

Example Results

Evaluation of T cell activation during target cytotoxicity induced by a CD3xCD19 Bispecific T cell Engager (BiTE) antibody

Some antibody-based therapies have been specifically designed to induce T cell-mediated killing of target cells. These include bispecific T cell-redirecting antibodies (bsAb) and smaller, engineered bispecific T cell engagers (BiTEs). BiTE antibodies, such as Blinatumomab, are used clinically for the treatment of B cell-acute lymphoblastic leukemia (B-ALL) and other late-stage cancers. This CD3xCD19 construct, simultaneously engages the CD3 on T cells and the tumor-associated antigen, CD19, present on B cells. This interaction causes the clonal expansion and activation of T cells as well as direct contact between CD3+ T cells and CD19+ tumor cells, resulting in tumor-specific cell lysis.

In this study, the iQue® TCA kit was used to examine whether CD8+ T cells were activated when PBMCs were co-cultured *in vitro* with Incucyte® Nuclight Green, CD19+ Ramos target cells in the presence of the CD3xCD19 BiTE (10 ng/mL, 5-fold serial dilution). Incucyte® Nuclight reagents are a collection of reagents (Sartorius) that aid in the production of cell lines expressing a

nuclear-restricted fluorescent protein. Control antibodies were assessed in parallel (control Ab 1: anti-hCD3xβGAL) or CD19 alone (control Ab 2: anti-hCD19xβGAL) at 10 ng/mL (5-fold serial dilution). In addition, stimulation with CD3/CD28 Dynabeads™ (75K/well, 3-fold serial dilution) was analyzed as a positive control. Supernatant samples were taken daily for cytokine analysis and endpoint subset analysis occurred at 72 hours.

Cytokine production was evaluated from the supernatant samples taken daily, enabling comparison to target cell killing (Figure 2). The CD3xCD19 BiTE antibody induced production of IFNγ and TNFα in a concentration dependent manner, reaching maximal concentrations of 1.3 ± 0.3 ng/mL and 1.5 ± 0.3 ng/mL, respectively. Interestingly, CD3/CD28 Dynabead™ stimulation evoked vastly increased production of both cytokines, with IFNγ reaching 14.4 ± 2.5 ng/mL and TNFα 3.8 ± 0.2 ng/mL. Comparison to the levels of cytotoxicity (Figures 2A,2B and 2C) highlight that despite the low levels of cytokines produced by the BiTE antibody, higher levels of target cell death was induced when compared to CD3/CD28 stimulation.

Endpoint subset analysis showed that, as expected, the CD3/CD28 Dynabeads™ induced concentration-dependent increases in the proportions of CD69, CD25 and HLA-DR-positive populations yielding comparable EC_{50} values of 4,690, 16,958 and 13,865 beads/well, respectively (Figure 3A). The maximal population percentages were 29% for CD69, 69% for CD25 and 32% for HLA-DR. In contrast, inclusion of the BiTE antibody displays a clear left shift in the CD69 expression pattern, with low concentrations (20 pg/mL) capable of inducing almost exclusive expression of this early activation marker (EC_{50} value of 5.5 pg/mL, Figure 3B). CD25 (mid activation) and HLA-DR (late activation) is induced, but at much higher concentrations of BiTE, yielding EC_{50} values of 87 pg/mL and 72 pg/mL, respectively. Maximal subset percentages were 45% for CD69, 92% for CD25 and 46% for HLA-DR. These data suggest different mechanisms of activation may cause different proportions of CD8-positive subsets.

Summary

The iQue® Human T cell Activation Kit is an optimized, high throughput, multiplexed assay which provides rapid and routine monitoring of *in vitro* T cell activation and proliferation. The assay uses only 5–10 µL of sample, saving precious cells and reagents. The sample acquisition and analysis time for a 96-well plate on the iQue® Platform is only 15 minutes. High content data are provided by the integrated software and assay template, which auto-generates all cell and bead gates, cell metrics, IC_{50} and EC_{50} curves, and quantitates secreted cytokine levels.

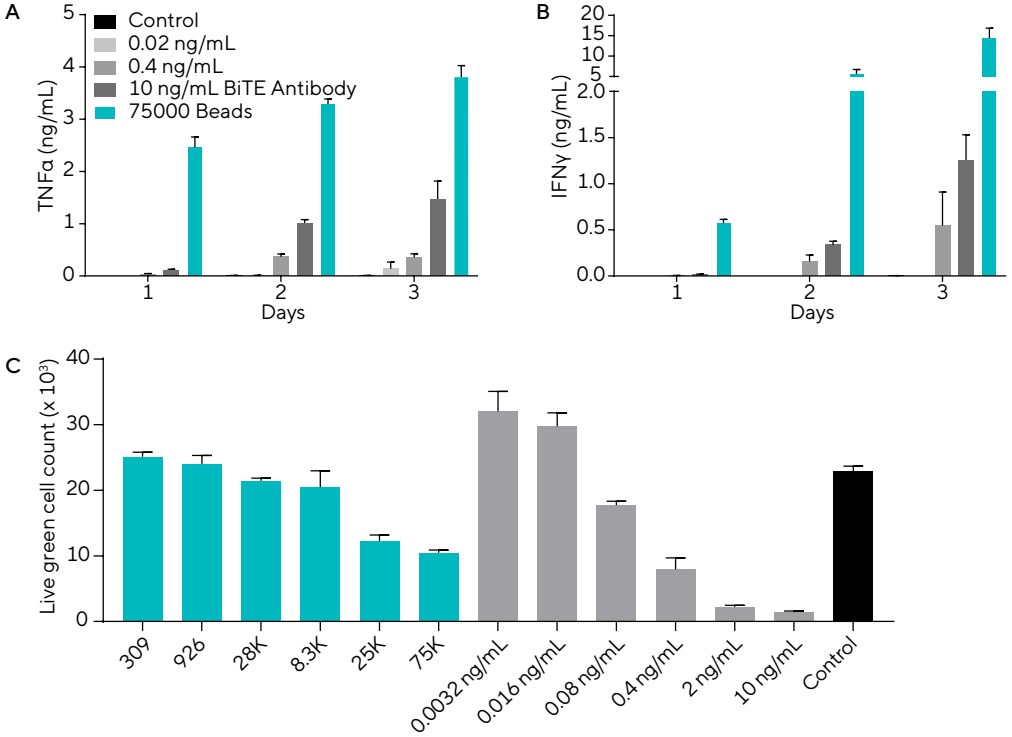


Figure 2

Temporal Cytokine Production and Immune Cell Killing

Note. Ramos Nuclight Green cells (15K/well) were seeded with PBMCs (1:5 Target to Effector) and either activated with increasing concentrations of BiTE antibody (anti-hCD3xCD19) or CD3/CD28 Dynabeads™ (75K/well). Daily supernatant samples (10 µL) taken for analysis of cytokine (IFNγ and TNFα) concentrations by the iQue® Platform (A and B). Temporal cytokine concentrations were compared to the level of immune cell killing as measured by the live green-labeled target cell count data in (C), with bars for CD3/CD28 Dynabeads™ in teal, BiTE in grey and no antibody control in black.

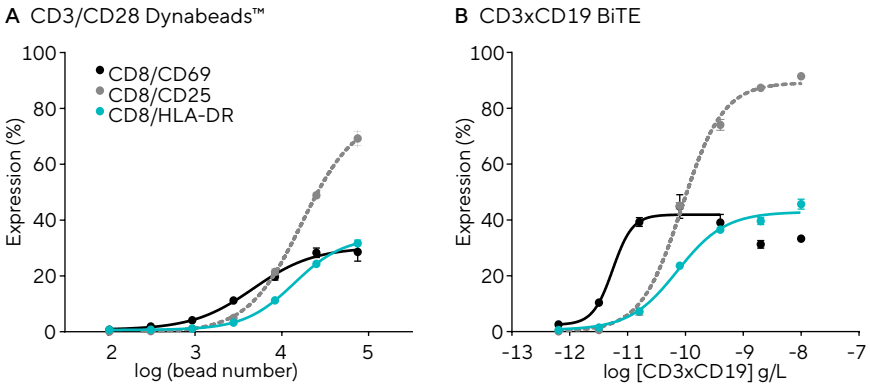


Figure 3

Subset Analysis of Activation Markers in CD8+ Immune Cells

Note. Concentration response curves to activation induced by (A) CD3/CD28 Dynabeads™ (100 to 75K beads/well) or (B) CD3xCD19 BiTE antibody (0.6 pg/mL to 10 ng/mL).

T Cell Killing

As mentioned in the introduction T cell mediated killing is a process by which T cells recognize and destroy infected or cancerous cells. By studying this process, researchers can identify ways to boost the immune system's ability to recognize and attack cancer cells. For example, researchers use T cell mediated killing data to identify the specific antigens or proteins on cancer cells that can be targeted by CAR-T cells. This can lead to the development of more effective immunotherapies that can improve outcomes for cancer patients.

The iQue® Human T Cell Mediated Killing Kit—Assay Principles

The iQue® Human T Cell Mediated Killing Kit was designed for ease of use in multiplexing cell phenotype and function markers along with bead-based, secreted protein profile measurements in the same assay. This assay, optimized for suspension cell cultures, offers analysis of killer T cell phenotypes (CD3, CD8), and functions at different stages including activation marker (CD25), exhaustion marker (PD-1), cell membrane integrity, cell count, secreted pro-inflammatory cytokine IFNγ, and pro-apoptotic serine protease Granzyme B with the use of a 2-plex Qbeads® (Figure 4). This is a simplified 'plug-and-play' assay workflow with no additional color compensation, and pre-mixed reagents for CD antibody staining and for secreted protein detection. Total assay time is approximately 3 hours, with a hands-on time of about 30 minutes. An included template with pre-set compensation matrices enables data acquisition of the multiplexed, phenotyping assay without the need for single stain color compensation.

Example Results

Specific Killing Profile of CAR-T Cells Targeting CD19

CAR-T cells are designed to selectively target and kill tumor cells through interaction with a specific surface antigen, while limiting off-target side effects. To demonstrate this specificity *in vitro*, anti-CD19 CAR transduced T cells or donor-matched mock-transduced T cells were used in an immune cell killing assay. CD19 antigen positive Ramos or CD19 antigen negative Jurkat cells were seeded in combination with T cells at various target to effector ratios (T:E).

On Days 2, 4, and 7, samples were analyzed on the iQue® Platform to assess phenotype and function using the iQue® Human T Cell Killing Kit. Results show that, when combined with antigen positive Ramos cells, there was a rapid upregulation of T cell activation marker CD25 and exhaustion marker PD-1 (Figure 5A and B) on the CD8+ cytotoxic T cells. This upregulation demonstrated some time dependence, with the highest levels observed on Day 7, but there was little difference between CAR-T cell densities (data not shown). Expression of all three activation markers was low in co-cultures with antigen-negative Jurkat cells or in the presence of mock-transduced T cells (< 7%). In the presence of Ramos cells, concentrations of secreted cytokine IFNγ (indicator of activation) and release of Granzyme B, an indicator of cell killing,

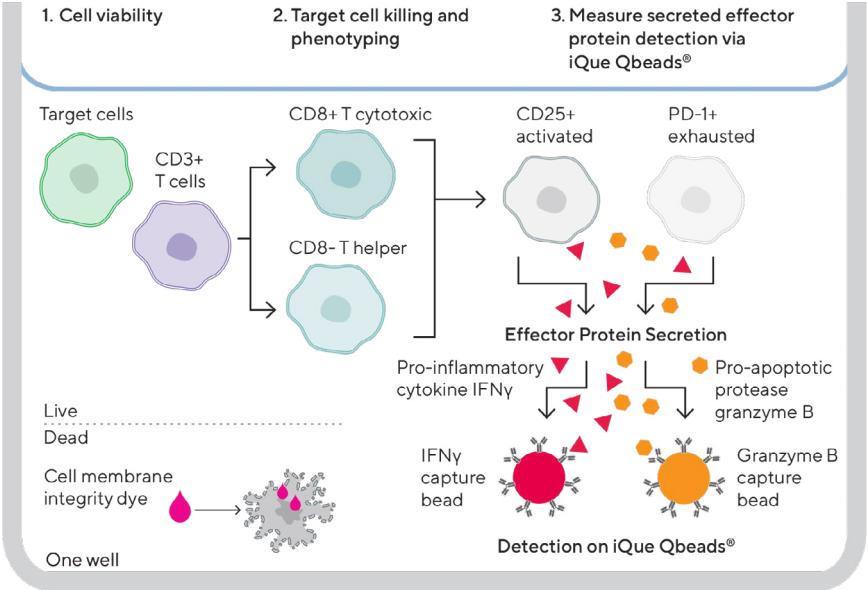


Figure 4

Illustration of iQue® Human T Cell Killing Kit Assay Principles

Note. Simultaneous measurement of T cell proliferation or encoded target cells is possible but is not included in this illustration.

increased in co-cultures containing CAR-T cells, but only in the presence of antigen-positive Ramos cells (Figure 5C and D). For all secreted proteins, there was a general increase in levels with increasing CAR-T density (not shown). Overall, this complete quantification demonstrates a clear antigen-specific activation of anti-CD19 CAR-T cells as measured by both surface markers and secreted proteins.

Summary

The iQue® Human T Cell Killing kit enables the simultaneous, high throughput analysis of target cell killing, T cell phenotypic, and activation marker expression, and quantification of secreted effector proteins and cytokines. Combining the power of iQue® Advanced High-throughput Flow Cytometry with real-time data analysis using integrated iQue Forecyt® software provides a simplified solution to enhance immuno-therapeutic drug discovery processes.

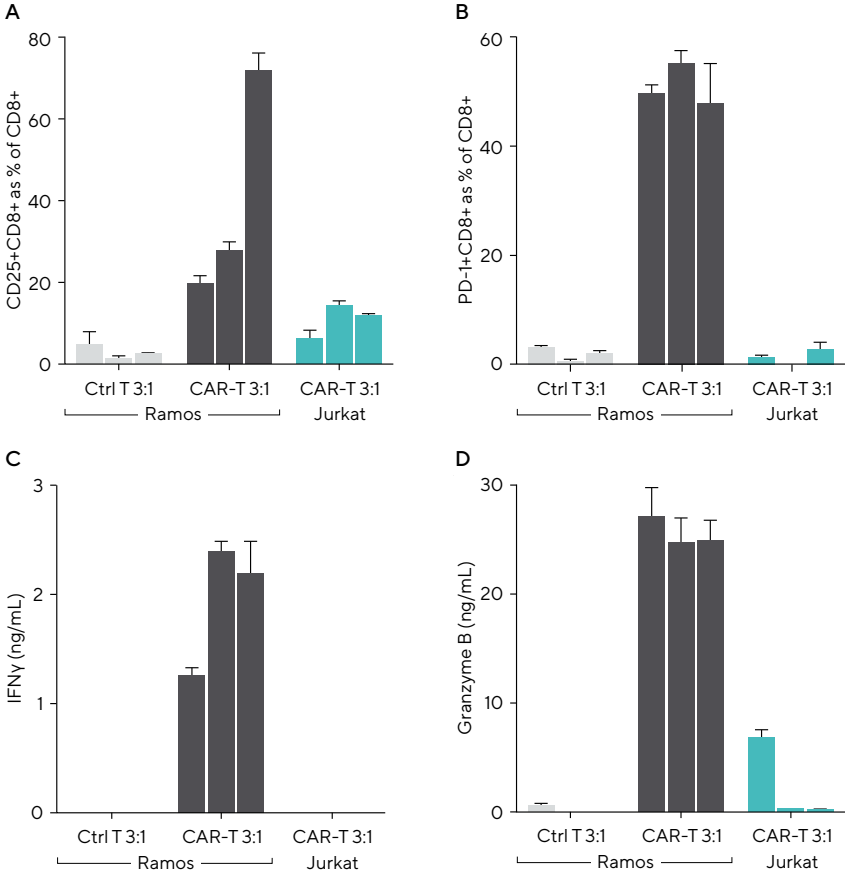


Figure 5

Antigen Specific Activation of Anti-CD19 CAR-T

Note. Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using the iQue® Human T cell Killing Kit. (A, B) Expression levels in CD8+ T cells of CD25 and PD-1. (C, D) IFNγ and Granzyme B. Grey bars represent Ramos with mock transduced T cells, black bars are CD19 CAR-T with Ramos cells, and teal bars are CAR-Ts in combination with Jurkat cells. The three bars represent the three timepoints and all data shown as mean ±SEM of three wells.

T Cell Exhaustion

T cell exhaustion is a broad term used to describe loss of the CD8 cytotoxic effector function due to chronic antigen stimulation. This phenomenon was initially thought to involve the clonal deletion of virus specific CD8 T cells that occurs during high-grade chronic infections.[6] However we now understand that these cells are not actually deleted under conditions of antigen persistence, but instead become functionally inept and incapable of elaborating the usual array of effector activities typically associated with robust protective effector and memory T cell populations.[7,8] This state generally manifests with several characteristic features, such as; progressive and hierarchical loss of effector functions, sustained upregulation and co-expression of inhibitory receptors, altered expression, use of key transcription factors, metabolic derangements, and a failure to transition to quiescence and acquire antigen-independent memory T cell homeostatic responsiveness. More specifically, this dysfunction, or exhaustion is associated with expression of high levels of inhibitory receptors, including PD-1, CTLA-4, TIM-3, LAG-3 and CD244.

Affected cells also exhibit impaired effector cytokine production, such as IL-2, TNFα, IFNγ, and Granzyme B. As such, exhausted T cells are recognized as a distinct subgroup from prototypic effector and memory T cells.

In disease states, exhaustion plays an important part in the tumor microenvironment (TME) where it has been shown to be detrimental to the immune response against cancer.[9] When effector T cells enter the TME, they are regulated by a complex immunosuppressive network that consists of cancer cells, inflammatory cells, stromal cells, and cytokines. These TME components have crucial roles in regulating T cell phenotype and function, and can drive T cells to terminally differentiate into 'exhausted' T cells. Preventing exhaustion, for example in the development of autologous T cell therapy, has therefore become a very important area of focus. Being able to detect and quantify exhaustion in relevant cell models will enable a better understanding of how exhaustion develops and provide a platform for testing out therapeutic solutions. There is also a need for high throughput workflows which allow users to quickly achieve their results with minimal sample usage or manipulation.

A major type of cancer immunotherapy is the use of checkpoint inhibitors that target these T cell exhaustion or 'inhibitory' receptors. Blocking these checkpoint receptors can reinvigorate the T cells to continue killing. The most commonly used immune checkpoint inhibitors that have shown clinical success include antibodies that target the programmed cell death 1 (PD-1) protein or cytotoxic T lymphocyte associated protein 4 (CTLA-4) on CTLs, or the expression of the PD-1 Ligand (PDL-1) on tumor cells. Targeting these inhibitory receptors with anti-CTLA-4 and anti-PD-1 monoclonal antibodies reduces immunosuppression by blocking the interaction of the receptors with their ligand. This type of therapy has been shown to be beneficial in several types of cancer.[1]

Assay Principles

The iQue® Human T Cell Exhaustion Kit contains mix and read reagents to allow for the capture of both phenotypic and cytokine data simultaneously. The patented sampling micro-volume system allows for fast and efficient data acquisition, while the profiling kit with pre-designed gating strategy working in conjunction with the iQue Forecyt® software enables easy analysis. Immunophenotyping using various T cell exhaustion markers and iQue Qbeads® swiftly provides a wealth of information on T cell immunophenotyping, exhaustion status, secreted cytokines (IFNγ and TNFα), cell count, viability, and proliferation (Figure 6). The three exhaustion markers measured by the kit are associated with different stages of T cell exhaustion, from early (PD-1) to mid (LAG-3) to late (TIM-3), giving the potential to track the temporal progression of exhaustion. Simultaneous measurement of T cell proliferation or encoded target cells is optional. The high-throughput capabilities of the iQue® flow cytometry platform allows for thousands of wells to be analyzed with minimal sample use or manipulation.

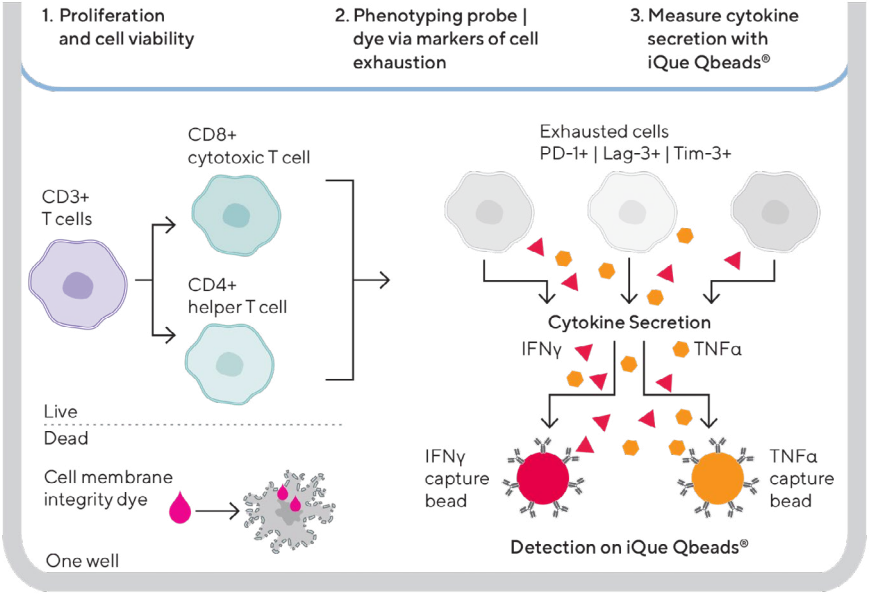


Figure 6

Illustration of iQue® Human T Cell Exhaustion Kit Assay Principles

Example Results

Exhaustion Markers Are Maintained with Continued Stimulation

To confirm that exhaustion could be quantified using the iQue® Human T Cell Exhaustion Kit, unstimulated PBMCs were compared to those that were stimulated once, or continuously re-stimulated with Dynabeads™ CD3/CD28 over a 9-day time course. Using TIM-3 as an example (Figure 7A-C), PBMCs that had been continually stimulated had a significant and sustained upregulation of TIM-3 throughout the time course (Day 0–0.4 ± 0.1%; Day 2–35.0 ± 0.7%; Day 5–42.0 ± 1.1%; Day 9–94.4 ± 0.7%; data for Day 5 has not been included in this figure). PBMCs that were exposed to a single Dynabead™ CD3/CD28 stimulation displayed an initial elevation of TIM-3 early on in the time course however this diminished to low levels after Day 5 (Day 0–0.4 ± 0.2%; Day 2–36.0 ± 1.9%; Day 5–34.8 ± 1.1%; Day 9–18.1 ± 0.5%). Non-stimulated controls were considered negative for this exhaustion marker and throughout the experiment levels remained consistently low (Day 0–0.4 ± 0.2%; Day 2–4.6 ± 0.2%; Day 5–4.2 ± 0.0%; Day 9–4.2 ± 0.8%).

Continuous stimulation of PBMCs produced a general pattern for all three markers (PD-1, LAG-3 and TIM-3) were increased and sustained expression was observed over the time course (Figure 7D-F). This condition simulated the chronic antigen exposure, which are the typical conditions for inducing a state of exhaustion in T cells. The single stimulation caused an initial up-regulation of expression in all three markers on Day 2 when compared to controls, which then decreased over the course of the 9 days. Non-stimulated PBMC controls only produced low expression of exhaustion markers, and this was consistent throughout. These multiple time points also indicate the timescale and order as to which markers are expressed first. It was observed that TIM-3 took considerably longer to reach high levels of expression when compared to PD-1 and LAG-3.

Cytokine Production Is Reduced in Exhausted T Cells

Exhausted T cells are expected to have aberrations in cell signalling and function. To assess changes in the release of cytokines upon T cell exhaustion, non-exhausted T cells or pre-exhausted T cells (repeatedly stimulated with CD3/CD28 Dynabeads™) were seeded (200K/well) into an assay plate and stimulated T=0 with increasing concentrations of Dynabeads™ CD3/CD28 (390-200K). Supernatant samples (10 µL) were assayed every 24 hours over the course of 3 days using the iQue® Human T Cell Exhaustion Kit.

Exhausted T cells secreted significantly lower concentrations of TNFα when compared to the stimulated non-exhausted T cells (Figure 8). On Day 3 of the assay, a bead density of 200K/well in non-exhausted cells induced an 11.5-fold greater release when compared to the same bead density in exhausted cells. Stimulating non-exhausted T cells with Dynabeads™ induced a concentration-dependent increase in TNFα secretion which peaked at 100K beads, and this pattern was constant across the time course. Exhausted T cells exhibited a more bell-shaped distribution in secretion levels,

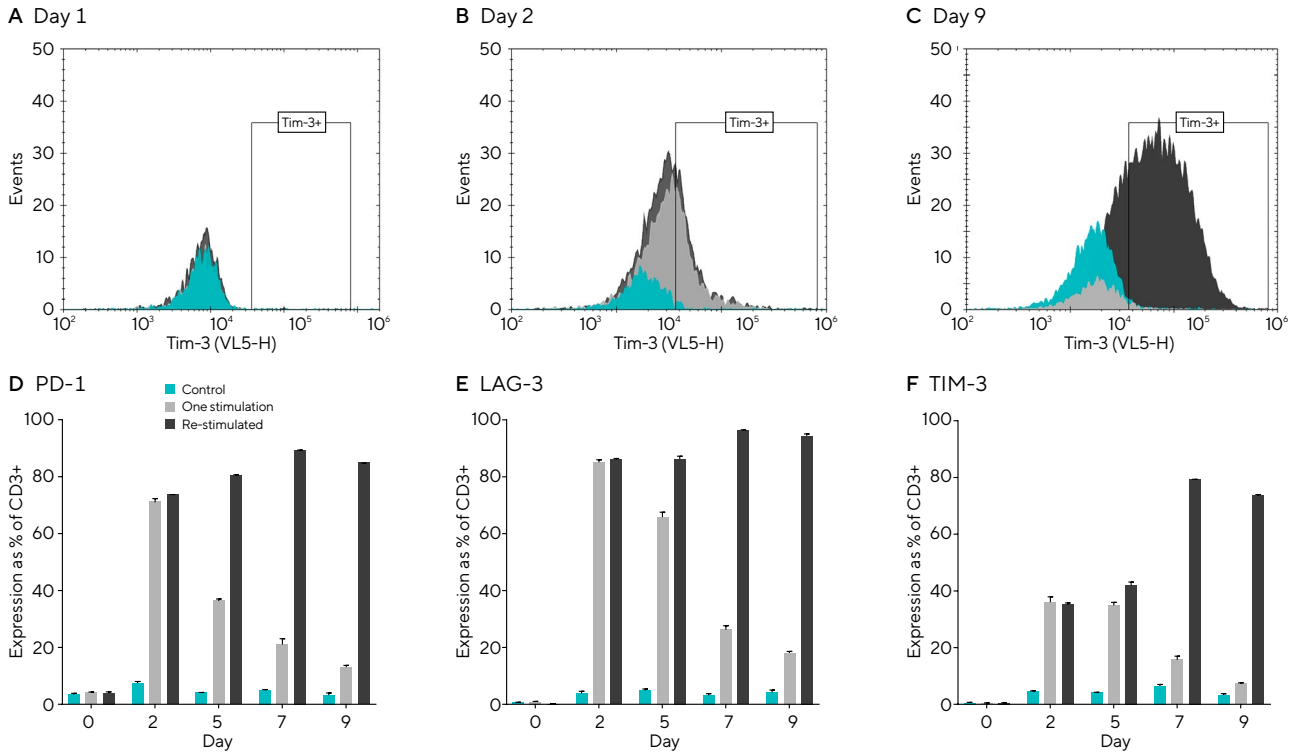


Figure 7

Exhaustion Marker Expression is Maintained on Re-Stimulated PBMCs Only.

Note. PBMCs were treated using one of the following three conditions: 1) No stimulation controls (teal), 2) Single stimulation (stimulated with 1:1 Dynabeads™ CD3/CD28 on Day 0) (light grey) or 3) Restimulated (stimulated every 2-3 days with 1:1 Dynabeads™ CD3/CD28) (dark grey). 10 µL samples were analyzed using the iQue® Human T Cell Exhaustion Kit over the course of the 9-day experiment. (A-C) Histograms depicting the population shifts of each of the PBMC treatments. (D-F) PD-1, LAG-3, and TIM-3 expression for each treatment over the full-time course. Each data point represents mean ± SEM, n=3 wells.

which saw the low secretion levels peak at a density of ~12.5K beads, higher bead concentrations produced similar secretion levels to the exhausted control wells. No stimulation exhausted controls still produced a cytokine response in contrast to non-exhausted controls which was expected due to the pre-stimulation.

Summary

The iQue® Human T Cell Exhaustion Kit enables the simultaneous quantification of exhaustion markers and cytokines in a high-throughput system and is applicable to therapeutic drug testing.

The easy-to-use kit, which consists of ready-to-use reagents including optimized antibodies and cell health dyes enables users to concentrate on assay plate design and coupled with iQue Forecyt® software, plate to decision making time is rapidly reduced. The iQue® Platform can be incorporated into existing workflows used to analyze pharmacological drug effects on the immune system. The flexible and innovative design of both instrument and kit allows users to effectively characterize therapeutic drugs.

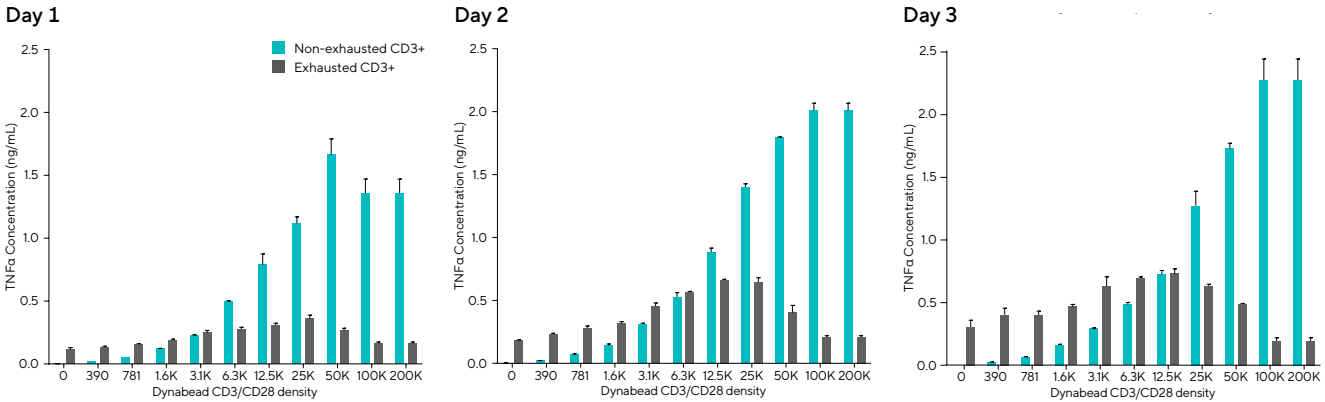


Figure 8

TNFα Production is Reduced in Exhausted T Cells

Note. Non-exhausted (i.e. no prior stimulation) or pre-exhausted CD3+ T cells were seeded at 200K/well. Exhausted T cells had been repeatedly stimulated (5 times total, every 2–3 days) with Dynabeads™ CD3/CD28 (1:1 bead:cell ratio). In-well, activation was induced with Dynabeads™ CD3/CD28. Every 24 hours, 10 µL samples were analyzed using the iQue® Human T Cell Exhaustion Kit. Each data point represents mean ± SEM, n=2 wells.

T Cell Memory

Following the primary response and termination of the foreign body, an extreme decline in the T cell population occurs. At this point, some T cells differentiate into memory T cells that have an ability to 'remember' the foreign body they helped remove, with a half-life of around 8–15 years. These cells are divided into two main subsets, central memory (T_{CM}) and effector memory (T_{EM}) cells, and can be activated more easily, with increased proliferative potential, for a more rapid response.[2]

T_{EM} cells have a rapid effector function, producing Granzyme B and IFN-γ, but limited proliferation. They control initial exposure to the reinfection or re-emergence of the foreign body. TCM cells display an increased proliferation potential following antigen re-encounter, but require more time to proliferate and induce the production of more effector cells that can eliminate the target. While these two subsets give a broad overview, memory cells show a huge plasticity, and should be seen as a spectrum rather than two distinct classes (Figure 9), offering huge potential for the development of T cell therapies.[2]

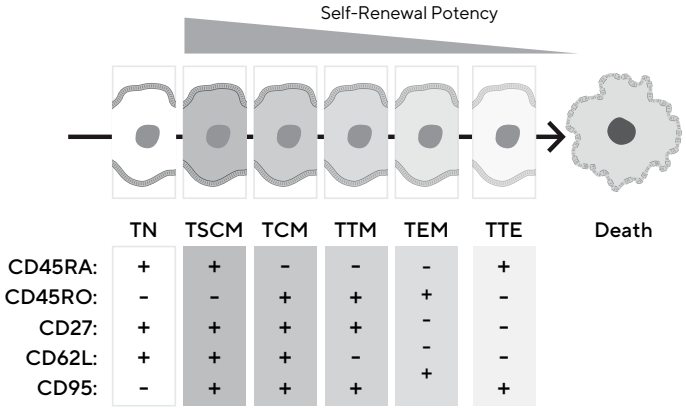


Figure 9

T Cell Memory Development

Note. Schematic showing surface marker expression changes on T cells as they develop into different T memory phenotypes. From naïve (T_N) to stem cell memory (T_{SCM}) to central memory (T_{CM}) to transitional memory (T_{TM}) to effector memory (T_{EM}) to terminal effector (T_{TE}) T cells through to cell death.

Assay Principles

The iQue® Human T Cell Memory Kit multiplexes analysis of T cell phenotype and cytokine secretion to determine the proportions of T cell memory populations in samples (Figure 10). In each assay well, live immune cells are distinguished from dead cells by staining with a fluorescent membrane integrity dye. The dye enters only dead cells or those with a compromised membrane, staining the nucleic DNA by intercalation. Live cells are immunophenotyped by staining with a fluorescent antibody panel to separate CD3+ (T cells), CD3- (non-T cells), CD4+ (T-helper cells) and CD8+ (T-cytotoxic cells). The panel also includes five different T cell-surface markers (CD45RA, CD45RO, CD27, CD62L and CD95) for distinguishing naïve, memory and effector T cells. Secreted effector cytokines,

pro-inflammatory cytokine IFNγ and anti-inflammatory cytokine IL-10, are measured in a "sandwich" immune assay format by two different iQue Qbeads® in the same well. If necessary, up to six additional cytokines may also be quantified in a multiplexed assay format by combining the iQue® Human T Cell Memory Kit with the iQue® Human T Cell Companion Kits of user's choice (IL-2, IL-6, IL-13, IL-17A, TNF, or GM-CSF).

Example Results

The data below, demonstrates the use of the iQue® Human T Cell Memory Kit to determine how CD3+ T memory cell development varies between donors under identical stimulation conditions. PBMCs from three separate donors were plated (120 K/well) and activated with increasing concentrations of Dynabeads™ CD3/CD28 (60–480 K/well) over a period of 72 hours. CD3+ T cells were then analyzed on the iQue® using the T Cell Memory Cell and Cytokine Profiling Kit. Upon stimulation with Dynabeads™, T_N cell numbers decreased, as cells were pushed down the development pathway. The Dynabeads™ CD3/CD28 activate through TCRs to produce an effector type function as opposed to memory function, and this was

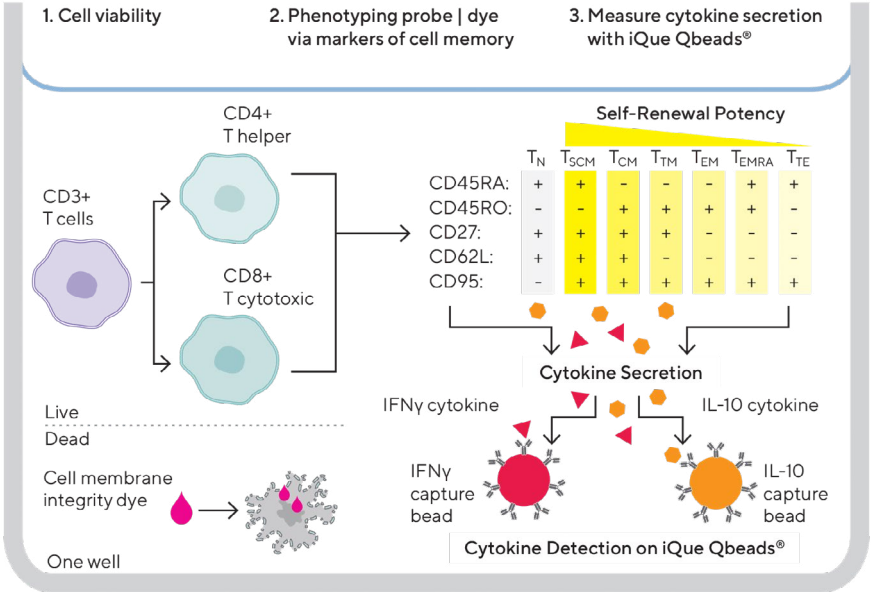


Figure 10
Illustration of iQue® Human T Cell Memory Kit Assay Principles

reflected in a concentration-dependent increase in the percentage of T_{EM} cells observed across all donors (Figures 11A to C). However, Donor 2 (D2) had at least 10% greater expression of this subtype when compared to the other donors. This high level appears to be in correlation with IL-10 secretion, which was also significantly higher in D2, and is known to be heavily involved in controlling the immune response. There was also a notable concentration-dependent increase in

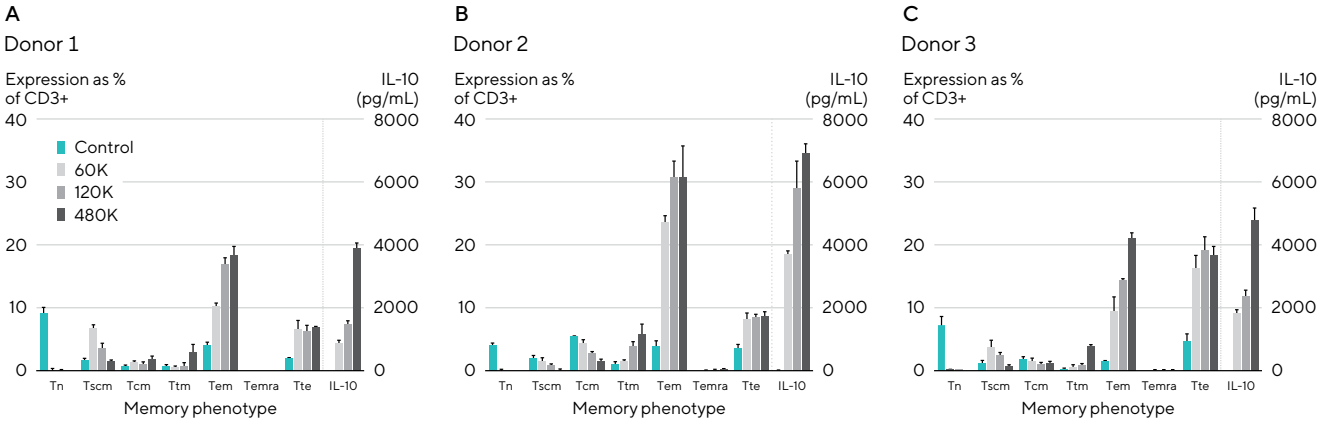


Figure 11

T Cell Memory Development Varies Between Donors

Note. (A) Donor 1, (B) Donor 2, (C) Donor 3 graphs showing % of CD3+ cells expressing proteins from each stage of the T cell memory development. IL-10 release is also shown. Teal bars are controls with no activator. Grey bars represent three ascending concentrations of Dynabeads™ (light to dark): 60 K, 120 K and 480 K beads/well.

the T_{TE} cell population across all donors, suggesting this mechanism of activation through the TCR pushes memory T cells towards a more terminally differentiated phenotype, allowing for CD8+ cytotoxic killing.

Summary

The iQue® Human T Cell Memory Kit can be used for simultaneous, high throughput analysis of T cell memory phenotype and secreted effector proteins and cytokines. Combining the power of iQue® Advanced Flow Cytometry Platform with real-time data analysis using integrated iQue Forecyt® software provides a simplified solution for quantifying memory T cell populations that can be applied to immuno-oncology therapy development and investigation. Spatial-temporal analysis of T memory cell phenotypes and functions are performed using a single, high-content miniaturized assay. This format saves precious samples, decreases reagent costs, and enhances data integrity.

T Cell Killing of Tumor Cells in Advanced 3D Cell Models

A crucial stage in the development of novel T cell therapies, or T cell targeting therapeutics is to characterize their function using *in vitro* assays. Much of this work, particularly in the field of flow cytometry, has been conducted using suspension cells or cell monolayers (2D). However, increasing evidence supports the use of advanced 3D tumor cell cultures, such as spheroids and organoids, as models that more closely reflect the *in vivo* scenario to enable improved clinical translation. For example, a spheroid model has more complex cell-cell interactions; optional extracellular matrix proteins and a layered structure, with zones undergoing proliferation and quiescence, as well as an inner necrotic core.[10,11] These structural features have a multitude of effects on cells, including graduated distribution of nutrients and gases alongside the added requirement for immune cells to penetrate the tumor during killing.

Traditional methods for measuring T cell response in 3D models, such as imaging-based techniques, are often limited because they:

- Provide bulk measurement of infiltration or killing without a deeper investigation into effects on cell marker expression or cytokine release.
- Require multiple workflows for quantification of different parameters, often using multiple instruments.
- Involve lengthy, time-consuming workflows, which require multiple rounds of protocol optimization, fixation, and repetitive washes.
- Necessitate correlation of data from several different assays for each treatment condition, increasing the risk of data variability.

Assay Principles

The iQue® spheroid immune cell killing (ICK) workflow measures subset analysis and cytokines quantification to examine the activation, killing, exhaustion, and memory profiles of T cells during co-culture with single spheroids. The workflows utilize the iQue® Advanced High-Throughput Flow Cytometer with associated suite of T cell characterization reagent kits, and validated spheroid washing and dissociation protocols to provide an end-to-end solution for the evaluation of T cell response in advanced 3D tumor models (Figure 12).

Incucyte® Nuclight Green labeled tumor cells are seeded in 96-well Ultra-Low Attachment (ULA) plates and incubated for 72 hours to promote formation of spheroids before unlabeled PBMCs and activators are added. At assay endpoint, spheroids and immune cells are dissociated and labeled using either the iQue® T Cell Activation Kit, iQue® Human T Cell Killing Kit, iQue® Human T Cell Exhaustion Kit or iQue® Human T Cell Memory Kit depending on the desired outputs. Supernatant samples (10 µL) are also taken for cytokine analysis using iQue Qbeads®. Removal of these low-volume samples is non-perturbing and can be taken daily to facilitate temporal cytokine analysis. Each T cell characterization kit contains a pre-set gating template, which is imported into the iQue Forecyt® software to facilitate instantaneous data readouts, including pharmacological readouts, such as EC_{50} values.

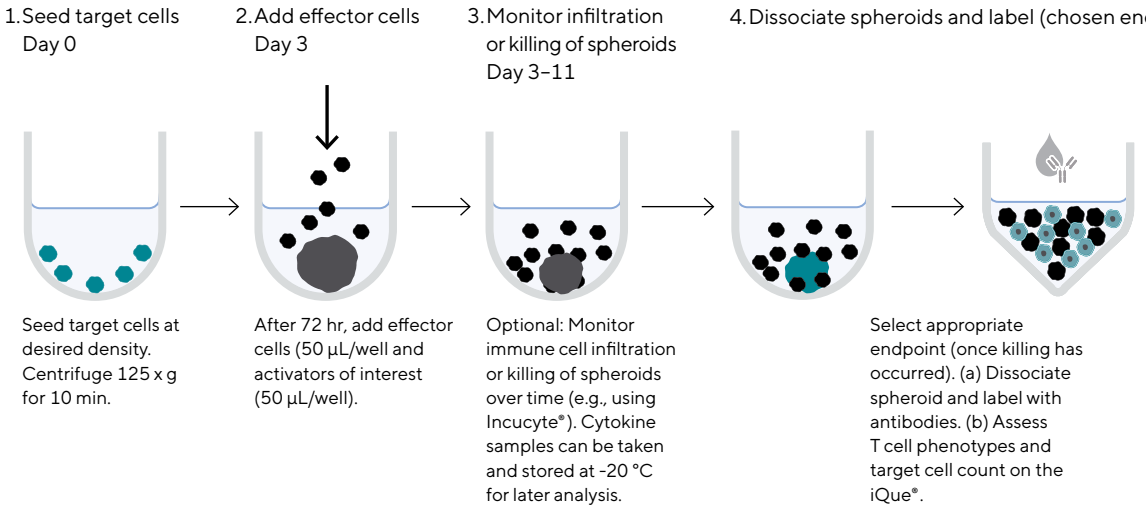


Figure 12
Schematic Outlining the Protocols for Analysis of ICK in 3D Tumor Spheroids Using the iQue® Platform

Example Results

Activation and Exhaustion Status of T Cells Changes Throughout Spheroid Killing

To exemplify the use of the iQue® 3D ICK workflow, BT474 spheroids were co-cultured with PBMCs and CD3/CD28 Dynabeads™. Replicate plates were set up and dissociated on Days 1, 4 and 8, allowing temporal assessment of immune and target cell populations using the iQue® Human T Cell Activation and iQue® Human T Cell Killing kits. Prior to dissociation, 10 µL supernatant samples were taken from each plate for cytokine analysis using iQue Qbeads® from the chosen kits. Monitoring the viability of the green target cells indicated that, even by Day 1, there had been a considerable level of immune cell killing at the top concentration (50K) of Dynabeads™, with only 26 ± 4% live target cells remaining compared to 68 ± 2% in the non-activated control (Figure 13B). By Day 4, there were very few live target cells remaining at any Dynabead™ density, suggesting the immune cells had largely acted to eliminate the spheroid. This increase in killing was supported by the Granzyme B data (not shown), which increased to high levels over the time course, suggesting that the Granzyme B and perforin cytolytic pathway was contributing to the killing of the spheroids.

Throughout the assay, we observed changes in the activation status of the immune cells, with consistent Dynabead™ concentration dependent increases in activation marker expression. As expected, expression of early activation marker, CD69, peaked on Day 1, with 98 ± 0.5% expression at the top Dynabead™ density (50K). CD69 expression then decreased by Day 4 and 8 (Figure 13C). Expression of mid-stage activation marker CD25 peaked on Day 4 (98 ± 0.7%, 50K Dynabeads™) and remained high on Day 8, while late-stage activation marker HLA-DR expression hit a peak on Day 8, with 77 ± 2% expression with 50K Dynabeads™ (data not shown). These data fit the expected temporal shift between the early-, mid- and later-stage activation markers throughout immune cell killing and highlight the importance of monitoring the progression through these phenotypic changes in immune cells over time in order to build up a fuller picture of their killing profile and how it is affected by immuno-therapeutics. PD-1 expression increased from Day 1 to 4, peaking at 76 ± 3% with 50K Dynabeads™, then decreased again by Day 8 to 36 ± 5% (Figure 13D). This, perhaps, suggests that during the peak of killing through Days 1 to 4 the cells began to display early indicators of exhaustion, but that after spheroids were eliminated, some T cells were 'rested' and retrieved from the exhaustion pathway by Day 8.[12]

Stimulated T cells Shift from Early to Late-Stage Memory Cell Development

In vitro analysis of T-memory cell populations is critical during vaccine development and in production of adoptive cell therapies, such as CAR-T cells. The bracket 'T memory cell' envelops multiple, functionally distinct subsets each of which are characterized by differences in expression of key surface markers (Figure 14A). As T cells progress through the T-memory development pathway, they gain effector function, but lose the ability to proliferate. For applications such as adoptive cell therapies, earlier phenotypes such as the stem-cell memory (T_{SCM}) and central memory (T_{CM}) are critical for persistent, long-term therapeutic effectiveness, while effector phenotypes such as Effector Memory (T_{EM}) and Terminal Effector (T_{TE}) cells are likely to result in a transient response. With replicate plates to those used in the experiments in Figure 13, we also looked at the changes in CD8+ memory T cell development during Dynabead™ activated killing of spheroids (Figure 14B-D). There was a general shift across the killing time course from the earlier stage memory phenotypes such as T naïve (T_N) on Day 1 to the later-stage phenotypes, such as T_{EM} and T_{TE} by Days 4 and 8. Production of IL-10 increased from Day 1 to 4 and remained high through to Day 8. Both the progression through to the later stages of development and the production of IL-10 were enhanced by Dynabeads™ in a concentration-dependent manner.

Unexpectedly, the proportion of T_N cells in the nonactivated PBMC control on Day 1 was very low (3 ± 0.4%) compared to at the lowest Dynabead™ density (50 ± 4%), however, these levels appeared to recover by Day 4 and 8, which saw 32 ± 4% and 29 ± 3% T_N cells in the non-activated control. Despite the very high level of killing by the immune cells (Figure 13B), only a very small proportion of them progressed to the final stage of the differentiation pathway (T_{TE}), suggesting that many of the cells may have maintained their self-renewal potency, despite their high activity.

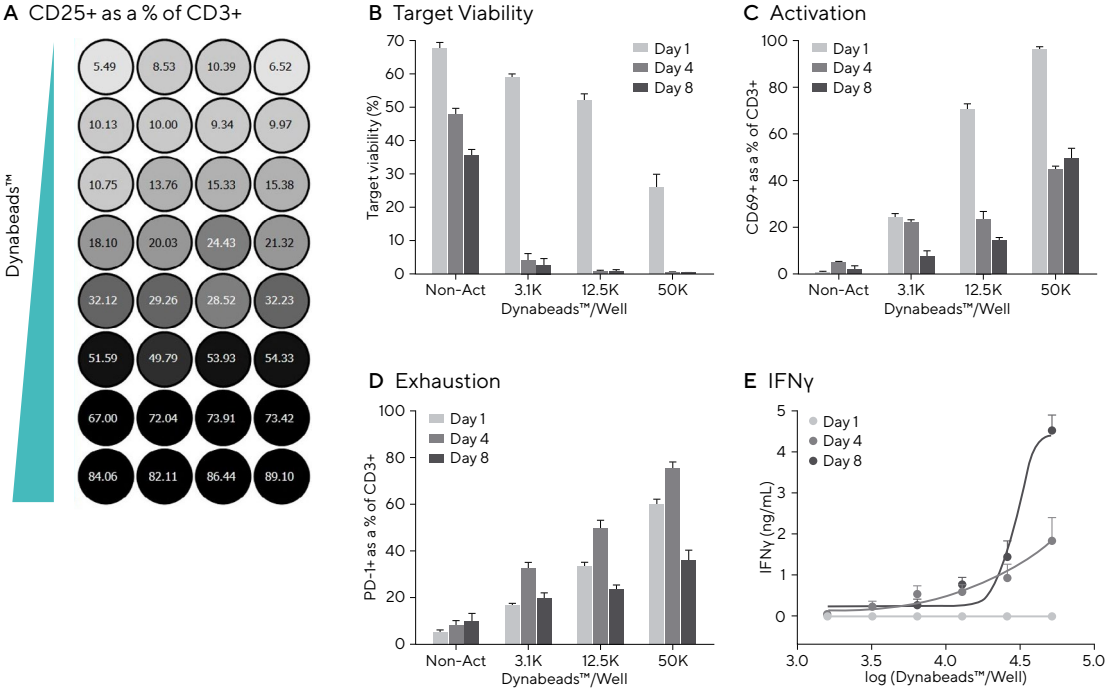


Figure 13

During Spheroid Killing T Cell Cytokine Release and Expression of Markers of Activation and Exhaustion Change Over Time

Note. Incucyte® Nuclight Green labeled spheroids were incubated with unlabeled PBMCs (5:1 E:T) and Dynabeads™. On Days 1, 4, and 8, 10µL supernatant samples were removed for cytokine analysis before spheroids were dissociated and immune cell subsets analyzed using the iQue® Human T Cell Killing and Activation kits. (A) Heat map showing changes in % of CD25 expression with Dynabead™ density on Day 1. (B) Temporal changes in target cell viability as measured by the TCK kit. (C) Early activation marker CD69 expression changes over time. (D) Expression of PD-1 as an indicator of early-stage exhaustion. (E) Concentration response curves showing temporal changes in the cytokine IFNγ.

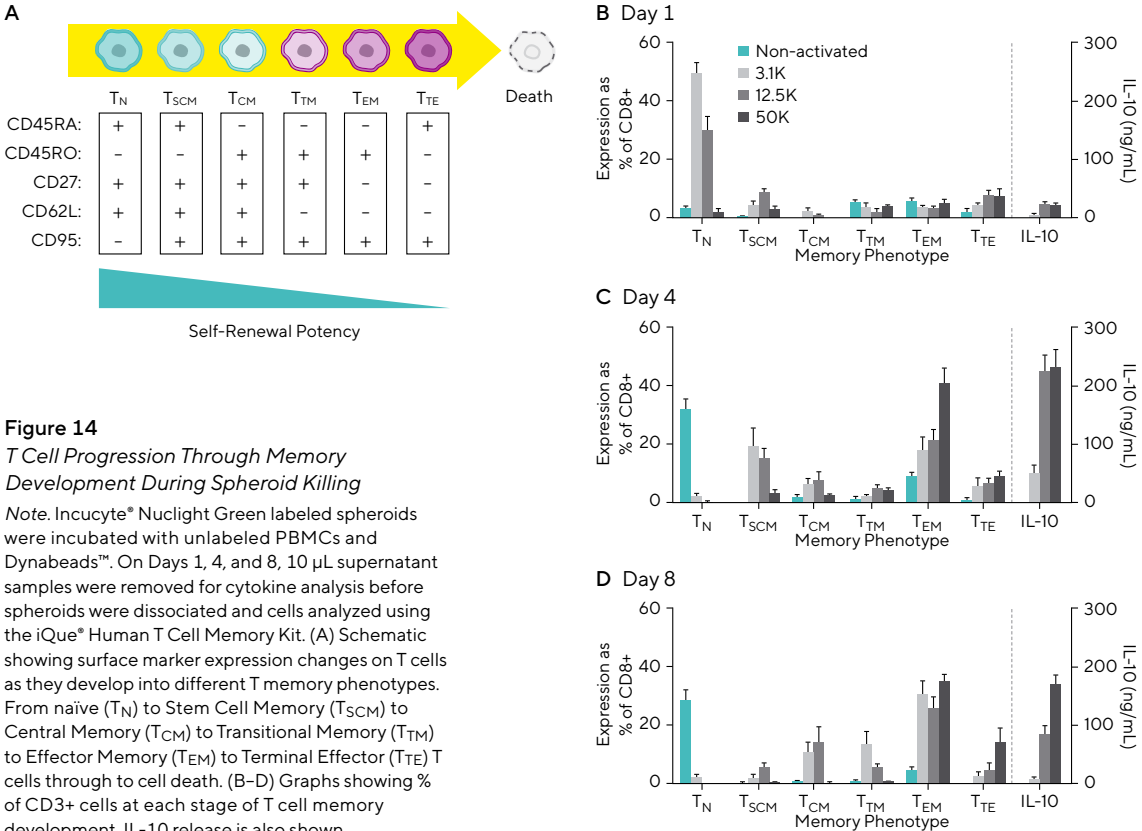


Figure 14

T Cell Progression Through Memory Development During Spheroid Killing

Note. Incucyte® Nuclight Green labeled spheroids were incubated with unlabeled PBMCs and Dynabeads™. On Days 1, 4, and 8, 10 µL supernatant samples were removed for cytokine analysis before spheroids were dissociated and cells analyzed using the iQue® Human T Cell Memory Kit. (A) Schematic showing surface marker expression changes on T cells as they develop into different T memory phenotypes. From naïve (T_N) to Stem Cell Memory (T_{SCM}) to Central Memory (T_{CM}) to Transitional Memory (T_{TM}) to Effector Memory (T_{EM}) to Terminal Effector (T_{TE}) T cells through to cell death. (B–D) Graphs showing % of CD3+ cells at each stage of T cell memory development. IL-10 release is also shown.

Summary
The iQue® Platform in conjunction with the validated suite of iQue® T cell characterization kits can be used to assess immune cell phenotype and function in 3D advanced cell models. Importing the pre-set gating templates into the iQue Forecyt® software facilitates quick, multi-parameter analysis, enabling broad *in vitro* characterization of a drug's effect on killing of tumors can be profiled in minimal time. Spheroids are dissociated using a reproducible, easy-to-follow workflow which facilitates the evaluation of 3D models using advanced flow cytometry. This enhances the wealth of information obtained, compared to traditional techniques such as imaging, and allows investigation of solid tumor therapies, whereas in the past we were limited to liquid tumors.

Tumor Infiltrating Lymphocytes

Tumor infiltrating lymphocytes (TILs) elicit altered responses (anti- or pro-tumorigenic) depending on the microenvironment and stage of disease. Immune cells must maintain the ability to survive, proliferate, and kill the cancerous cells once within the tumor microenvironment, if effective treatment is to occur. Individuals with high numbers of TILs are expected to be more responsive to neoadjuvant chemotherapy and have improved pathological complete response rates. Infiltration is affected by a multitude of environmental factors and the ability to study this *in vitro* could lead to the development of new therapeutics. Utilizing flow cytometry alongside advanced assays has the potential to offer translational models for the development of therapeutics modulating T cell infiltration.

Assay Principles
Here, we present an advanced *in vitro* 3D model of TILs, enabling the simultaneous quantification of phenotypes, cell type ratios and activation status of infiltrated and non-infiltrated cells. Unlabeled target cells are seeded in 96-well Ultra-Low Attachment (ULA) plates and incubated for 72 hours to promote formation of spheroids. Incucyte® Cytolight Rapid Green reagent is used to label PBMCs, which are then added alongside activators before seeding. This allows us to distinguish the tumor cells from the immune cells during analysis. At assay endpoint (up to 48 hours), non-infiltrated immune cells are washed off before the spheroids and TILs are dissociated. T cell subsets are then analyzed using the iQue® Human T Cell Activation Kit (Figure 15).

Comprehensive *in vitro* analysis of TILs using advanced high-throughput flow cytometry provides a turnkey solution for the *in vitro* quantification and phenotyping of TILs and non-infiltrating lymphocytes in advanced cell models. The simple, ease of use workflow utilizes a 96-well plate format from start to finish and can increase confidence by minimizing variability and facilitating the inclusion of more replicates without increasing acquisition time.

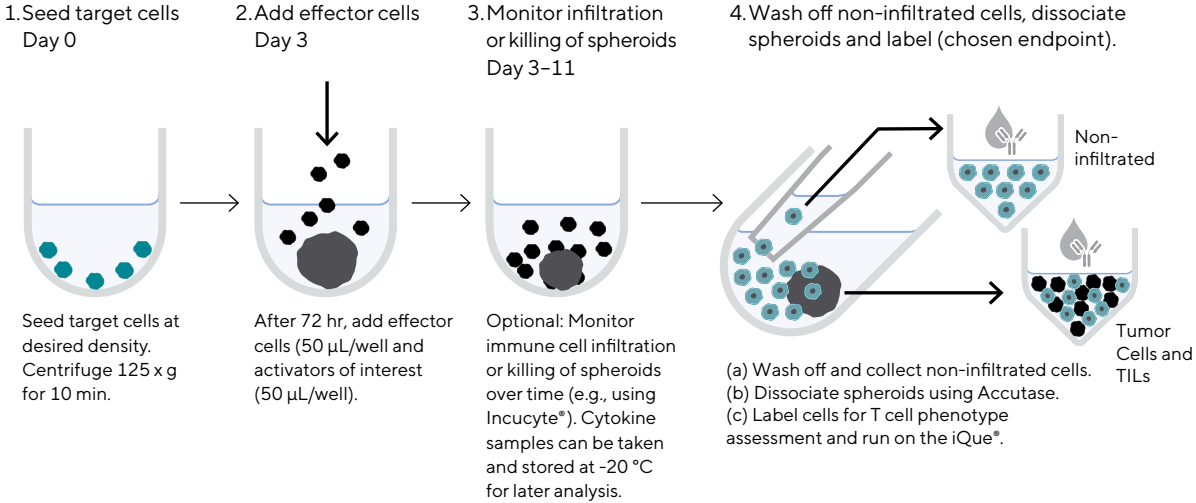


Figure 15
Schematic Outlining the Protocols for Analysis of TILs in 3D Tumor Spheroids Using the iQue® Platform

Example Results

Activation Marker Expression on TILs Is Greater Than Non-Infiltrated T Cells

It is widely accepted that increased numbers of TILs within a tumor are often associated with improved clinical prognosis.[13] growing evidence suggests that the composition of the TILs, in terms of the cell subsets present and their activation status, is also highly important in denoting the quality of the anti-tumor response the TILs can exert.[13–15] Initial experiments aimed to investigate the phenotypic profile of these cells *in vitro* using the TILs assay model with wild type BT474 (breast cancer) spheroids and Incucyte® Cytolight Rapid Green labeled PBMCs, activated with a range of concentrations of CD3/CD28 Dynabeads™. After 24 hours in co-culture, the non-infiltrated tumor cells were washed off and transferred into a separate plate, leaving only the spheroids and Green-labeled TILs in the assay plate. This three-step washing protocol was validated by taking images after each wash using the Incucyte® Live-Cell Imaging system (Figure 16A). The images clearly show a reduction in the number of immune cells suspended

around the spheroid with each progressive wash step, finally resulting in negligible remaining non-infiltrated cells after the third wash. Once the non-infiltrated cells had been separated, the spheroids and TILs were dissociated to create a single cell suspension suitable for analysis using the iQue® Human T Cell Activation kit.

The relative number of CD3 positive TILs per well is shown in a plate view diagram generated using the iQue Forecyt® software (Figure 16B). This shows a Dynabead™ concentration-dependent increase in infiltration of T cells into the spheroid. Figures 16C and D compare the expression of early-, mid- and late-stage activation markers (CD69, CD25, and HLA-DR) between the non-infiltrated and infiltrated T cells. At all but the highest concentration of Dynabeads™, the expression of the three activation markers was higher on the infiltrated T cells than on the non-infiltrated. On the infiltrated cells, high expression of CD69 was maintained, regardless of the density of Dynabeads™ present, with an average of 67.1 ± 0.6%. Non-infiltrated cells displayed increased sensitivity to changes in external stimuli with CD69 expression increasing in a Dynabead™ concentration-dependent manner, from 2.3 ± 0.2% to 86.9 ± 0.7% when the Dynabead™ density was increased from 247 to 20K per well.

Summary

The iQue® Platform with iQue® T Cell Activation Kit can be used to quantify TILs in a 3D single spheroid model. Infiltrated immune cell populations can be analyzed and compared to their non-infiltrated counterparts. Both the numbers and phenotypes of TILs can be important indicators of clinical outcomes and are impacted by many environmental and physiological factors. The ability to analyze TILs *in vitro* could aid the development of novel cancer therapeutics.

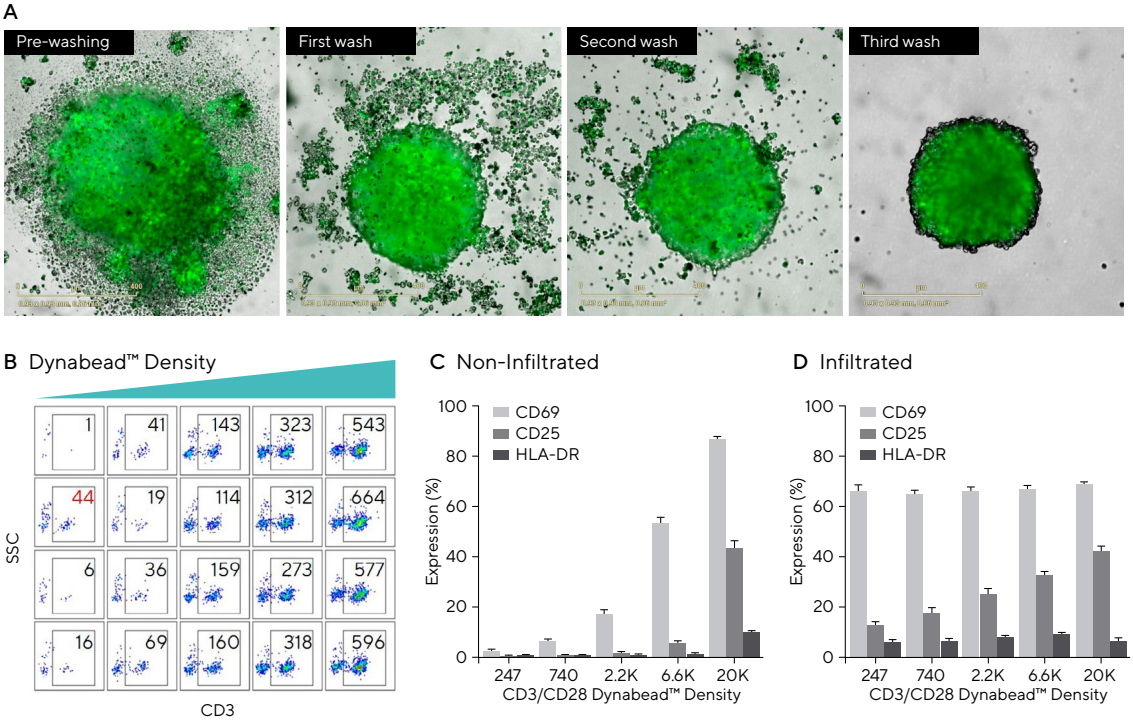


Figure 16: *Comparison of the Activation Status of Infiltrated and Non-infiltrated T Cells*

Note. BT474 spheroids were formed in ULA plates for 72 hours before Incucyte® Cytolight Rapid Green-labeled PBMCs (5:1 effector-to-target ratio (E:T)) and CD3/CD28 Dynabeads™ were added for 24 hours. (A) Incucyte® images used to validate the washing protocol for separation of non-infiltrated immune cells from the spheroid and TILs. (B) Spheroids and TILs were dissociated to a single cell suspension and analyzed using the iQue® Human T Cell Activation Kit. Plate view shows individual well plots of side scatter (SSC) vs. CD3 with gates highlighting the number of CD3 positive TILs per iQue® sip from each well. Each column represents a different Dynabead™ density (n = 4). An outlier (highlighted in red) was excluded from subsequent analyses. (C) and (D) Activation marker expression comparison between the non-infiltrated and infiltrated T cells.

Mixed Lymphocyte Reaction (MLR)

Immune checkpoints are regulatory signals that control the synapse between T cells and antigen presenting cells (APCs), such as dendritic cells (DCs). The balance between these stimulatory and inhibitory signals plays an important role in the tumor microenvironment, ensuring T cells can be 'switched-on' to increase tumor cell killing, but also 'switched-off' to prevent over activation of the T cells and attacking of healthy cells. Immunotherapies, termed checkpoint inhibitors, have been developed that block the immune checkpoints that usually 'switch off' T cells. This block tips the balance of the checkpoint regulatory signals, increasing T cell activation and resulting in enhanced tumor cell killing. Currently approved checkpoint inhibitor therapies include monoclonal antibodies that target PD-1, PD-L1 and CTLA4, whilst the continued exploration of additional immune checkpoint targets is a hot topic in the drug discovery field.[16]

Mixed lymphocyte reaction (MLR) assays mimic dendritic cell activation of T cells *in vitro* to provide a model for investigation of potential checkpoint inhibitor therapies. Immune cells from two individuals are cultured together and the detection of 'non-self' antigens presented by the DCs triggers a T cell activation response. This relies on differences in the donors' human leukocyte antigen (HLA) haplotypes. A potential therapeutics' ability to potentiate T cell response in an MLR assay can be evaluated through measurements of cell marker expression, proliferation, and cytokine release. Conventional techniques for analysis of MLR assays, such as traditional flow cytometry and ELISA are often limited because they:

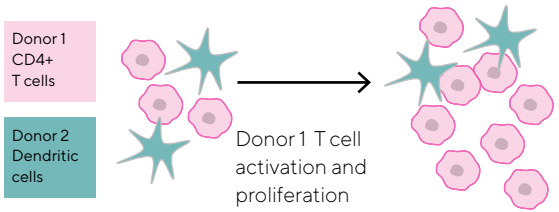
- Require separate assays to measure cytokines, proliferation, and activation marker expression. This means readouts for each treatment are taken from different sample populations and analysis timepoints, which can introduce variance in data.
- Necessitate manual correlation of data for a single treatment from multiple assay platforms, such as a plate reader and an ELISA assay.
- Use instrumentation with low-throughput acquisition and large sample volume requirements.
- Are laborious and time-consuming.

Assay Principles

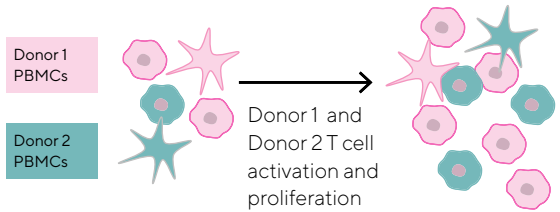
We have developed a simple, high-throughput workflow for quantifying T cell response in an MLR assay. There are two versions of MLR assay: one-way and two-way MLR. One-way MLR involves co-culturing of CD4+ T cells from one donor with DCs from another donor, resulting in unidirectional T cell activation. Two-way MLR is a co-culture of PBMCs from two different donors, and results in stimulation of T cells from both donors.[17]

Samples (10 µL) are collected and analyzed using a range of validated reagents from compatible iQue® kits, including the iQue® Human T Cell Activation Kit, the iQue Qbeads® Human Inflammation Panel Kit and a Custom iQue Qbeads® PlexScreen Kit (Figure 17). This allows for measurement of marker expression, proliferation and cytokine release from a single assay plate, collapsing the more traditional workflows. The integrated iQue Forecyt® software provides instantaneous pharmacological readouts for T cell activation response to checkpoint inhibitor drugs.

One-way MLR
Co-culture isolated CD4+ T cells from Donor 1 with dendritic cells (DCs) from Donor 2



Two-way MLR
Co-culture PBMCs from Donor 1 with PBMCs from Donor 2



Measure T cell response using the iQue® and associated kits to quantify cytokine release, proliferation and activation marker expression. Low sample volume requirements (10 µL) enable a single assay plate to feed multiple kits and timepoints.

Kit	iQue ® Human T Cell Activation Kit	iQue Qbeads® Human Inflammation Panel Kit	Custom iQue Qbeads® PlexScreen Kit
Outputs	▪ Proliferation ▪ CD69 ▪ CD25 ▪ HLA-DR ▪ TNFa ▪ IFNγ	▪ IFNγ ▪ IL-2 ▪ IL-6 ▪ CCL2 ▪ CCL3 ▪ CXCL9 ▪ CXCL10	Mix and match from a huge range of human secreted proteins to create a panel to suit your needs. Choose to measure a variety of cytokines, chemokines and interleukins.

Figure 17

Illustration of the MLR Assay Principles

Note. Analysis of T cell response from one or two-way MLR can be performed using the iQue® and a range of associated kits, including the iQue® Human T Cell Activation Kit, the iQue Qbeads® Human Inflammation Panel Kit and a Custom iQue Qbeads® PlexScreen Kit. These kits can be used alone or in combination to measure proliferation, marker expression and cytokine release as required to suit user needs, all from a single assay plate.

Example Results

T Cell Activation in MLR is Enhanced in the Presence of the Checkpoint Inhibitor Pembrolizumab

The iQue® MLR assay was used to investigate the effects of a checkpoint inhibitor drug on the T cell activation response. T cells and DCs from donors with highly mismatched HLA alleles were incubated with varying concentrations of Pembrolizumab, which is an anti-PD-1 monoclonal antibody indicated for treatment of cancers, such as non-small cell lung cancer (NSCLC) and melanoma.[18,19] The PD-1 ligand, PD-L1, is highly expressed on DCs and together these proteins represent a major checkpoint for immune regulation. Blocking this interaction with drugs such as Pembrolizumab 'Removes the brakes' and enhances T cell activation and anti-tumor activity.

Cytokine samples were analyzed on Days 2 and 6 for IFNγ and TNFα concentrations using Qbeads® from the iQue® Human T cell Activation Kit. Pembrolizumab induced a concentration-dependent increase in the release of both cytokines, indicating an increase in T cell activation, but the temporal profile of production of each cytokine was different (Figure 18A and B). TNFα release increased in a drug-dependent manner on Day 2 (EC_{50} = 0.54 µg/mL) then decreased by Day 6. Conversely, IFNγ release was low on Day 2 and increased by Day 6, with an EC_{50} of 0.44 µg/mL; very similar to the EC_{50} for Day 2 TNFα.

In monoculture, the checkpoint inhibitor did not affect the level of T cell activation, with Day 6 CD25 expression of 21.9 ± 9% and 23.2 ± 5% in the absence and presence of 10 µg/mL Pembrolizumab (Figure 18D and E). In the MLR co-culture, CD25 expression was 61.9 ± 2%; about 3-fold greater on CD4+ T cells in monoculture (Figure 18F). Pembrolizumab induced a further concentration dependent increase in CD25 expression, with a maximum of a 12% increase in the presence of drug compared to the co-culture alone (Figure 18C and 18G). This increase in activation is less stark than the increase due to the addition of DCs, which may reflect the clinical situation, where the drug is designed to delicately tip the balance towards enhanced T cell activation, without inducing overactivation. If T cells become highly activated, an inflammatory response can be triggered, leading to a loss of specificity of immune cell killing of cancer cells and resulting in killing of healthy cells.

Summary

The iQue® used in conjunction with a validated suite of reagent kits provides a simple and flexible workflow for measuring T cell response in an MLR assay. Combining fast sample acquisition by the iQue® Platform with pre-set gating and analysis using the inbuilt iQue® Forecyt software means libraries of potential checkpoint inhibitor therapeutics can be profiled for their activity in minimal time. Advantages of the iQue® MLR workflow also include:

- Cell markers and cytokines are measured in multiplex, speeding up time to actionable results and negating the need to correlate data from multiple platforms. This improves data coherence with all readouts for each treatment provided by the same population of cells at a single time point.
- Small sample volume (10 µL) requirements mean a single assay plate can feed multiple different kits and time points; allowing the user to generate a wide range of assay outputs with minimal usage of precious sample.
- Pharmacological readouts, such as EC_{50} values, generated using the iQue Forecyt® software, can be used to rank checkpoint inhibitor drugs based on their ability to induce T cell response.

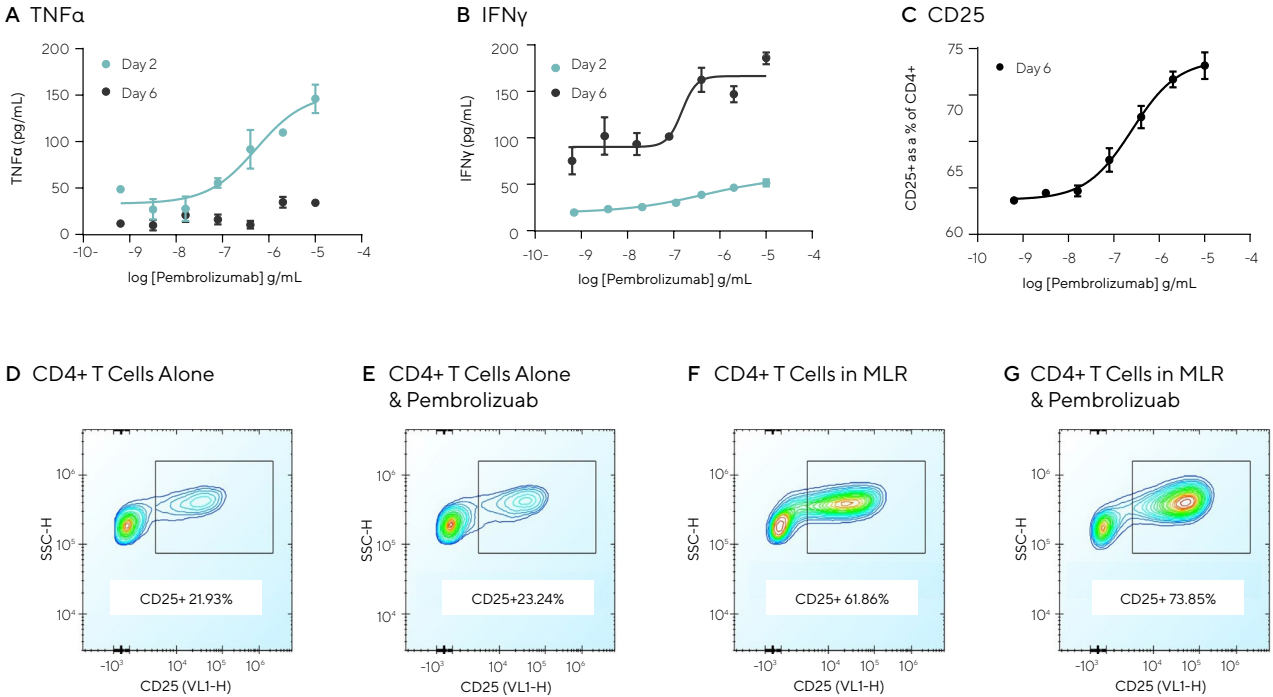


Figure 18

Pembrolizumab Induces a Concentration Dependent Increase in Activation Markers and Cytokines by T Cells in the Presence of DCs.

Note. DCs were thawed and activated overnight (with IL-4, Granzyme B and LPS) prior to plating at 40K/well in a 96-well plate. CD4+ T cells were added at a 3:1 T cell-to-DC ratio. Checkpoint inhibitor Pembrolizumab was added to enhance T cell activation. Cytokine samples (Day 2 and 6) and marker expression (day 6) were analyzed using the iQue® Human T Cell Activation Kit. (A) and (B) Concentration response curves show day 2 and 6 release of cytokines, TNFα and IFNγ, in response to Pembrolizumab. (C) Day 6 CD25% expression in CD4+ T cells from MLR. (D-G) Contour plots show % CD25 expression in T cell monoculture controls and MLR co-cultures, both with and without 10µg/mL Pembrolizumab.

Natural Killer Cells

Natural Killer (NK) cells are an essential part of the innate immune system. They interface with the adaptive immune system and play a critical role in tumor immune surveillance and anti-tumor responses. Although chimeric antigen receptor (CAR)-modified T cells have proven to be highly effective as an anti-cancer therapeutic, there are potential problems associated with their use, particularly the risk of inducing graft-versus-host disease (GVHD) and aberrant cytokine release. In contrast to T cells, NK cells are potent cytotoxic effector cells that do not require MHC restriction and have a low risk of inducing GVHD. Recent advances in the development of CAR-modified NK cells have shown great promise for clinical utilization in cancer immunotherapy with capability for both direct tumor killing as well as for use in combination therapy with monoclonal antibodies to boost NK cell antibody-dependent cell-mediated cytotoxicity (ADCC). A critical process in the development of new NK cell-mediated therapeutics is the ability to expediently assay, screen, and analyze data for NK cell activation and tumor killing.

More information on NK cells and their analysis using iQue® Human NK Cell Killing Kit can be found in **Chapter 5** Assays for antibody discovery.

Example Results

Cytokine Activation Increases NK Cell Cytotoxic Activity

NK cell cytotoxic activity was assessed in a direct tumor cell killing model utilizing the K562 human chronic myelogenous leukemia cell line as the tumor target cells. The K562 cells were first stained with the iQue® Cell Proliferation and Encoding (V/Blue) Dye, and then combined (20K/well) with unlabeled PBMCs or enriched (negatively selected) human NK cells that had been incubated for 16–18 hours in either media alone (non-activated NK cells) or media containing 200 units/mL of IL-2 + 100 ng/mL of IL-15 (cytokine-activated NK cells) at Effector:Target (E:T) ratios of 1:1 or 5:1.

The results in Figure 19 show that there was a clear increase in death of tumor cells in the presence of cytokine-activated NK cells when compared to non-activated NK cells. Target cell killing was enhanced at the higher E:T ratio of 5:1 when compared to the 1:1 ratio, regardless of whether the NK cells were activated or non-activated. Death of K562 in target cell-only wells was minimal. Death of K562 cells in most conditions was comparable between the 4 hour and 24 hour timepoints, aside from the increase in tumor cell killing by the cytokine-activated NK cells at the 1:1 E:T ratio.

Expression of NK Cell Activation Markers is Increased Upon Interaction with Tumor Cells

NK cells from the co-cultures analyzed in the above example were also assessed for their activation marker expression using the iQue® Human NK Cell Killing Kit (Figure 20). There was a clear increase in the expression of activation markers CD69 and CD25 in NK cells that had been incubated with cytokines, which fits the observed increase in target cell death by the cytokine activated NK cells. The inclusion of K562 tumor cells induced an increase in activation marker expression both in the non-activated and cytokine activated cells. This may suggest that cell signaling and increased cytokine release during NK cell killing of target cells leads to an overall increase in the activation status of NK cells in the co-culture.

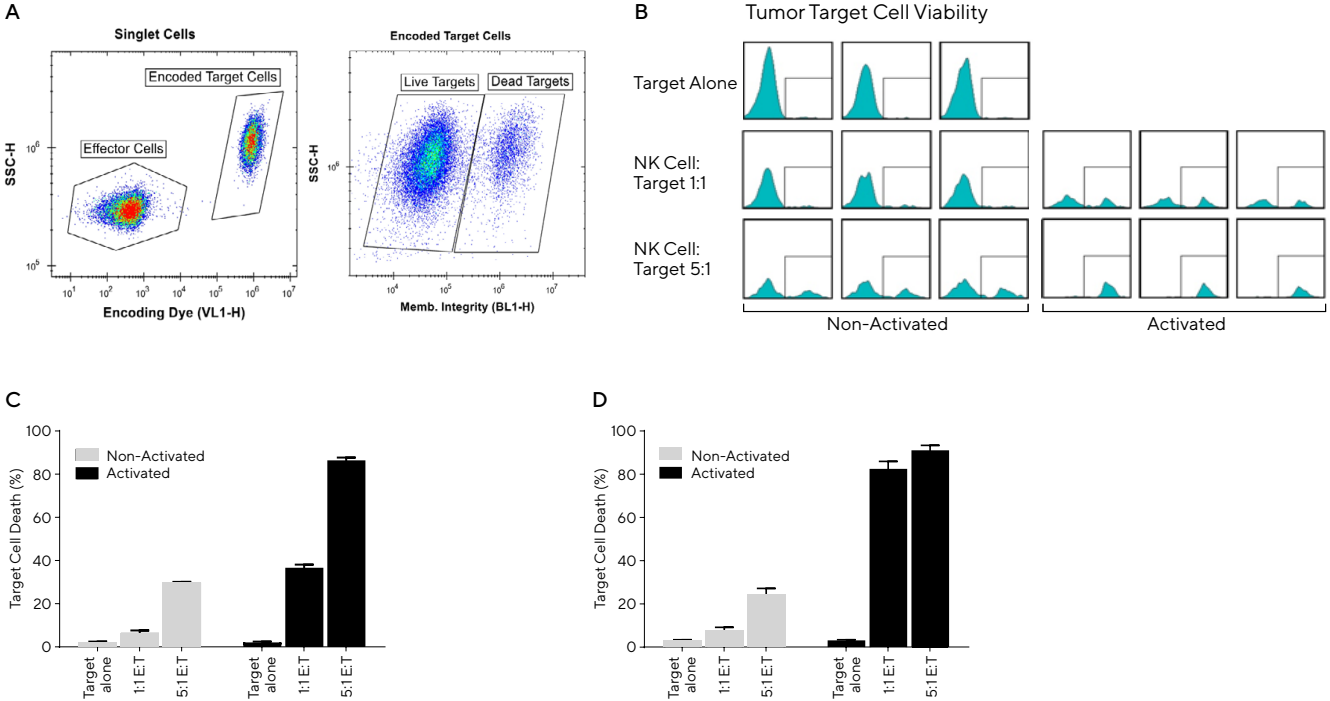


Figure 19

Comparison of Direct Tumor cell (K562) Killing by Non-Activated vs. Cytokine-Activated Human Enriched NK cells

Note. (A) Dot plot showing separation of target cells from effector cells with the use of a fluorescent encoder dye. Target cell killing was then determined with the use of a cell membrane integrity dye to distinguish live and dead targets cells. (B) Histogram depicting target cell viability following a 4 h co-culture with non-activated or cytokine activated NK cells. (C, D) Percent tumor cell killing after co-culture of tumor cells with non-activated or cytokine activated NK cells for (C) 4 h or (D) 24 h. Co-culture groups were plated in triplicate. Data represents average/group ±1 SD.

Summary
The advantage of the iQue® Kits over conventional assays is that they can assess multiple parameters using very few cells in a single multiplexed assay compared to common immunology research workflows, which generally require multiple assays and many different specialized machines and laboratory equipment. The assays can be performed in 96- or 384-well microtiter plates and are performed using pre-mixed reagents in a fast, simple workflow. In addition, the low-volume sample requirement of these assays allows for measuring more endpoints with smaller amounts of precious or rare samples. Data acquisition is performed on the iQue® Advanced Flow Cytometry Platform using the integrated iQue Forecyt® software package with pre-set compensation matrices that enable data acquisition of the multiplexed, phenotyping assay without the need for single stain color compensation and allows for real-time data analysis and visualization. Thus, the multiplexed iQue® Kits collapse traditional workflows into a single assay platform, which allows for streamlined and rapid data acquisition. For more information in reference to NK assessments please see **Chapter 5** Assays for antibody discovery

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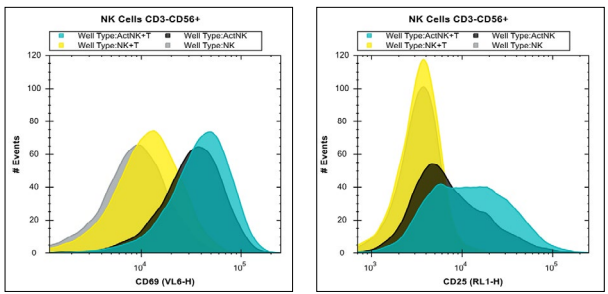

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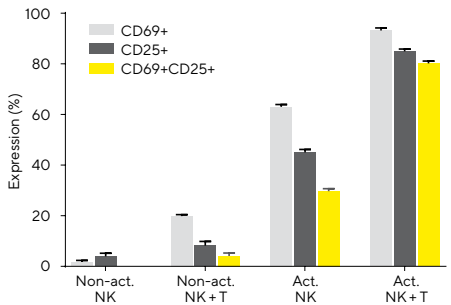


Figure 20
Expression of the Activation Markers

Note. CD69 and CD25 were analyzed following co-culture of non-activated or cytokine-activated NK cells with K562 tumor cells (T). (A) Overlay histograms depicting CD69 and CD25 expression on NK cells after 4 h co-culture. (B) Summary of activation marker expression following 24 h of co-culture (n = 3/group, NK, or Non-act. NK = non-activated NK cells, Act. NK = cytokine-activated NK cells).

References

1. Durgeau A, Virk Y, Corgnac S, Mami-Chouaib F. Recent Advances in Targeting CD8 T cell Immunity for More Effective Cancer Immunotherapy. Front Immunol, 9 (2018)
2. Pennock N, White J, Cross E, Cheney E, Tamburini B, Kedl R. T cell responses: naïve to memory and everything in between. Adv Physiol Educ, 37(4);273-283 (2013)
3. Chen EW, Brzostek J, Gascoigne N, and Rybakin V. Development of a Screening Strategy for New Modulators of T Cell Receptor Signaling and T Cell Activation, Scientific Reports (8) 2018.
4. Reinhold SE, Grimbacher B, and Witte T. Autoimmunity and Primary Immunodeficiency: Two Sides of the same Coin, Nature Reviews Rheumatology (14) January 2018
5. Dufva O, Koski J, Maliniemi P, et al. Integrated drug profiling and CRISPR screening identify essential pathways for CAR-T cell cytotoxicity. Blood, 135(9);597-609 (2020)
6. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature 1993 362: 758-61.
7. Brooks DG, Teyton L, Oldstone MB. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. J Virol. 2005; 79: 10514-27.
8. Fuller MJ, Zajac AJ. Maintenance, loss and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol. 2004 172:4204-14.
9. Jiang Y, Li Y, Zhu B. T cell exhaustion in the tumor microenvironment. Cell Death dis. 2015 6:e1792.
10. Cui X, Hartanto Y, Zhang H. Advances in multicellular spheroids formation. J R Soc Interface. 2017;14(127).
11. Zanoni M, Piccinini F, Arienti C, et al. 3D tumor spheroid models for in vitro therapeutic screening: A systematic approach to enhance the biological relevance of data obtained. Sci Rep. 2016;6(1).
12. Weber EW, Parker KR, Sotillo E, et al. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. Science. 2021;372(6537).
13. Antohe M, Nedelcu RI, Nichita L, et al, Tumor infiltrating lymphocytes: The regulator of melanoma evolution (Review). Oncol Lett. 2019;17(5).
14. Ladányi A, Somlai B, Gilde K, Fejös Z, Gaudi I, Tímár J. T cell Activation Marker Expression on Tumor-Infiltrating Lymphocytes As Prognostic Factor in Cutaneous Malignant Melanoma. Clin Cancer Res. 2004;10(2).
15. Hillen F, Baeten CIM, van de Winkel A, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. Cancer Immunol, Immunother. 2007;57(1).
16. Qin, S., Xu, L., Yi, M. et al. Novel immune checkpoint targets: moving beyond PD-1 and CTLA-4. Mol Cancer 18, 155 (2019)
17. Nicolaidou V., Stylianou C., Koumas L. et al. Gene expression changes in HLA mismatched mixed lymphocyte cultures reveal genes associated with allorecognition. Tissue Antigens;85(4):267-277. (2015)
18. Dang TO, Ogunniyi A, Barbee MS, Drilon A. Pembrolizumab for the treatment of PD-L1 positive advanced or metastatic non-small cell lung cancer. Expert Rev Anticancer Ther.;16(1):13-20. (2016)
19. du Rusquec P., de Calbiac O., Robert M. et al. Clinical utility of pembrolizumab in the management of advanced solid tumors: an evidence-based review on the emerging new data. Cancer Manag Res.; 11:4297-4312. (2019)

For further supporting information (videos and literature), see Appendix.

Chapter 7

Advanced Flow Cytometry Assays to Support Cell Therapy

Cell-based therapies hold great potential to revolutionize medicine and provide new therapeutic options for various diseases, including rare diseases and cancer. In regenerative medicine, cell therapies can harness the power of living cells to regenerate damaged or diseased tissues and organs. Despite rapid growth, companies face high costs and technical difficulties when it comes to phenotyping and profiling cell products.

Cell therapies involve living cells that are either derived from the patient (autologous), or a donor (allogenic). For example, stem cell therapies are used to treat diseases of the bone marrow and blood. CAR T cells are of high interest in biomedical research at the intersection of cell and gene therapy. CAR-T cells are gene-modified cell therapies that are designed to interact with a specific surface protein on the tumor cell, triggering cell death. This targeted approach to cancer treatment is preferred to traditional chemotherapies and has worked remarkably well in treating blood cancers like leukemia and lymphoma.

Bringing a new treatment to market requires expertise and innovative tools that accelerate progress from R&D to Process Development through Manufacturing. Some of the unique workflow challenges in cell therapy discovery involve the thorough characterization and quality control of cells for desired stability, potency, purity, and functional attributes. Additionally, digital tools are needed to help turn complex datasets into actionable insights that inform clinical outcomes.

Flow cytometry is used frequently during cell therapy development. Early on, flow cytometry can be used to identify specific populations of the donor T cells that are associated with a better prognosis for CAR-T cell therapy. The cells are then continuously characterized for quality, safety, and function until transfer to patient, and through follow-up monitoring.

Phenotyping, purifying, and expanding immune cells from patients is both expensive and technically difficult. From a single sample, there is a need to connect insights from immune cell health, phenotype and proliferation to cytokine secretion profiles. Advanced flow cytometry systems provide throughput, speed and more data per assay well to support these workflows. With the iQue® Advanced Flow Cytometry Platform, you can get both immunophenotyping data with measurements of cell health, and bead-based cytokine secretion, in a single experiment; this saves sample and avoids the need for multiple assays. Advanced flow technologies also integrate powerful software for instant access to real-time data, better visualization tools and dynamic

gating capabilities, making it easy to interpret complex datasets.

Advanced flow cytometry adds value at multiple stages in the development and expansion of cell therapy products, like induced pluripotent stem cells (iPSCs) and CAR-T cells. By combining multiplexed analyses, scientists can simultaneously learn about immune cell phenotype and function in a single well.

Induced Pluripotent Stem Cells (iPSCs) Maintenance and Differentiation

In 2006, Japanese researchers published an important discovery outlining a method for creating iPSCs from primary mouse fibroblasts by activating the expression of key transcription factors.[1] Since this discovery, the field of stem cell biology has rapidly expanded and iPSCs form the basis of many new areas of research. They are intrinsically valuable due to their unique characteristics and the control they afford to researchers and clinicians over the building blocks of the body. The major benefits of the use of iPSCs are the number of different cell types that can be differentiated from them and their capacity for infinite expansion.[2] This flexibility provides many opportunities for the development of specific cell and tissue models, both in 2D and 3D, for pharmacological testing,[3] cancer research,[4] organoid modeling of tissues,[5] and neurodevelopmental biology.[6] In addition, iPSCs are increasingly used in translational applications, targeting eventual use in the clinic via autologous cell therapies and for individualized medicine approaches.[7] Increasingly, there is a need to improve the culture and expansion methods of iPSCs away from 2D plate-based methods and towards more physiologically relevant methods such as 3D culture. In particular, 3D suspension cultures, like cell aggregates and spheroids, can maintain greater cell-to-cell contact, produce endogenous extracellular matrix to promote growth conditions similar to *in vivo*, and are readily available for downstream applications.[8]

Some limitations are inherent in any system, however, and iPSCs are high maintenance, expensive, and require constant monitoring to ensure they maintain pluripotency, viability, and homogeneity.[9] Long-term culture of iPSCs can result in genotypic and phenotypic heterogeneity, even in a cell line derived from a single source cell; therefore, it is vital that methods for monitoring, detecting, and reducing heterogeneity in iPSC lines are developed.[10]

Increasing use of stem cells in both clinical and research settings necessitates fast, reliable, and relatively inexpensive solutions for the growth, characterization, and maintenance of this valuable biological resource. This is important during monitoring growth and tracking successful differentiation. Conventional methods for monitoring iPSC characteristics during culture, such as traditional flow cytometry, can:

- Be labor-intensive and time-consuming, requiring multiple steps including fixation, staining, and washing
- Require large sample volumes, necessitating the use of more precious cells and expensive consumables, reducing the amount of sample remaining for downstream expansion, characterization, and differentiation
- Demand in-depth manual manipulation and analysis of raw data and require compensation optimization
- Involve low throughput instrumentation, increasing workflow time and reducing capacity for intra- and inter- experiment replication

iQue Advanced Flow Cytometry Pluripotency Maintenance and Growth at a Glance

When generating iPSCs for use in downstream applications, it is commonplace to perform initial checks for key attributes such as viability and pluripotency.[11] This allows for assessment of the quality of the produced iPSC culture, which may differ due to numerous factors such as the reprogramming technology used. For example, lentiviral miRNA infection is known to produce a much higher yield of pluripotent cells compared to adenoviral methods.[12] Some studies suggest that the somatic cell source from which an iPSC was derived can impact its ability to differentiate into certain lineages, potentially due to epigenetic variances.[13] It is therefore important that there are simple, robust, and standardized techniques for evaluating and comparing individual iPSC lines to ensure we choose the best lines for lengthy and expensive studies.

Here we describe a combined iQue® Advanced Flow Cytometry and Incucyte® Live-Cell Analysis approach for stem cell evaluation during cell line maintenance (Figure 1). Fast sample acquisition, low volume requirements (10 µL), and plate-based data analytics for the iQue® conserve precious time and sample during iPSC phenotyping. Meanwhile, Incucyte® images provide continuous morphological assessment for easy monitoring of iPSC colonies throughout differentiation.

Stem cell pluripotency can be assessed using a combination of recognized surface and intracellular markers. Figure 2 shows data generated using a panel of antibodies against SSEA-1, a marker of normal, non-pluripotent cells, and SSEA-4, TRA-1-60, Sox 2, and Oct 3/4 to characterize pluripotent cells. This assessment was completed in one well alongside the iQue® Fixable Cell Viability Dye. Histograms and dot plots created in the iQue Forecyt® software show the expression of various surface and intracellular markers in iPSC and control cells. THP-1 cells were included as a positive control for SSEA-1 expression and negative control for the other markers.

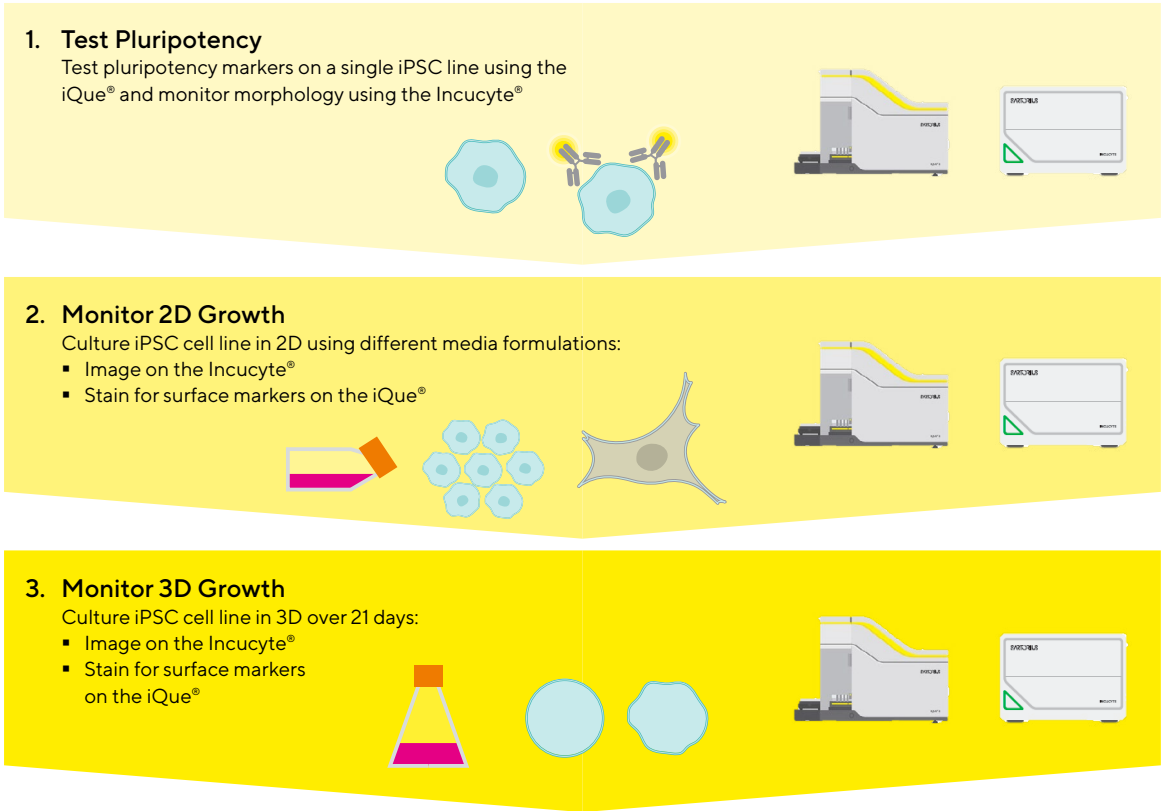


Figure 1

Schematic Highlighting the Combined iQue® and Incucyte® Workflow for iPSC Monitoring

Note. Using a combination of instruments, researchers can test iPSC pluripotency and monitor iPSC growth in both 2D and 3D culture.

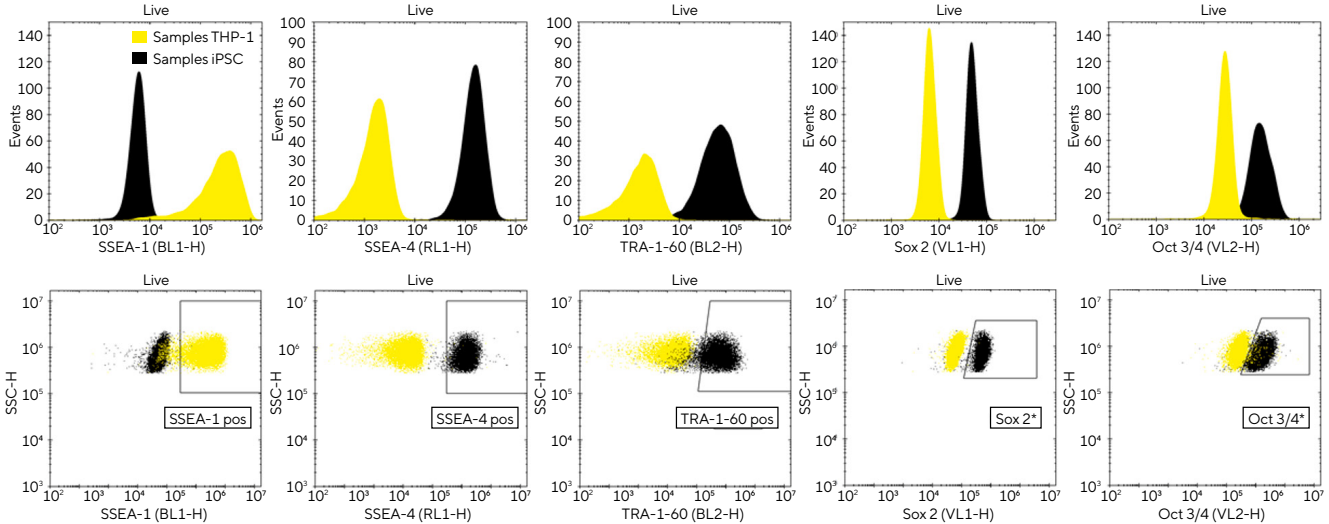


Figure 2

Pluripotency Marker Assessment for iPSCs

Note. * intracellular markers that were analyzed in fixed samples.

Example Results

Scaling up an iPSC culture requires changes to the conditions under which the cells are grown. Due to the relative flexibility of iPSCs, they can be grown both as 2D monolayers and 3D suspension cultures, facilitating scale-up in bioreactors of good quality iPSC lines for a range of downstream processes.

As previously mentioned, maintaining the health and pluripotency of the cultures is of high importance. To investigate the effects on the expression of surface markers for pluripotency of non-optimized and optimized growth conditions in suspension growth, the following experiment was set up. iPSCs were grown as monolayers for at least two passages before transfer into suspension culture (125 mL shaker flasks, 150K cells per mL in 20 mL media) for at least one passage before they were seeded and subjected to Optimized (passaging at each timepoint) and Non-optimized (no passaging) conditions over 7 timepoints covering a total of 21 days. Cells were

imaged using the Incucyte® Live-Cell Analysis System and surface markers were analyzed using the iQue® Advanced Flow Cytometry platform.

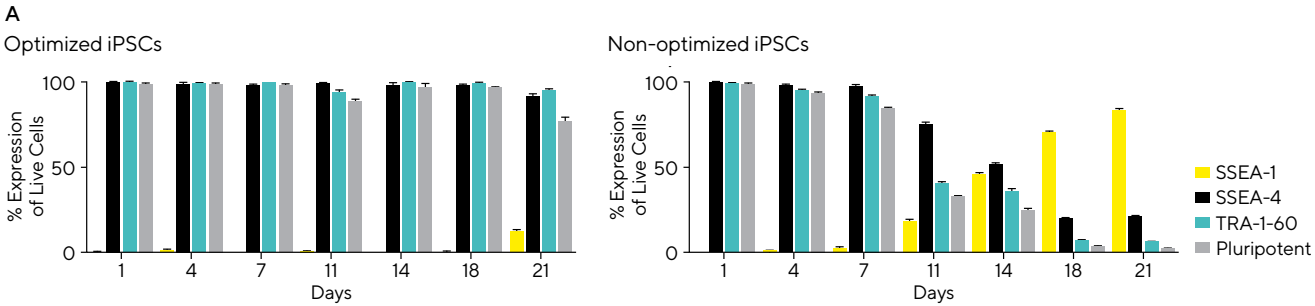
When iPSCs are grown in suspension, marker expression analysis shows that dramatic changes in phenotype only began to present on Day 11 in non-optimized conditions, (Figure 3A, 18.5 ± 0.39% increase in SSEA-1, 75.2 ± 1.2% reduction in SSEA-4, 40.5 ± 0.5% TRA-1-60 pluripotent marker expression, 32.9 ± 0.5% overall pluripotency reduction). This trend continued until the endpoint at Day 21 resulting in a final SSEA-1 expression of 83.5 ± 0.7%, SSEA-4 at 20.7 ± 0.3%, TRA-1-60 at 5.9 ± 0.1%, and the pluripotent population at 2.2 ± 0.1%. This data indicates a loss of pluripotency throughout the time course in contrast to the results with the expression profile of Optimized iPSC culture, which stay consistently pluripotent until Day 21. At this timepoint, we start to see an increase in SSEA-1 expression (12.4 ± 0.6%) and a decrease in the pluripotency markers, SSEA-4 (91.1 ± 1.8%), and the pluripotent population (76.5 ± 2.6%). Heatmaps produced in iQue Forecyt® software present the same data for SSEA-1 and pluripotent expression for two representative timepoints at Day 1 and Day 18, providing a snapshot of the changes in expression during the experiment (Figure 3B). Control cells, THP-1 and NCCIT, represented a non-pluripotent control line and a line expressing pluripotent surface markers, respectively.

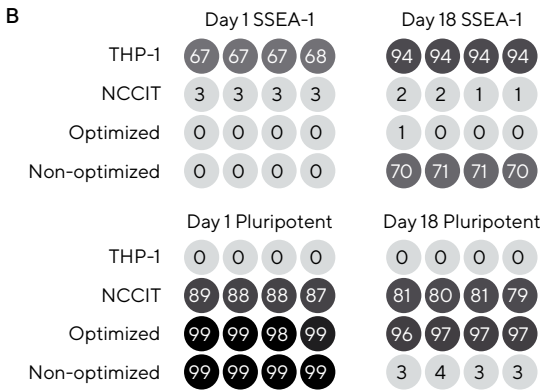
Suspension grown iPSCs were sampled at each timepoint, plated into a 24-well plate, and visualized on the Incucyte® Live-Cell Analysis System (Figure 3C). The images show the morphological differences after 18 days between the Optimized (top) and Non-optimized (bottom) culture conditions. When iPSCs are grown in suspension in Optimized conditions, we observe a very distinct, compact spheroid with high levels of circularity, compared to the Non-optimized conditions, in which the spheroids are much larger, far less compact, and lack the circularity of the Optimized spheroids (0.59 vs 0.49 eccentricity, respectively, data not shown).

iQue Advanced Flow Cytometry Tracking Differentiation at a Glance

Similar assays can be used to exemplify the combined use of the iQue® and Incucyte® systems in an integrated workflow for iPSC line assessment and monitoring during differentiation (Figure 4). This builds on the assays already described to assess iPSC pluripotency, tracking marker changes during directed differentiation. Further analysis of key markers linked to the desired final phenotype can also be tracked during the process. By combining with the Incucyte® Live-Cell Analysis Platform, any associated morphology or proliferation changes can also be monitored.

To ensure the optimal iPSC line is used for the extended differentiation protocol, lines can be pre-evaluated for their pluripotency via assessment of marker expression and colony morphology. The preferred cells can then be differentiated, a process which can be monitored over time by measuring pluripotency and lineage-specific markers using the iQue® and observing changes in morphology using the Incucyte® system. At the endpoint of any differentiation protocol, functional analysis of the derived cells can also be performed.





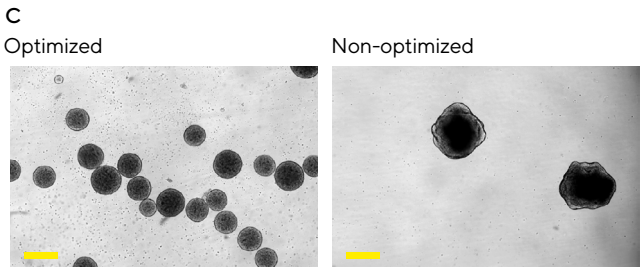


Figure 3

Monitoring of Pluripotency of iPSCs grown in Suspension Using Optimized and Non-Optimized Media

Note. (A) Pluripotency marker expression was tracked over 21 days (± SEM, n = 4), (B) heatmaps produced in the iQue Forecyt® software show the changes in marker expression at 1 and 18 days. (C) images show 3D iPSC spheroids grown in Optimized and Non-optimized growth conditions, images (10X) at the 14-day timepoint, scale bar indicates 400 µm.

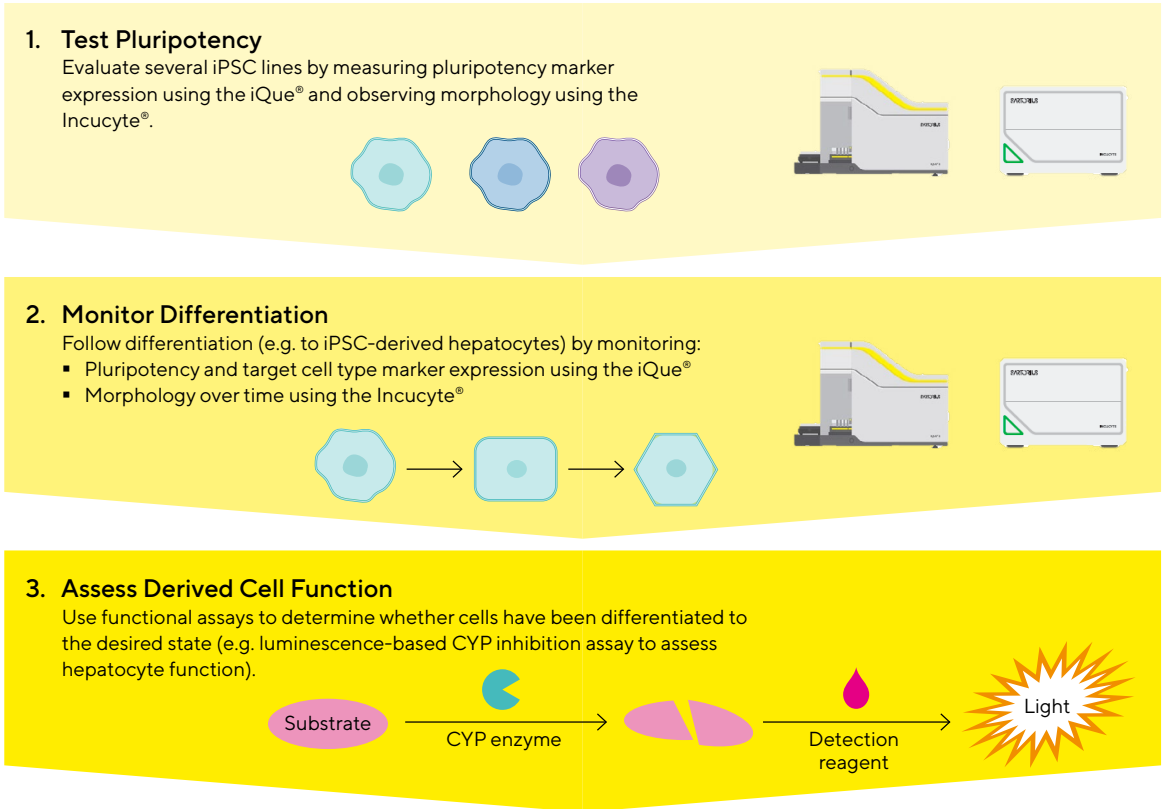


Figure 4
Schematic Highlighting the Combined iQue® Platform and Incucyte® System workflow for iPSC Selection and Differentiation Monitoring

Example Results

Data is presented to show a directed differentiation to hepatocyte-like cells using Cellartis® iPS Cell to Hepatocyte Differentiation System (Takara Bio). This is a forty-day culture protocol involving expansion and differentiation of iPSCs in a series of cell culture mediums and extracellular matrices to generate iPSC-derived hepatocyte cells (Figure 5).

Cell surface marker expression during differentiation was monitored as described previously for pluripotency assessment (SSEA-1, SSEA-4, and TRA-1-60) with an extended panel to include markers from different stages in hepatocyte development: CD184 (CXCR4) and CD99. Viability assessment was performed using iQue® Cell Membrane Integrity (B/Red) Dye. In addition, phase images (10X) were taken using the Incucyte® Live-Cell Analysis System, so that characteristics such as cell morphology and confluency could be observed throughout the differentiation process. The Incucyte® System can acquire phase images from an extensive range of culture vessels, meaning the cells could be monitored throughout the expansion process, from 24-well dishes to T75 flasks. THP-1, AU565, and HepG2 cells were included as non-pluripotent cell controls. HepG2s are a liver cell line, expected to display similarities in phenotype and function to the derived hepatocytes.

The three control cell types expressed the marker of differentiated cells SSEA-1 and did not express the pluripotency markers SSEA-4 and TRA-1-60 (Figure 6A). Conversely, the undifferentiated iPSCs did not express SSEA-1, but were highly pluripotent, with 99% of cells expressing the fully pluripotent phenotype (SSEA-1-/SSEA-4+/TRA-1-60+). From Day -14 to Day 0, cells were adapted to and expanded in the Cellartis DEF-CS culture system. This system is designed for high-density iPSC culture in a tightly packed monolayer, rather than in the standard iPSC colony morphology. SSEA-4 and TRA-1-60 expression remained high throughout this adjustment period (Figure 6B); however, compared to the undifferentiated iPSCs grown in mTESR

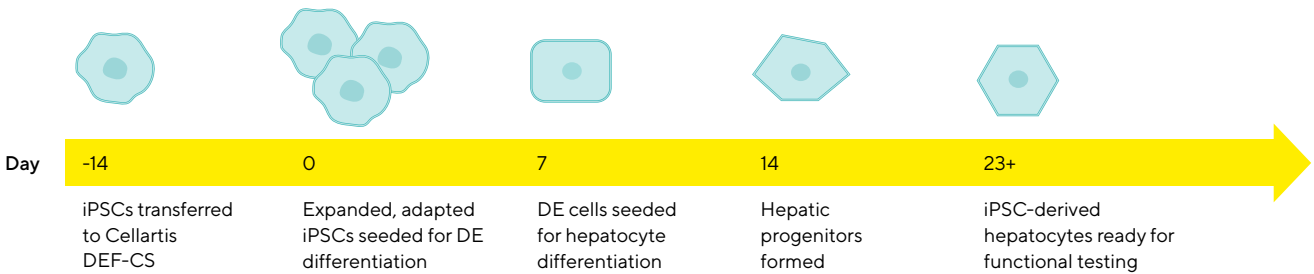


Figure 5: *Summary of the iPSC-Hepatocyte Differentiation Protocol*

Note. Definitive Endoderm (DE).

media, there was an increase in SSEA-1 expression in the Cellartis culture system, implying there may have been some spontaneous differentiation.

For the remainder of the time course, SSEA-1 expression was largely varied, peaking at 54.8 ± 0.6% on Day 7. Expression of both SSEA-4 and TRA-1-60 declined throughout the differentiation process, with SSEA-4 decreasing more rapidly, leaving only 7.6 ± 0.1% expression remaining by Day 7 compared to 54.8 ± 0.9% expression of TRA-1-60.

Incucyte® images facilitated visual monitoring of changes in phenotype and loss of pluripotency over time (Figure 6C). After 3 days in the Cellartis DEF-CS culture system (Day -11), the morphology of the cells had changed compared to the iPSCs grown in mTESR, becoming more spread out and losing the colony-based formation. On Day 2, the cells regained the 'colony-like' spatial distribution, but this was lost once they differentiated to definitive endoderm by Day 7. From Day 7, the cells became increasingly tightly packed in a monolayer, with the ability to distinguish individual cell features lost, as is evidenced in the image from Day 25.

To ensure that the differentiation process is successfully progressing towards the desired lineage, it is important to monitor the gain in specific characteristics over time, concurrently with the loss in pluripotency. For this study two additional markers were included with the aim of measuring the gain of hepatocyte-like phenotype over time. Both CD184, a marker for definitive endoderm cells, and CD99, hepatocyte marker, were seen to increase, indicating a successful differentiation.

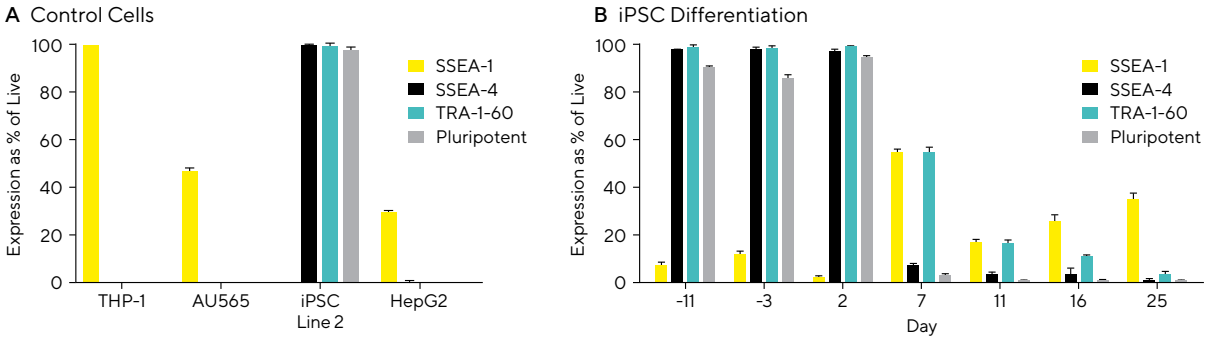
Summary

Overall, these data exemplify the ease with which the iQue® and Incucyte® Platforms can be used to monitor the temporal progression of differentiation. The development of simple, robust methods such as these for monitoring iPSC differentiation has promise to enhance the scalability and throughput of applications utilizing iPSCs, such as liver toxicity screening.

The experiments shown have highlighted some of the advantages of this workflow:

- Pluripotency marker expression and viability of iPSCs are easily measured using the iQue® Platform with simple, one-wash labeling protocols
- The miniaturized assay format due to the low volume requirements of the iQue® Platform reduces sample wastage, meaning more cells are available for downstream analysis, expansion, and differentiation
- High-throughput sample acquisition using the iQue® Platform (15 minutes for a full 96-well plate) reduces workflow time and facilitates enhanced replication, enhancing data quality
- Plate-level, real-time data visualization tools via advanced flow cytometry enable rapid comparison between cell lines, resulting in the fastest path to actionable results
- Monitoring cultures using the Incucyte® provides an easy way to check for pluripotency, confluency, and differentiation over time
- Measuring differentiation marker expression over time using the iQue® complements endpoint functional analysis to provide full confidence in the progression of your differentiation workflow

Together, this creates a streamlined workflow for iPSC characterization with potential applications in research and drug discovery.



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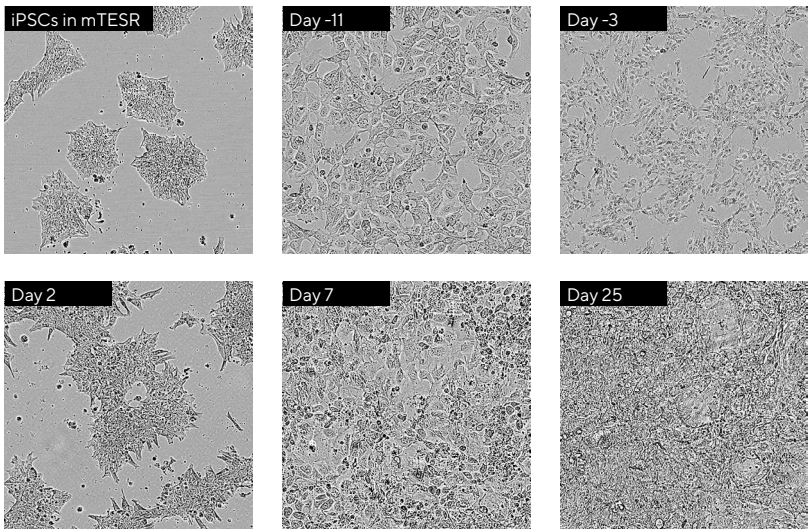

Figure 6

Monitoring Loss in Pluripotency Markers and Morphology Over Time

Note. (A) Control cell expression (± SEM) of pluripotency markers (n = 4); (B) Marker expression (± SEM) on iPSCs during differentiation (n = 4); (C) Incucyte® phase images (10X) of differentiating iPSCs in comparison to iPSCs grown conventionally in mTESR.

Chimeric Antigen Receptor (CAR) T Cells

The successful use of immunotherapies to help combat cancer has expanded rapidly in the last few years, with many therapies now approved for clinical use. The precision of the immune system allows a more targeted approach to killing cancer cells, while sparing healthy cells, when compared to traditional chemotherapeutic strategies. One key area of advance has been in the use of gene-modified cell therapies with the introduction of chimeric antigen receptor (CAR) T cells leading the field. The CAR construct is designed to interact with a specific surface epitope or antigen present on the tumor cell, which once in close proximity, enables the T cell to kill the tumor cell. Where specific antigens can be identified on the tumor cells, CAR-T cells display targeted effects and, as they are sourced from the patient (known as autologous therapy), there is a lack of rejection. CD19 targeted CAR-T cell therapies, for example Kymriah® (Novarits) or Yescarta® (Kite/Gilead),[14] have shown clinical success against liquid tumors common in lymphoblastic leukemia and non-Hodgkin's lymphoma. Despite this progress, obstacles remain, such as, the high cost and technical difficulties of phenotyping, profiling and purifying immune cells.[15,16] Also, while some patients have been highly responsive to treatment, others were refractive, and uncovering the mechanistic basis for these differing outcomes is an active area of research. In more recent years, research has progressed to explore the introduction of CAR constructs into alternative immune cells, for example CAR-NK or CAR-macrophages,[17-19] and to investigate gene modified cells that target solid tumors.

This chapter will touch on the process of manufacturing and expanding cell therapy products with a focus on CAR-T cells. Key *in vitro* assays used to phenotype and assess function of these modified cells will be introduced with three case studies illustrating the utility of the iQue® Advanced Flow Cytometry Platform.

Manufacture and Expansion of CAR-T

In recent years, there has been a large focus on improving the efficiency and quality of CAR-T cells to support their continued clinical use. As shown in Figure 7, there are multiple stages involved in the development and expansion of autologous CAR-T material. At all stages, the resulting product needs to be assessed for quality and functionality, while reducing time from initial donation to re-introduction to the patient.

During early development, the CAR construct is optimized to ensure its specificity and engagement with the target of interest. Constructs typically include a recognizable marker which serves as an easy identifier of transduced cells. This marker is used following T-cell reprogramming to assess transduction efficiency and can be used for CAR-T enrichment during downstream processing. Once CAR-T cells have been transduced, *in vitro* assays are used to profile the cells and to assess their functional reactivity to the target of interest. For example, flow cytometry can be used to

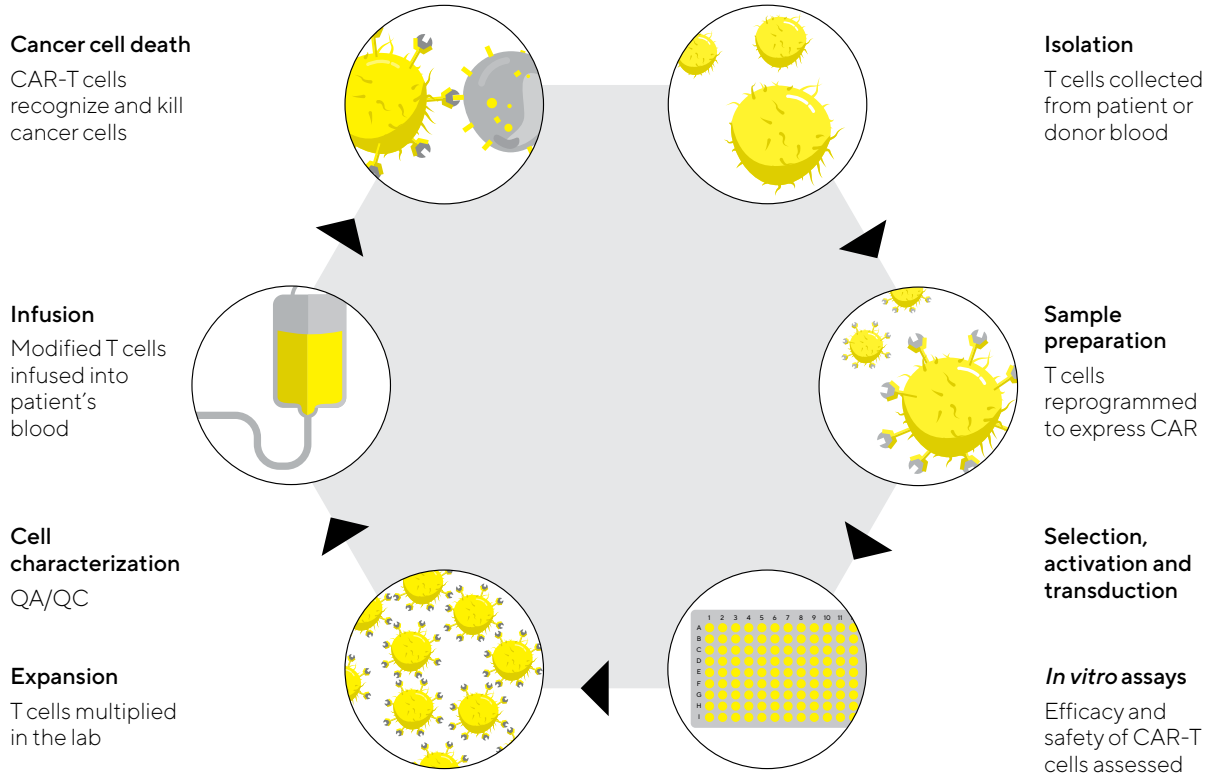

Figure 7
Development Cycle of CAR-T Material

assess the phenotype of cells in combination with functional readouts following a tumor killing assay.[20] These assays need to deliver reproducible and biologically relevant results.

iQue Advanced Flow Cytometry Phenotypic and Functional Testing at a Glance

The phenotype of the final CAR-T cell product is of great importance because it strongly links to its clinical potency. Much of the interest in this field has been focused on the influence of exhaustion and memory phenotypes on CAR-T function.[21] For a prolonged anti-tumor response, it is critical that populations of functioning CAR-T cells are maintained once re-introduced into the patient. This relies on preservation of the cells' self-renewal potency coupled with a lack of exhaustion, meaning their ability to kill tumor cells is sustained. There are key phenotypes that can be tracked *in vitro* to determine that these parameters have been maintained. Large populations of memory T cells such as stem central memory (TSCM) or central memory cells (TCM) are desirable as they have high self-renewal capabilities. Terminally-differentiated cells, such as terminal effector cells (TTE), are undesirable because they have lost their ability to self-renew. Markers such as PD-1, LAG-3 and TIM-3 are important indicators of exhaustion. Expression of these markers will often fluctuate during the expansion phase due to the stimulation added to drive activation and expansion of the T cells.[22] More recently, interest has also been directed towards determining the optimal ratio of CD4 and CD8 cells in a CAR-T product.[23,24]

The data below (Figure 8) shows an example phenotype profile of CAR-T cells during a 10-day expansion process with anti-CD3/anti-CD28 activation beads as a static culture in flasks (cells supplied by Dr. Qasim Rafiq's lab at University College London). Samples were analyzed on Day 3 and Day 10 post CAR transduction using the iQue® Human T Cell Kits previously described. The data quantifies the general T-cell population for CD3, CD4 and CD8 alongside viability and transduction efficiency across the sample days. For this example, the Day 10 memory phenotypes display a higher proportion of the desired TSCM and TCM cells with negligible populations of the more differentiated phenotypes of effector memory (TEM), TTE and effector memory cells re-expressing CD45RA (TEMRA). The activation profile shows that early activation markers, CD69 and CD25, are more highly expressed on Day 3 and reduce by Day 10, while HLA-DR, a later marker for activation, increases from Day 3 to Day 10. PD-1 and LAG-3 display a similar trend to the early activation markers, in that they are initially high, but lower by Day 10. Interestingly, TIM-3 expression remains high throughout. Both IFNγ and TNFα concentrations are high in the Day 3 sample, but dramatically drop by Day 10.

This type of profiling data can help support optimization of expansion processes and the complete understanding of the phenotype ratios present in the final product.

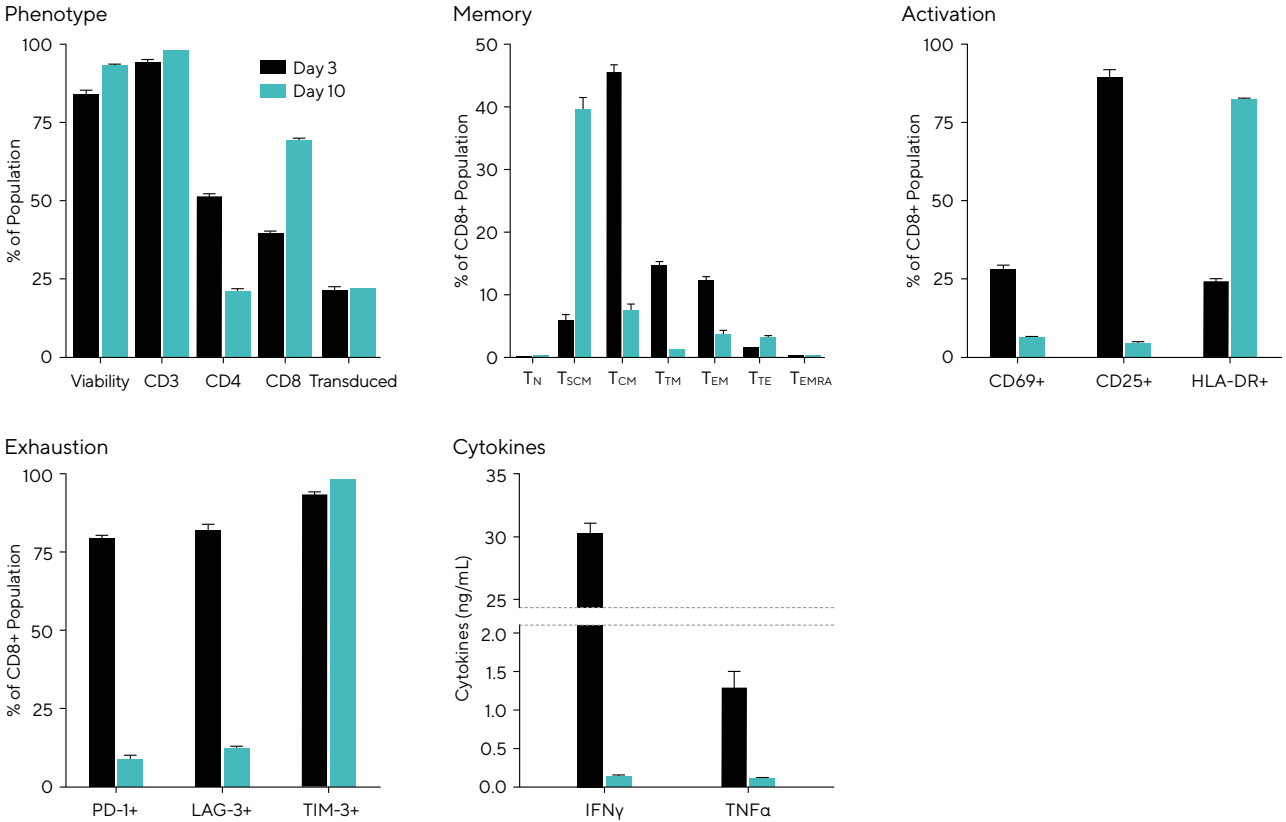

Figure 8

CAR-T Phenotyping using iQue® T Cell Characterization Kits During an Anti-CD3/Anti-CD28 Driven Expansion of Transduced T Cells

Example Results

Exhaustion Profiling of CAR-T Cells Under Antigen Challenge

Repeated exposure to tumor cell antigens can lead to CAR-T cell exhaustion. Examining the phenotypic profile of exhausted CAR-T cells can help to improve our understanding of how this exhaustion affects the longevity of the clinical response. To investigate this *in vitro*, CAR-T cell exhaustion was induced by continuously challenging anti-CD19 CAR-T cells with an antigen-positive Ramos cell line. Every 2–3 days for 10 days, the CAR-T cells were counted and re-stimulated with fresh Ramos cells (1:1 T:E). On Day 11, the stimulated CAR-T cells were counted and seeded into a 96-well plate with Ramos cells (1:1 T:E). A fresh batch of non-exhausted CAR-T cells were plated both in co-culture with Ramos and as a monoculture for comparison. Daily cytokine samples (10 µL) were taken from all wells of the assay plate and, after 72 hours, cells and supernatants were quantified using the iQue® Human T Cell Exhaustion Kit.

Cytokine secretion, both of IFNγ and TNFα, was low in wells containing the exhausted CAR-T and Ramos cell co-culture (Figure 9A and B). Comparatively, fresh CAR-T cells co-cultured with Ramos cells secreted significantly higher levels of IFNγ and TNFα, with peak concentrations at 48 hours of 3.5 ± 0.2 ng/mL and 0.6 ± 0.1 ng/mL, respectively. Fresh CAR-T cells in monoculture produced low levels of each cytokine. This distinct loss of cytokine secretion in wells with the repeat antigen challenged T cells is a clear sign of their exhaustion.

After 72 hours, expression of the LAG-3 exhaustion marker was highly elevated in the challenged CAR-T cells, with 86 ± 0.3% of the CD4+ population positive for this phenotype, whereas the freshly stimulated CD4+ CAR-T cells had just 31 ± 0.6% expression (Figure 9C). The CAR-T cell monoculture had a small population positive for LAG-3 (17 ± 0.8%).

A reduction in the exhausted CAR-T cells' ability to kill the target cells was also observed in the co-culture incubations (Figure 9D). Non-exhausted CAR-T cells were able to reduce Ramos cell numbers more effectively than exhausted CAR-T cells, with iQue® acquired values having an average of 4,606 ± 463 cells per sip from wells containing non-exhausted CAR-T cells compared to 8,483 ± 688 cells per sip with the exhausted CAR-T cells. The reduced ability of T cells to kill target cells is another hallmark of exhaustion.

Overall, this complete quantification demonstrates a clear antigen-specific driven exhaustion profile in these anti-CD19 CAR-T cells. The data demonstrates the utility of the exhaustion profiling kit in this type of cellular profiling.

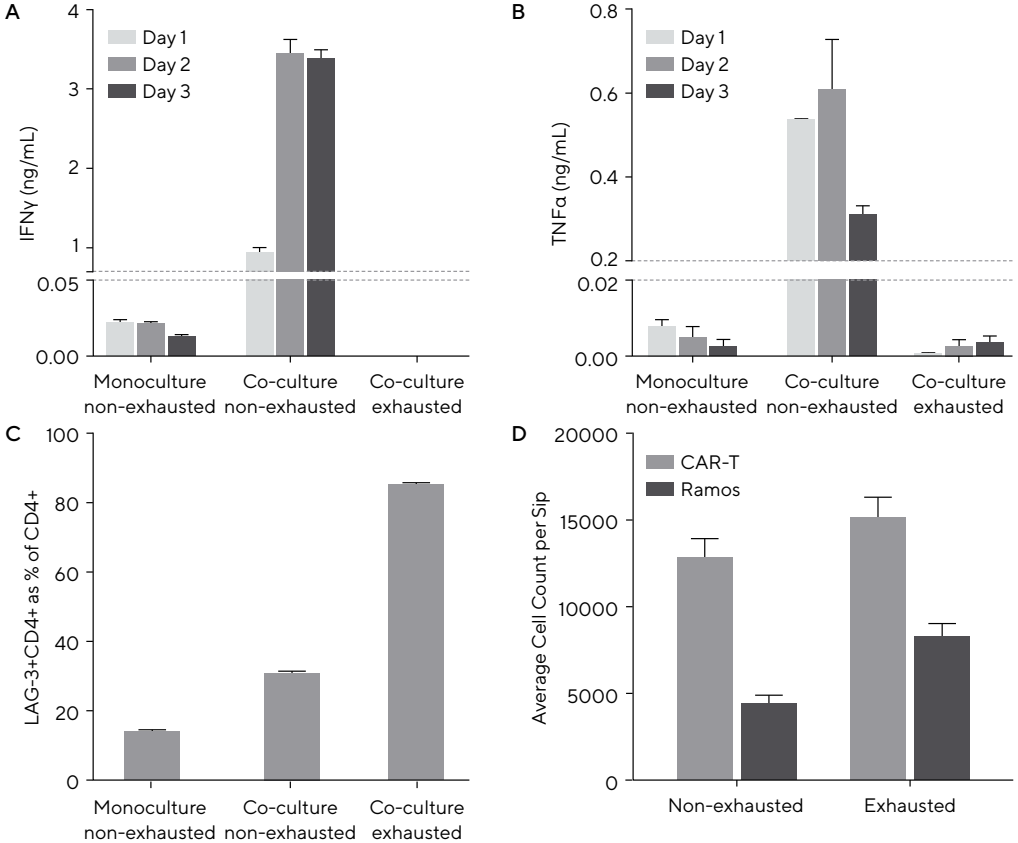

Figure 9: *Anti-CD19 CAR-T Cells Challenged with the CD19+ Ramos Cell Line Exhibited A Clear Exhaustion Phenotype*

Note. Each data point represents mean ± SEM, n = 4 wells.

Anti-HER2 CAR-T Killing is Specific to HER2-Positive Tumor Spheroids

So far, clinical success with CAR-T cells has been focused on treatment of liquid tumors, for example with CD19 targeted CAR-T approved for treatment of acute lymphoblastic leukemia, but much research is still ongoing to find suitable targets for solid tumors, which would hugely open up the potential uses of CAR-T therapy.[14]

To assess the specificity of the anti-HER2 CAR-T cells towards HER2 overexpressing tumor cells, target spheroids were created using both a HER2 high expressing cell line, AU565 cells, and a HER2 'negative' cell line, MDA-MB-468 cells. These cell types gave median fluorescence intensity (MFI) values (with IgG background control subtracted) of 1.0×10^6 and 4.2×10^3 for the BV421 labeled HER2 antibody, respectively, when measured using the iQue® Platform (Figure 10A and B).

The spheroids were incubated with anti-HER2 CAR-T cells (2:1 E:T) or non-transduced control T cells from the same donor in replicate plates which were analyzed using the iQue® Human T cell Killing Kit on Days 1, 4, and 7. There was a clear, temporal increase in death of the HER2-positive AU565 spheroids in the presence of the anti-HER2 CAR-Ts, up to 89 ± 0.4% by Day 7 (Figure 10C). This increase was not seen with the control T cells or with the HER2 negative MDA-MB-468 cells, which were only 23 ± 2% dead in the presence of the CAR-Ts on Day 7. This indicated that the anti-HER2 CAR-Ts had specifically killed the HER2 positive tumor cells, suggesting an interaction between the anti-HER2 CAR-T construct and the overexpressed HER2. This was supported by the activation marker expression (CD25) data, which saw a specific upregulation on the CAR-Ts across the time course in the presence of the AU565s, but much lower-level expression in the presence of the MDA-MB-468s (Figure 11D). Moreover, cytokines IFNγ and Granzyme B were only produced in the AU565 and anti-HER2 CAR-T co-culture (Figure 11E and F).

Anti-HER2 CAR-T killing of another HER2 'low' cell type, MDA-MD-231 cells (MFI 1.1×10^4) (data not shown) was also tested. In contrast to the HER2 'negative' MDA-MB-468s, very high levels of killing, activation marker expression, and cytokine release by the CAR-Ts were measured in the presence of this cell type, suggesting that very low HER2 expression on targets can initiate the killing response. This may explain some of the catastrophic effects that occurred when anti-HER2 CAR-Ts were tested in clinical trials.[13] Many body cells express low levels of HER2, which meant that 'on-target, off-tumor' events of the CAR-Ts resulted in one patient experiencing multiple organ failure, which ended the trial. Research is being carried out into how to develop clinically viable HER2 targeting CAR-Ts, for example by looking at reducing the affinity of the HER2 construct so it only affects HER2 overexpressing tumor cells.[14] The experiments in Figure 11 highlight how the specificity and activity of CAR-T cells can be tested *in vitro* using a 3D immune cell killing advanced flow cytometry workflow.

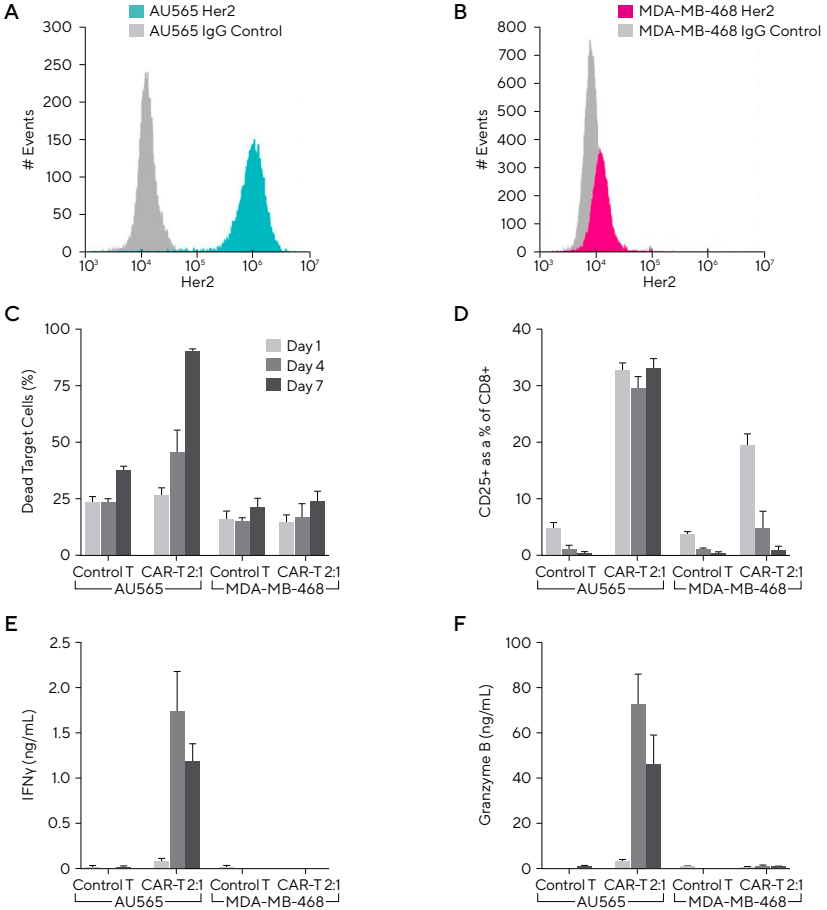

Figure 10

Targeted Activation and Killing of HER2-Positive Tumor Cells by Anti-HER2 CAR-T Cells

Note. (A) and (B) AU565 or MDA-MB-468 cells were incubated with BV421 labeled IgG control or HER2 antibodies and their fluorescence measured using the iQue® Platform. (C)-(F) Incucyte® Nuclight Green labeled HER2-positive AU565 or HER2-negative MDA-MB-468 spheroids were incubated with either anti-HER2 CAR-T cells or non-transduced T cells from the same donor at a 2:1 ratio. On Days 1, 4, and 7, 10 µL supernatant samples were taken for cytokine analysis before spheroids were dissociated and cells were analyzed using the iQue® T Cell Killing Kit.

Summary
The use of advanced cell therapies is rapidly developing, with increased focus on improving the efficiency of cell production for use in the clinic. Development is focusing on improving construct longevity, selectivity, manufacturing, and delivery to the patient. The potential switch to the use of allogeneic, off-the-shelf products offers a number of potential benefits for the clinic, such as reduced cost of manufacturing, improved long-term storage of cells, and increased consistency of larger batches.

The data examples shared in this chapter demonstrate how the use of advanced flow cytometry can add value when developing and characterizing T-cell therapies. These techniques have value at multiple stages in the development and expansion of cell products.

For further supporting information (videos and literature), see Appendix.

References

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76. DOI: 10.1016/j.cell.2006.07.024. Epub 2006 Aug 10. PMID: 16904174.
2. Liu G, David BT, Trawczynski M, Fessler RG. Advances in pluripotent stem cells: History, mechanisms, technologies, and applications. Stem Cell Rev Rep. 2020 Feb;16(1):3-32. DOI: 10.1007/s12015-019-09935-x. PMID: 31760627; PMCID: PMC6987053.
3. Gutbier S, Wanke F, Dahm N, et al. Large-scale production of human iPSC-derived macrophages for drug screening. Int J Mol Sci. 2020 Jul 7;21(13):4808. DOI:10.3390/ijms21134808. PMID: 32645954; PMCID:PMC7370446.
4. Karagiannis P, Kim SI. iPSC-Derived natural killer cells for cancer immunotherapy. Mol Cells. 2021 Aug 31;44(8):541-548. DOI: 10.14348/molcells.2021.0078. PMID: 34373366; PMCID: PMC8424143.
5. Silva AC, Matthys OB, Joy DA, et al. Co-emergence of cardiac and gut tissues promotes cardiomyocyte maturation within human iPSC-derived organoids. Cell Stem Cell. 2021 Dec 2;28(12):2137-2152.e6. DOI: 10.1016/j.stem.2021.11.007. PMID: 34861147.
6. Benito-Kwiecinski S, Lancaster MA. Brain organoids: Human neurodevelopment in a dish. Cold Spring Harb Perspect Biol. 2020 Aug 3;12(8):a035709. DOI: 10.1101/cshperspect.a035709. PMID: 31767649; PMCID:PMC7397826.
7. Song B, Cha Y, Ko S, et al. Human autologous iPSC-derived dopaminergic progenitors restore motor function in Parkinson's disease models. J Clin Invest. 2020 Feb 3;130(2):904-920. DOI: 10.1172/JCI130767. PMID:31714896; PMCID: PMC6994130.
8. Centeno EGZ, Cimarosti H, Bithell A. 2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling. Mol Neurodegener. 2018 May 22;13(1):27. DOI: 10.1186/s13024-018-0258-4. PMID: 29788997; PMCID: PMC5964712.
9. Doss MX, Sachinidis A. Current challenges of iPSC-based disease modeling and therapeutic implications. Cells. 2019 Apr 30;8(5):403. DOI: 10.3390/cells8050403. PMID: 31052294; PMCID:PMC6562607.
10. Hayashi Y, Ohnuma K, Furue MK. Pluripotent stem cell heterogeneity. Adv Exp Med Biol. 2019;1123:71-94. DOI: 10.1007/978-3-030-11096-3_6. PMID: 31016596.
11. Sullivan S, et al. Quality control guidelines for clinical-grade human induced pluripotent stem cell lines. Regenerative Medicine, 2018;13(7), pp.859–866.
12. Rao M, Malik N. Assessing iPSC reprogramming methods for their suitability in translational medicine. Journal of Cellular Biochemistry. 2012;113(10), pp.3061–3068.
13. Sanchez-Freire V, et al. Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells. Journal of the American College of Cardiology. 2014;64(5), pp.436–448.
14. Han D, Xu Z, Zhuang Y, Ye Z, Qian QJ. Current Progress in CAR-T Cell Therapy for Hematological Malignancies. Cancer. 2021;12(2):326-334.
15. Hardy IR, Schamel WW, Baeuerle PA, Getts DR, Hofmeister R. Implications of T cell receptor biology on the development of new T cell therapies for cancer. Immunotherapy. 2020 Jan;12(1):89-103.
16. Gomes-Silva D, Ramos CA. Cancer Immunotherapy Using CAR-T Cells: From the Research Bench to the Assembly Line. Biotechnol J. 2018 Feb;13(2):10.1002.
17. Chen Y, Yu Z, Tan X, et al. CAR-macrophage: A new immunotherapy candidate against solid tumors. Biomedicine and Pharmacotherapy. 2021;139:111605.
18. Wrona E, Borowiec M, Potemski P. CAR-NK Cells in the Treatment of Solid Tumors. Int. J. Mol. Sci. 2021;22(11):5899.
19. Gong Y, Klein Wolterink RGJ, Wang J, Bos G, Germeraad W. Chimeric antigen receptor natural killer (CAR-NK) cell design and engineering for cancer therapy. J Hematol Oncol. 2021;14(1):73.
20. Martinez EM, Klebanoff SD, Secrest S, et al. High-Throughput Flow Cytometric Method for the Simultaneous Measurement of CAR-T Cell Characterization and Cytotoxicity against Solid Tumor Cell Lines. SLAS Discov. 2018 Aug;23(7):603-612.
21. McLellan AD, Rad SMAH. Chimeric antigen receptor T cell persistence and memory cell formation. Immunol Cell Biol. 2019 Aug;97(7):664-674.
22. Gargett T, Yu W, Dotti G, et al. GD2-specific CAR T Cells Undergo Potent Activation and Deletion Following Antigen Encounter but can be Protected From Activation-induced Cell Death by PD-1 Blockade. Mol Ther. 2016 Jun;24(6):1135-1149.
23. Stock S, Schmitt M, Sellner L. Optimizing Manufacturing Protocols of Chimeric Antigen Receptor T Cells for Improved Anticancer Immunotherapy. Int J Mol Sci. 2019 Dec 10;20(24):6223.
24. Turtle CJ, Hanafi LA, Berger C, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J Clin Invest. 2016 Jun 1;126(6):2123-38.

Chapter 8

Appendix

Advanced Flow Cytometry Application Notes, Product Guides, and Other Resources

Chapter 1 Introduction

Videos:

Instrument Introduction
Flow Cytometry Solutions with iQue®

Introduction to iQue®
iQue®3 Faster, Smarter Flow Cytometry

Traditional *vs.* Advanced Flow Cytometry
Outlining the Speed of Advanced Flow Cytometry Workflows

Software: Panorama
Creating a Multiplate Analysis using the Panorama Feature of iQue Forecyt® Software

Software: 21 CFR Part 11
iQue® 21 CFR Part 11 Software Module

Software: EC_{50}/IC_{50}
iQue Forecyt® Software: EC_{50}/IC_{50} Curves and iQue® Human NK Cell Killing Kits

Chapter 2 Advanced Flow Cytometry Cell Health Assays

iQue® Human 4-Plex Apoptosis Kit Using Advanced High-Throughput Flow Cytometry Application Note

iQue® Apoptosis Kits Product Guide

iQue® Cell Proliferation and Encoding Dyes Product Guide

iQue® Cell Proliferation and Encoding Dyes (V/Blue) Dye Product Guide

iQue® Fixable Viability Kits Product Guide

iQue® Reagent Kits High-Throughput, Multiplexed Solutions for Faster Time to Actionable Answers Brochure

Membrane Integrity Kits Product Guide

Chapter 3 Advanced Flow Cytometry for Secreted Protein Quantification

iQue Qbeads® Devscreen SH Kits Product Guide

iQue Qbeads® Devscreen Streptavidin Kits Product Guide

iQue Qbeads® Plexscreen Secreted Protein Assay Kit Product Guide

iQue Qbeads® Human Inflammation Panel Kit Product Guide

Inflammation Panel
Use of Human iQue Qbeads® Inflammation Panel Kit with iQue Forecyt® Software

Chapter 4 Advanced Flow Cytometry Assays to Support Cell Line Development

Cell Line Development: High-Throughput Viable Cell Density Determination Using the iQue® Advanced Flow Cytometer Application Note

iQue® Mouse IgG Type and Titer Kit Product Guide

iQue® Human IgG Titer & Viability Kit Product Guide

iQue® Cell Count and Viability Kit Product Guide

Use of Advanced Flow Cytometry to Accelerate Antibody Screening and Characterization and Reveal Deeper Biological Insights Whitepaper

iQue® Advanced Flow Cytometry Platform Application Posters Poster Compendium

Cell Line Development
iQue® Advanced Flow Cytometry Platform for Efficient Cell Screening

Chapter 5 Advanced Flow Cytometry Assays for Antibody Discovery

Utilizing Advanced High Throughput Flow Cytometry to Quantify Direct and Competitive Live-Cell Antibody Binding Application Note

Advanced Flow Cytometry Applications for Antibody Small Molecule and Phenotypic Screening Publications Review

Evaluating NK Cell Killing with Advanced Flow Cytometry Application Note

Evaluation of Complement-Dependent Cytotoxicity (CDC) using a Streamlined Miniturized Advanced Flow Cytometry Assay Application Note

Cross Platform Analysis of the Binding and Function of anti-HER2 Antibody-Drug Conjugates (ADCs) Application Note

Quantification of Antibody-Dependent Cellular Phagocytosis via a Streamlined Advanced Flow Cytometry Workflow Application Note

Antibody Internalization: Advanced Flow Cytometry and Live-Cell Analysis Give Rich Insights During Antibody Profiling Whitepaper

High Throughput, Multi-Parametric Analysis Accelerates Antibody Discovery Workflows Whitepaper

iQue® Human NK Cell Killing Kit Product Guide

iQue® Human NK Cell Companion Kits Product Guide

iQue® Antibody Internalization Reagents Product Guide

iQue® Human Antibody Dependent Cellular Phagocytosis (ADCP) Kit Product Guide

Poster: Antibody Internalization
A High-Throughput Multiplex Antibody Internalization Asssay

Chapter 6 Advanced Flow Cytometry Assays for Immune Cell Profiling

Understanding T Cell Phenotype and Function to Enable Improved Therapeutics Whitepaper

iQue® Human T Cell Activation Kit Protocol Optimization for Automated Liquid Handling Systems Technical Note

Combining Live-Cell Analysis and High-Throughput Flow Cytometry to Gain Additional Insights into the Mechanisms of Immune Cell Killing of Tumor Cells Application Note

Quantitative Analysis of CAR-T Cell Exhaustion Using Advanced Flow Cytometry Application Note

An Optimized, Multiplexed Assay for Screening *Ex Vivo* Conditions which Increase Memory T Cell Frequency Application Note

Quantifying T Cell Response in 3D Tumor Spheroids Using Advanced Flow Cytometry Workflows Application Note

Utilizing Mixed Lymphocyte Reaction (MLR) to Evaluate Checkpoint Inhibitor Therapies by Advanced Flow Cytometry Application Note

iQue® Human T Cell Activation Kit Product Guide

iQue® Human T Cell Exhaustion Kit Product Guide

iQue® Human T Cell Memory Kit Product Guide

iQue® Human T Cell Killing Kit Product Guide

iQue® Human T Cell Companion Kits Product Guide

iQue® Human General Immune Cell Killing Kit Product Guide

Phenotype and Function Kits
Multiplexing Phenoptype and Function for More Biologically Relevant Insights

T Cell Activation
iQue Forecyt® Software and T-Cell Activation Workflow

3D Models
T Cell Characterization in 3D Cell Models using Advanced Flow Cytometry

Poster: Natural Killer Cells (A)
Donor and Antibody Diversity in NK Cell-Mediated Antibody Dependent Cellular Cytotoxicity (ADCC), Detected Using an Optimized Multiplexed Assay and Advanced Flow Cytometry

Poster: Natural Killer Cells (B)
Assessment of NK Cell-Mediated Killing and Phenotypic Analysis Using Advanced Flow Cytometry and an Optimized Multiplexed Assay

Chapter 7 Advanced Flow Cytometry Assays to Support Cell Therapy

Intracellular Staining Assay for iQue® Platform Technical Note

Quantifying T Cell Response in 3D Tumor Spheroids Using Advanced Flow Cytometry Workflows Application Note

Characterization and Optimization of Induced Pluripotent Stem Cell Culture Using Advanced Flow Cytometry and Live-Cell Analysis Application Note

Phenotypic and Functional Characterization of CAR-T Cells with Advanced Flow Cytometry and Live-Cell Analysis Whitepaper

Utilizing Advanced Flow Cytometry and Live-Cell Imaging to Evaluate iPSC Pluripotency During Cell Line Selection and Differentiation Procedures Application Note

Top 5 Reasons to Choose Advanced Flow Cytometry:

1. **Throughput:** simple, scalable, multi-user environment features walkaway automation, comprehensive analysis and visualization tools that are not available in traditional flow cytometers
2. **Insight:** walk-up-and-use operation eliminates the need for time-consuming setup and so called “power users”; anyone can run an experiment and analyze their data in real time
3. **Multiplexing:** the ability to combine both cells and beads in the same assay allows for studying biologically relevant secreted proteins and cytokines at once
4. **Miniaturization:** sample volumes reduced to as little as a 1 µL conserve precious samples and reagents, bringing down overall costs
5. **Compliance:** iQue Forecyt® software supports 21 CFR Part 11 workflows with traceable electronic recordkeeping, export-ready audit reports, electronic signatures, and more

Built to Empower, Not Intimidate

Find out more
www.sartorius.com

Flow Cytometry by Sartorius

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iQue® Advanced Flow Cytometry Resources

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