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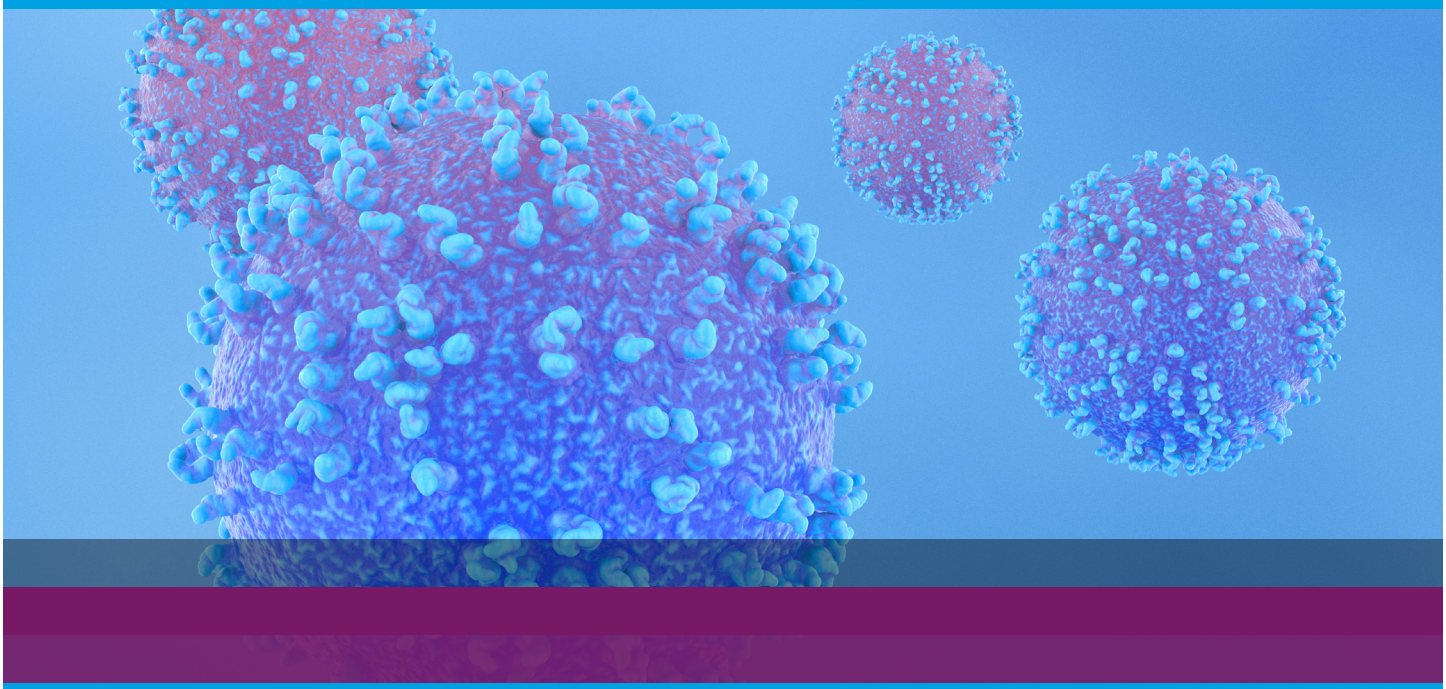
Trusted Answers

Robust Assays and Workflows To Meet the Demands of Cell & Gene Therapy Discovery, Process Development and Manufacturing

Agilent Technologies
Cell Analysis Division



Contents

Introduction

It has been over a decade since the approval of the first immune checkpoint inhibitor (ipilimumab) by the US Food and Drug Administration (FDA). This therapeutic antibody harnesses the intrinsic and complex ability of the immune system to fight cancer and began the modern age of immuno-oncology. Since then, the immuno-oncology field has witnessed a profound expansion of other immunotherapy modalities. These include engineered antibodies, such as bi-specific T-cell engagers (BiTEs), oncolytic viruses and adoptive cell therapies. Often, these therapies have changed the paradigm of cancer treatment and have quickly become the standard of care for the treatment of different types of malignancies.[1]

Cell therapy

Autologous cell therapies, including cells engineered to express chimeric antigen receptors (CARs) or engineered T-cell receptors (TCRs), represent the fastest-growing immuno-oncology (IO) sector and are at the forefront of the next generation of IO approaches. This growth has been fueled by the first CAR T-cell products targeting the B-lymphocyte antigen CD19 and the B-cell maturation antigen (BCMA). These cell therapy products have induced durable clinical responses in otherwise refractory B cell malignancies, leading to the FDA approval of seven different therapies within the last seven years (Table 1). According to the latest estimate, over 1,000 clinical programs are running globally.

Table 1. List of FDA-approved CAR T-cell therapies.[2]

Product	Trade name	Manufacturer	Year approved	Target	Indication
Idecabtagene vicleucel	ABECMA	Celgene (BMS)	2021	BCMA	r/r multiple myeloma
Lisocabtagene maraleucel	BREYANZI	Juno (BMS)	2021	CD19	Large B-cell lymphoma
Ciltacabtagene autoleucel	CARVYKTI	Janssen Biotech	2022	BCMA	r/r multiple myeloma
Tisagenlecleucel	KYMRIAH	Novartis	2017	CD19	r/r follicular lymphoma
Brexucabtagene autoleucel	TECARTUS	Kite Pharma	2020	CD19	r/r mantle cell lymphoma
Axicabtagene ciloleucel	YESCARTA	Kite Pharma	2017	CD19	Large B-cell lymphoma
Obecabtagene autoleucel	AUCATZYL	Autolus	2024	CD19	r/r B-cell precursor acute lymphoblastic leukemia

r/r: relapsed/refractory

Solid tumors represent approximately 90% of adult human cancers.[3] However, despite the impressive clinical results observed in hematological malignancies, progress for CAR T-cell therapy in solid tumors has met many obstacles. One such obstacle is the lack of solid tumor-specific cell-surface antigens. Solid tumors are heterogeneous and many of the target antigens are expressed in healthy tissue, leading to the high incidence of "on-target/off-tumor" toxicities. Moreover, the complex solid tumor microenvironment (TME) negatively affects both endogenous T-cells and transferred CAR T-cells. The TME is composed of soluble factors and suppressive cells – such as myeloid-derived suppressor cells (MDSCs) and Tregs – and contributes to the challenges for successful CAR T development for solid tumors.

CAR T-cell development and manufacturing

The development and manufacturing of safe and efficacious CAR T-cell therapies rests on four important pillars:

- The type and quality of the starting material
- The genetic engineering strategy for CAR design
- The expansion of engineered immune cells ex vivo
- The quality control (QC) and release criteria for manufactured products

While each of these important pillars has unique requirements and nuances, they all require the implementation of the appropriate analytical tools (Table 2).

Table 2. Critical steps in CAR T development and manufacturing.

Step	Important considerations
Starting cellular material	Understanding and defining the composition of the starting material is important to reduce variability and develop efficient CAR T-cell therapies. Cellular analysis tools, such as flow cytometry, are essential for providing incisive, population-based information regarding the identity and quality of the starting materials.
Cellular engineering strategy	Genetic engineering strategies for the design and delivery of CARs, as well as gene editing approaches (e.g., CRISPR) affect the potency, persistence and fitness of the immune cells. Using the appropriate analytical tools to guide decision making and selection of the optimal CAR for the disease of interest is paramount for CAR T development.
Ex vivo cell expansion	Design and selection of the optimal media conditions for *ex vivo* expansion of CAR T-cells are crucial during the process development and manufacturing of patient cells. Assessment of cell phenotype, cytokines and cell potency is critical to ensure safe and efficacious products.
QC release criteria	To guarantee the safety and efficacy of the cell therapy product, it is essential to measure its quality following strict safety, potency, purity and identity criteria.

Based on FDA guidelines for early drug development of CAR T-cells, few specifications are defined, and some tests may continue to be developed. Comprehensive product characterization studies are therefore valuable for identifying the right critical quality attributes (CQAs) that need to be assessed during manufacture and lot release. CQAs are defined as physical, chemical, biological or microbiological characteristics that should be within an appropriate limit, range or distribution to ensure the desired product quality. The precise nature of CQAs needs to be defined for each specific product. Table 3 shows CQAs generally considered for CAR T-cells.

Table 3. Measures of CQAs for CAR T-cell products.

CQA	Measure	Method of measurement
Identity	CAR expression	Flow cytometry or PCR
Safety	Sterility	Bacterial and fungal culture, Gram stain
	Mycoplasma	PCR or culture
	RCL/RCR	PCR or culture
	Vector copy number	PCR
	Bead content	Microscopy
Purity	T-cell content	Flow cytometry
	CAR T-cell content	Flow cytometry
	Contaminating cell content	Cell count flow cytometry
Potency	CAR T-cell content	Flow cytometry or PCR
	In vitro cytotoxicity	Cytotoxicity assays
	Cytokine secretion	Cytokine release assays
Durability	CAR T-cell persistence	Flow cytometry or PCR Metabolic profiling assays
	In vivo CAR T-cell expansion	Flow cytometry or PCR
Consistency	Measures include all CQAs but applied across different many CAR T-cell products	

PCR: polymerase chain reaction; RCL/RCR: replication competent lentivirus/replication competent retrovirus.

The FDA and other regulatory agencies recognize the inherent challenges in developing and manufacturing complex products, especially for autologous therapy, due to the limited availability of suitable animal models to test safety and efficacy.[4] However, preclinical testing of CAR T-cells is necessary before starting a clinical investigation. Critical process parameters (CPPs) should be established during process development to ensure the safety and efficacy of cell therapy products, with a particular emphasis on understanding and mitigating the inherent variability associated with starting materials and manufacturing processes.

Characterization assays

Current recommendations for CAR T-cell product development include the implementation of a matrix of characterization assays as early as possible during the discovery stage to obtain information about CQAs. These assays can be further refined, qualified and validated to meet the strict criteria during process development and ultimately as QC release assays (Figure 1).

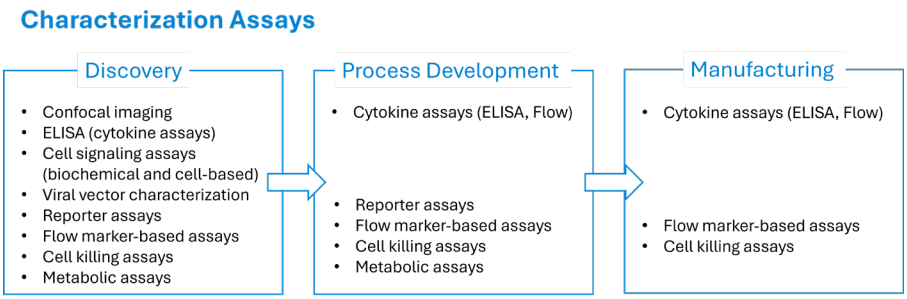
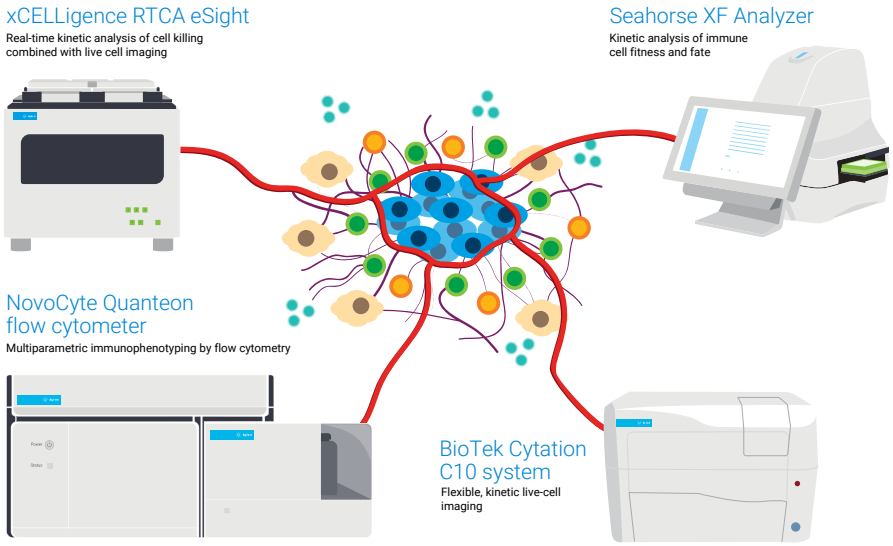


Figure 1. Cell-based characterization assays commonly used during the different stages of CAR T-cell product development.

Agilent Technologies has a comprehensive portfolio of innovative analytical, automation and reagent solutions to address the needs at each stage of cell therapy development (Figure 2). These analytical platforms will be discussed in depth, with a focus on the key assays and CQAs that can be measured.

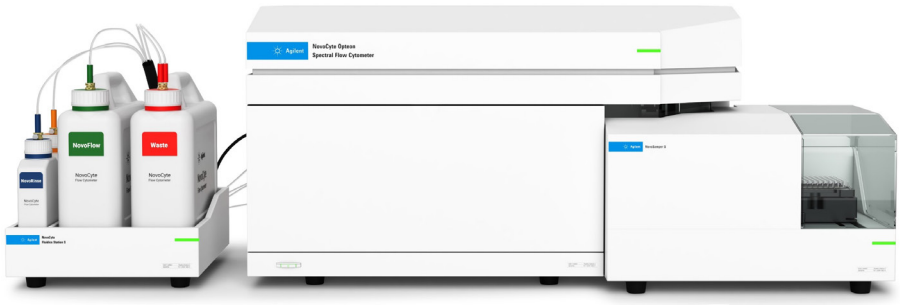


Instrument	Accessories	Critical parameters
NovoCyte flow cytometers	Autosampler, automation	Activation/exhaustion, proliferation, immunophenotyping, identity, potency
Seahorse XF analyzers	Assay plates, T-cell activation and metabolic profiling kits	Activation, metabolic profiling, persistence, metabolic fitness, ATP production
xCELLigence RTCA instruments	Biosensor E-Plates, immunotherapy kit, immunotherapy software module	Proliferation, potency (immune cell killing)
Cytation imaging plate readers	Automation	Activation, proliferation, cytotoxicity, cytokine profiling

Figure 2. Agilent's solutions for cell therapy analysis.

Analytical tools for CAR T-cell discovery, development and manufacturing

NovoCyte flow cytometers



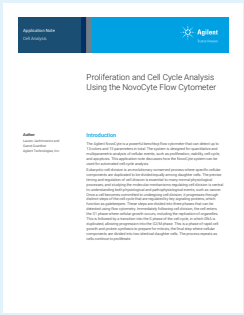
Flow cytometry, a proven technology to measure multiple single-cell parameters in a high-throughput manner, plays a crucial role in the development of cell and gene therapy in these key areas:

- Identification and characterization of immune cell subpopulation
- Evaluation of immune cell activation/expansion/exhaustion
- Detection of CAR at proteomic level
- Measurement of cytokine release
- In-process and quality control of CAR T-cell products
- Post therapeutic monitoring

However, the development of multi-parameter flow cytometry panels to assess the increasing diversity of engineered immune cells can be challenging. The NovoCyte flow cytometer series (Opteon, Penteon, Quanteon, Advanteon and the NovoCyte) provides an expanded set of capabilities that accommodate today's increasingly sophisticated multicolor flow cytometry assays for cell and gene therapeutic development. They provide users with the flexibility to choose from up to five lasers with up to 73 independent detectors. The newest NovoCyte Opteon spectral flow cytometer overcomes analytical limitations of conventional flow cytometry and the cost limitations of mass cytometry. All NovoCyte flow cytometers further benefit from hardware and software upgrades. The NovoSampler S/Q/Pro are automatic sample loading systems, which can be integrated into different laboratory automation platforms. The accompanying NovoExpress software also enables users to meet regulatory requirements defined in FDA 21 CFR Part 11 and Annex 11.

In-depth characterization of cell and gene therapies

NovoCyte flow cytometers can be used for a range of cell characterization assays. These assays provide researchers with valuable insights into the characteristics of their starting materials and final products, which can be informative to the development of more effective processes.



Application note:

Proliferation and Cell Cycle Analysis Using the NovoCyte Flow Cytometer

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T-cell proliferation assay

Flow cytometry is ideal for measuring T-cell proliferation and studying unique cell types within a heterogeneous population.[5] Different fluorescent dyes that incorporate into the cell membranes and intracellular structures of living cells (e.g., CFSE, PKH_{26}) can be used. As these dyes are evenly diluted into daughter cells with subsequent cell divisions, a decrease in fluorescence can be measured as distinct peaks. Each peak represents a successive generation of cell division (Figure 3). Advances in cytometer technology and integrated software have improved data collection and decreased hands-on time, but more importantly, enabled better quality and more in-depth information to be gathered for each sample.

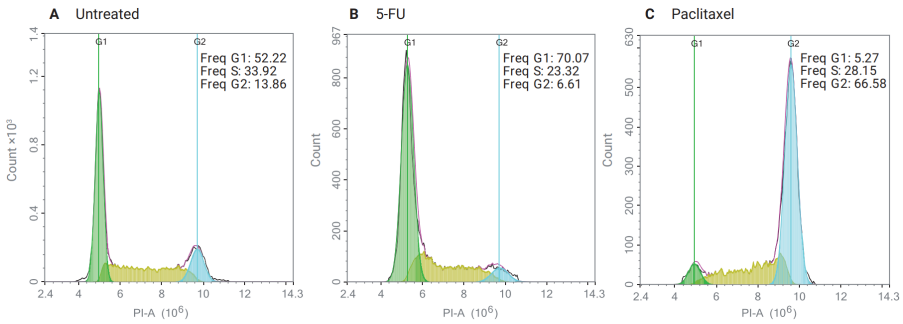
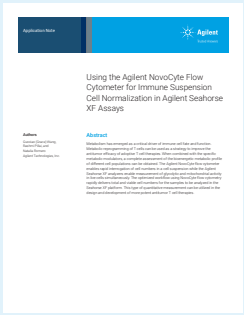


Figure 3. Representative cell cycle analysis in response to various treatments.



Application note:

Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Cell Normalization in Agilent Seahorse

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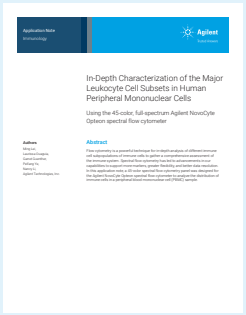
Absolute cell counting

Absolute cell counting can enhance cell proliferation analysis by obtaining the total increase in cell number during an experiment, which better determines how well cells are growing. Using an instrument with cell counting capabilities offers data about the total number of cells, in addition to the number of divisions. While many cytometers cannot determine the absolute cell count without using reference beads, direct cell count is available using all NovoCyte flow cytometers, as they are equipped with a syringe-driven pump for sampling. This system can conveniently and accurately determine the absolute cell count for every run without calibration beads.



Application note:

In-Depth Characterization of the Major Leukocyte Cell Subsets in Human Peripheral Mononuclear Cells

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Application note:

Sentinel Panel Design of 16-Color, 28-Markers for Immunophenotyping Peripheral Human Whole Blood

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Immunophenotyping

Immune status is associated with disease state, treatment efficacy and response to external stimuli. Immunophenotyping quickly identifies candidate cell types, subclasses and functions of specific cell subsets such as monocytes, NK cells, T-cells and B cells. Monitoring the frequency of these immune cell populations – as well as the differentiation, activation or exhaustion status – is essential to understanding therapeutic efficacy. The NovoCyte flow cytometer enables simultaneous quantification of multiple immune cell markers for better understanding of immune cell status and surveillance of the immune response (Figure 4).

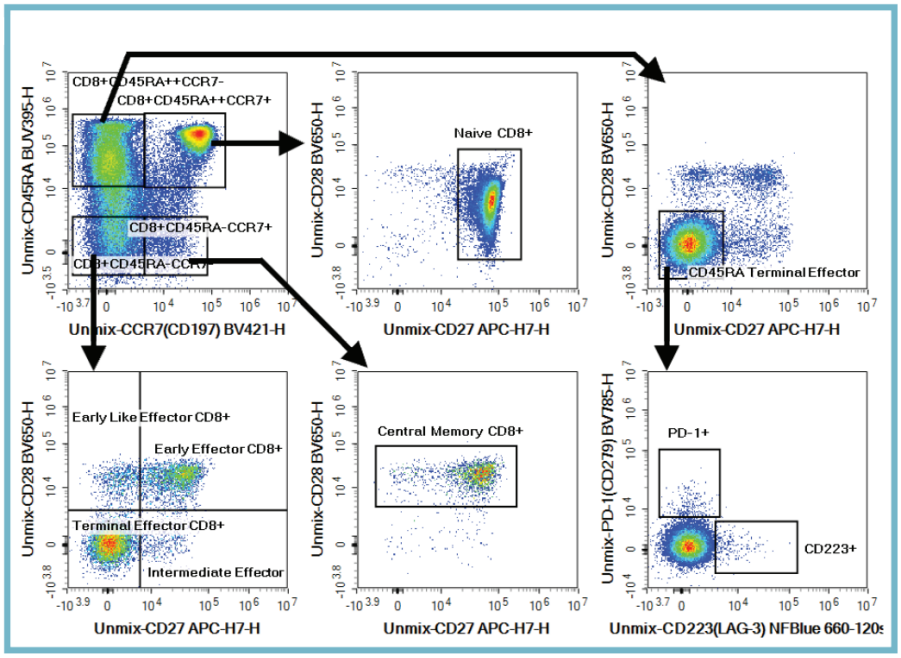
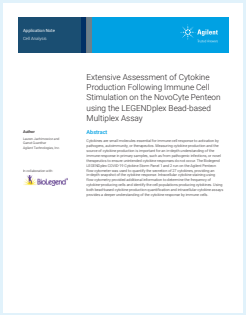


Figure 4. A representative CD8 T-cells from PBMCs, other immune cell subsets not shown.



Application note:

Extensive Assessment of Cytokine Production Following Immune Cell Stimulation on the NovoCyte Penteon using the LegendPLEX Bead-based Multiplex Assay

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Cytokine release measurement

Cytokines are small molecules essential for immune cell response to activation by pathogens, autoimmunity or therapeutics. Signaling by cytokines can modulate gene regulation, inflammation and innate and adaptive immune response. Measuring cytokine production and identifying the source of cytokine production are therefore important for an in-depth understanding of the immune response. Bead-based flow cytometric detection of cytokines is a highly effective method for measuring multiple soluble analytes in a single sample, using a mixture of bead populations with varying fluorescence intensities (Figure 5).

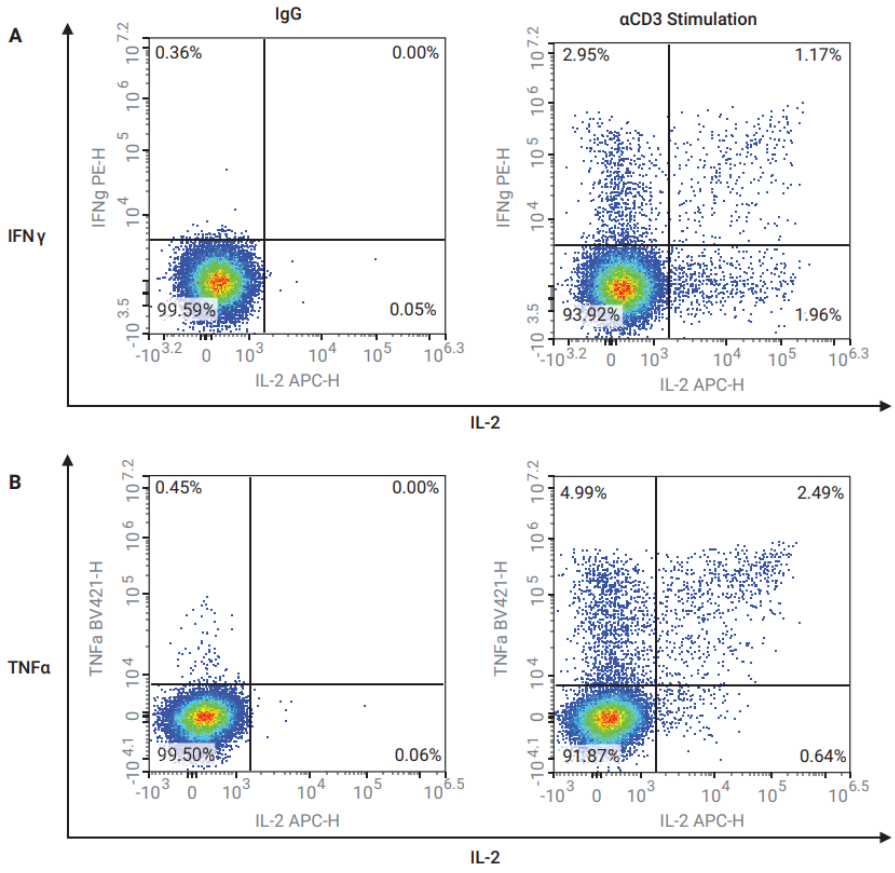
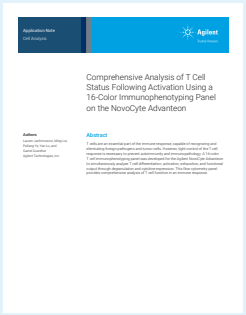


Figure 5. Cytokine production after αCD3 antibody activation.



Application note:

Comprehensive Analysis of T-cell Status Following Activation Using a 16-Color Immunophenotyping Panel

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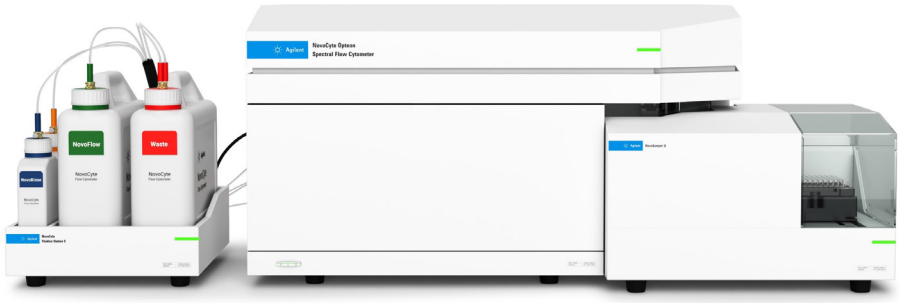
T-cell activation and exhaustion

In a normal immune response, T-cells are activated to rapidly divide and destroy all infected or cancerous cells. Activation through the T-cell receptor (TCR) and costimulatory receptors must be balanced with inhibitory receptors. This is needed to prevent the development of autoimmunity and immunopathology. When stimulation of T-cells is sustained, there is a progressive increase in the expression of inhibitory receptors and a decrease in T-cell effector function and proliferative capacity (T-cell exhaustion). This can be seen during a chronic infection or cancer. Inhibitory receptors have become a focus of cancer immunotherapy research because reversing inhibitory receptor expression through checkpoint inhibitor blockade has restored T-cell function and improved tumor immunity in the clinic. Measuring markers for T-cell differentiation, activation, previous antigen exposure, degranulation and cytokine production through the NovoCyte flow cytometer provides a more comprehensive understanding.

Find selected publications on this topic in the appendix.

Instrument models



The **NovoCyte Opteon Spectral Flow Cytometer** is a cutting-edge spectral flow cytometry solution designed to revolutionize your cell analysis research. The NovoCyte Opteon boasts up to 5 lasers and 73 detectors, a proprietary optics design and advanced electronics, enabling it to deliver data with high resolution and sensitivity. The wide dynamic range for both fluorescence detection and particle size measurement helps streamline experimental workflows. On-board temperature control, electronics and fluidics sensor circuitry provide real-time instrument status monitoring and ensure consistent, reliable data acquisition in different ambient environments.

The **NovoCyte Penteon Flow Cytometer** provides an expanded set of capabilities that accommodate high-end and increasingly sophisticated multi-color flow cytometric assays. Users now have the flexibility to choose from up to 30 fluorescence channels utilizing up to 5 lasers with up to 30 independent detectors.

The **NovoCyte Quanteon Flow Cytometer** is a 4 laser flow cytometer that can be configured with up to 25 independent photomultipliers for meeting the most demanding sample panels. The proprietary photomultiplier technology employed in the Quanteon provides excellent sensitivity, stability and a 7.2 log dynamic range. Very dim signals and very bright signals can all be captured and gated in the same view, therefore eliminating the need for laborious trial and error PMT tuning procedures.

The **NovoCyte Advanteon Flow Cytometer** provides an advanced set of capabilities while remarkably simple to operate. It can accommodate high-end and increasingly sophisticated multicolor flow cytometry assays. The system offers flexibility with 1, 2 or 3 laser options, up to 21 fluorescence channels and 23 independent detectors.

The **NovoCyte Flow Cytometer** is a high-performance benchtop flow cytometer designed for all levels of users and all types of laboratories. This budget-friendly instrument can detect up to 17 parameters with enhanced sensitivity and resolution. The customizable laser and optical configurations of the NovoCyte offer a high degree of flexibility while providing complex cell analysis capabilities.

Seahorse XF analyzer

It is now widely known that immune cells undergo major changes of energy metabolism during activation. Such changes determine cells' ability to proliferate, differentiate and carry out effector functions. During cell therapeutic development, various genetic engineering approaches are used to increase the activation, persistence, homing, efficacy and safety of immune cell therapies. Furthermore, metabolic poise is a major determinant of immune cell function and persistence that are intrinsically linked to anti-tumor efficacy, especially within the immunosuppressive TME. The analysis and optimization of key metabolic and bioenergetic parameters of immune cells are of critical importance, both for understanding the biology of the anti-tumor immune response and for therapeutic design.

Designed to simultaneously interrogate glycolytic and mitochondrial function in live cells, Agilent Seahorse XF technology is the leading platform used to study immune cell metabolism and is a key contributor to our current understanding

of immunometabolism and the recognition of the fundamental role of metabolic reprograming on fate and functions. Together with customized kits and assays, the Seahorse XF analyzer provides sensitive, real-time functional measurements that can quantitatively and objectively measure the metabolic activity of immune cells, thereby providing incisive information about critical quality attributes of immune cells throughout therapeutic design and manufacturing. In addition, the Seahorse XF provides insight for optimizing immune cell selection, to select optimal metabolic and design engineered immune cells with optimal tumor killing function. It can also be used for optimizing expansion conditions and immune cell components for cell therapy manufacturing.

How Seahorse XF technology works

The two major pathways to produce energy – mitochondrial respiration and glycolysis – involve cellular consumption of oxygen and efflux of protons, respectively. Seahorse XF technology uses solid-state sensors to detect extracellular changes in these analytes to measure rates of cellular respiration, glycolysis and ATP production (Figure 6). Cells are seeded in the assay wells of XF microplate at a confluency of 50–90%. Suspension cells are attached to the well bottom to maximize sensitivity. The instrument lowers the cartridge probes into the assay wells. The sensors are positioned 200 microns above the well bottoms, forming transient microchambers. As the oxygen and pH levels change, the changes in the sensors are read by the instrument. Measurements are typically made for 3 minutes and rates are calculated automatically. Upon completion of this measurement period the probes are raised, allowing the extracellular medium to come back to baseline conditions. The sensor cartridge also contains ports (4 per well) to enable injection of modulators into the cell wells during the assay. When specified by the instrument protocol, the system injects compound "A" into the assay wells and performs a gentle mixing step to ensure distribution of the compound throughout the assay medium. All wells are processed in this manner simultaneously. Subsequent measurement cycles, any additional injections specified by the protocol and rate calculations are performed automatically.

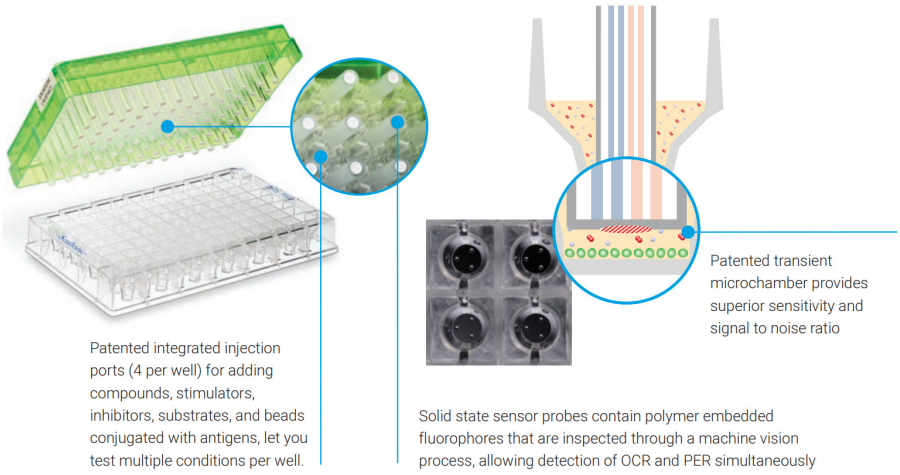
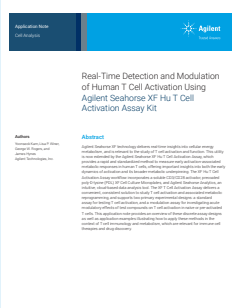


Figure 6. Seahorse smart plastic technology at a glance.

In-depth characterization of cell and gene therapies

The Seahorse XF analyzer works with several kits tailored for CAR T therapy discovery and development, enabling evaluation of CAR construct design, starting material selection, metabolic conditioning during *in vitro* cell expansion and the capacity of T-cells and NK cells to maintain metabolic fitness in restrictive microenvironments.



Application note:

Real-Time Detection and Modulation of Human T-Cell Activation Using Agilent Seahorse XF Hu T-Cell Activation Assay Kit

Download Here

Detection and modulation of human T-cell activation

The Seahorse XF Hu T-cell activation assay provides a rapid and standardized method to measure early activation-associated metabolic responses in human T-cells, offering important insights into both the early dynamics of activation and its broader metabolic underpinning. This assay delivers a convenient, consistent solution to study T-cell activation and associated metabolic reprogramming (Figure 7). It supports two primary experimental designs: a standard assay for testing T-cell activation, and a modulation assay for investigating acute modulatory effects of test compounds on T-cell activation in naïve or pre-activated T-cells.

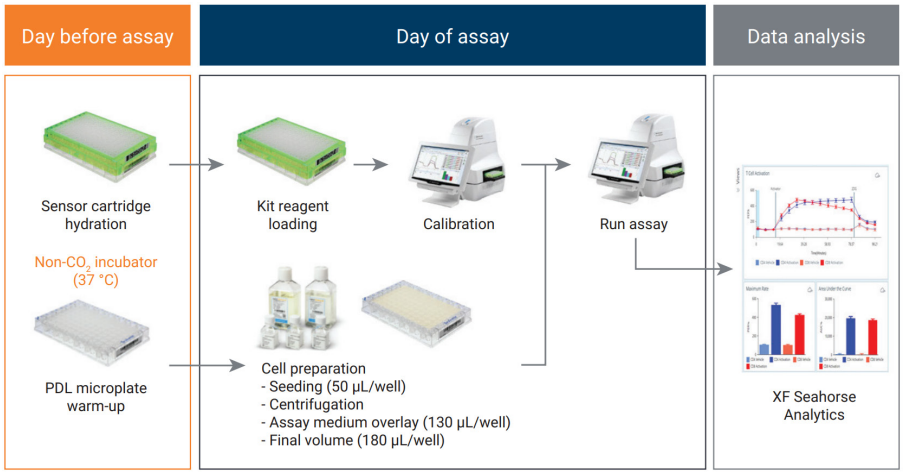
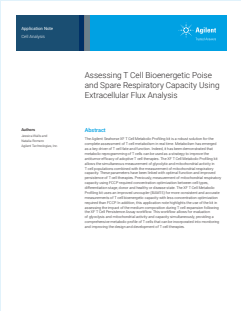


Figure 7. Summary of the Seahorse XF Hu T-cell activation assay workflow.



Application note:

Assessing T Cell Bioenergetic Poise and Spare Respiratory Capacity Using Extracellular Flux Analysis

Download Here

Assess T-cell bioenergetic poise and spare respiratory capacity

Metabolism has emerged as a key driver of T-cell fate and function. It has been demonstrated that metabolic reprogramming of T-cells can be used as a strategy to improve the antitumor efficacy of adoptive T-cell therapies. The Seahorse XF T-cell metabolic profiling kit allows the simultaneous measurement of glycolytic and mitochondrial activity in T-cell populations combined with the measurement of mitochondrial respiratory capacity. These parameters have been linked with optimal function and improved persistence of T-cell therapies (Figure 8).

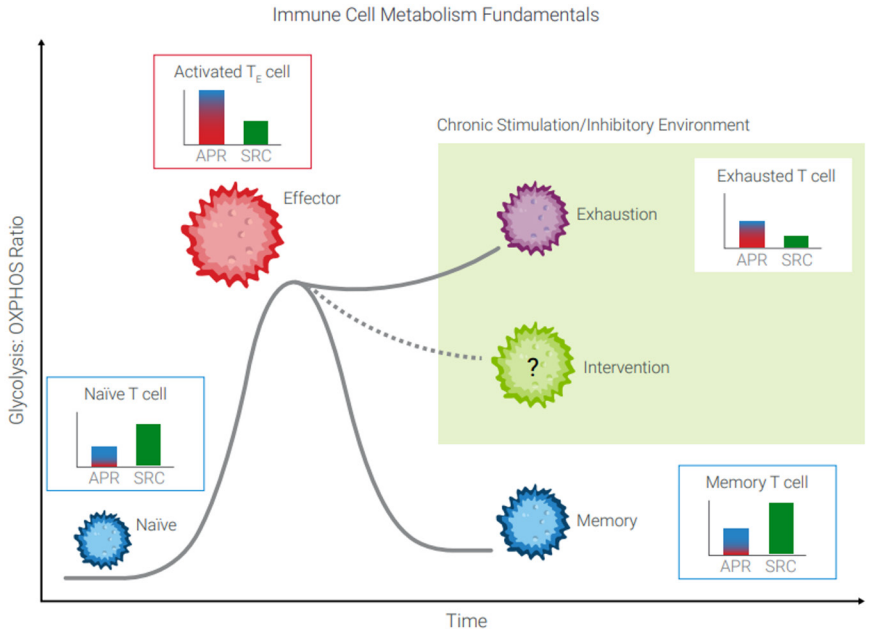


Figure 8. T-cell metabolic fundamentals, illustrating the evolution of metabolic phenotypes through the T-cell activation and proliferation cycle (gray lines), and their linkage to T-cell fate, fitness and function.

Application note:

Assessing NK Cell Bioenergetic Metabolism Using Extracellular Flux Analysis

Download Here

Assess NK cell bioenergetic metabolism

Modulating NK cell metabolism is a potential strategy to improve the efficacy of NK cell-based immunotherapy for cancer. The Seahorse XF NK cell metabolic profiling assay provides a complete assessment of NK cell metabolism in real time using the Seahorse XF analyzer and the Seahorse XF T-cell metabolic profiling kit (Figure 9). It allows for the simultaneous measurement of glycolytic activity, mitochondrial activity and bioenergetic capacity in NK cell populations. The Seahorse XF T-cell metabolic profiling kit uses an improved uncoupler (BAM15) for more consistent and accurate measurements of NK cell mitochondrial bioenergetic capacity, with minimal reagent optimization.

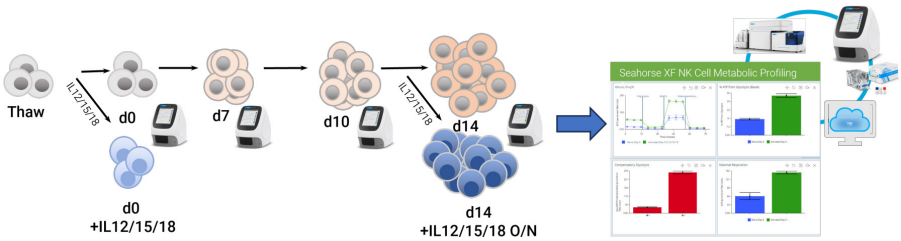


Figure 9. Experimental design to evaluate human peripheral blood NK cells during expansion.

Find selected publications on this topic in the appendix.

Instrument models

All Seahorse analyzers measure and report the oxygen consumption rate (OCR), proton efflux rate (PER) or extracellular acidification rate (ECAR), as well as quantify mitochondrial respiration, glycolysis and ATP production rates. With the ability to perform up to four independent injections per well with automatic mixing, all Seahorse analyzers perform label-free detection in live cells, in real time, with the sensitivity for small sample sizes.

The **Seahorse XF Pro Analyzer** (96-well format) is best for phenotypic screening, testing many conditions at once, and dose-response studies. It is also suitable for spheroids.

The **Seahorse XFe24 Analyzer** (24-well plate format) allows for automation and is tested for hypoxia. This instrument is best for islets and small model organisms, and it's also suitable for large study models (e.g., adipose).

The **Seahorse XF HS Mini Analyzer** (8-well miniplate format) is tested for hypoxia and is best for analyzing quantity-limited primary cells, low-respiring cell types and T-cell subsets. It is also suitable for use with limited biomaterials.

Related kits

The Seahorse XF T-cell Metabolic Profiling Kit allows for robust and quantitative measurements of both glycolytic and mitochondrial activities in T and NK cell populations, providing a complete picture of cell energy metabolism. The kit features improved reagents with minimal off-target effects and a streamlined assay workflow, reducing assay preparation time and minimizing the need for uncoupler optimization. The XF T-cell metabolic profiling kit is also integrated with Agilent Wave Pro and Agilent Seahorse Analytics software to simplify data analysis, visualization and interpretation.

The **Seahorse XF Hu T-Cell Activation Assay Kit** measures human (Hu) T-cell activation response within several minutes of stimulation using Seahorse XF analyzers. This is accomplished via measuring proton efflux rates (PER) associated with glycolytic pathway-dependent energy production and is built on the principle that T-cell activation is accompanied by a rapid switch in cellular energy production and intermediate demand to support effective proliferation, size expansion and differentiation.

BioTek cell imaging and microscopy solutions

Imaging approaches are essential in cell and gene therapy workflows, as they provide detailed insights into cellular processes within relevant model systems, enabling precise monitoring and analysis. These techniques facilitate the visualization of cellular behavior, gene expression and therapeutic efficacy in real time, ensuring accurate assessment and optimization of therapies.

The Agilent BioTek Cytation cell imaging multimode reader and Lionheart automated microscope provide flexible platforms that support a broad range of cell and gene therapy applications. These automated imaging systems enable quantification across different microplate formats and assay parameters. The onboard wide field-of-view camera sensor captures the entire well area of a 384-well microplate within a single image – supporting robust analysis for high-throughput studies. Also, the ability to capture multiple images per well provides whole-well imaging of lower-density microplates using the montage stitching feature, or strategic sampling of defined subregions of the culture area.

Flexible kinetic imaging protocols allow user-defined intervals and duration for generating immune cell activation and proliferation profiles over time. Brightfield and phase contrast modes support long-term live-cell analysis utilizing label-free approaches. Fluorescence-based imaging studies are supported by a full range of available fluorescence channels and both widefield and confocal imaging modes, enabling detailed characterization of immuno-oncology models. Onboard environmental controls maintain optimal conditions for live cell studies, while the incorporation of the BioSpa live-cell analysis system expands assay throughput to up to eight microplates in parallel.

Automated imaging solutions for measuring T-cell activation and proliferation

Cellular activation is a crucial initial step of a targeted immune response. Direct and indirect activation of T-cells *in vitro* causes increased cell proliferation and formation of cell clusters. This can be tracked and quantified by label-free and fluorescence assays using Agilent BioTek Lionheart FX and Cytation live-cell imaging systems maintaining cells under optimal conditions (37 °C and 5% CO_2) to ensure accurate and reproducible results. The system's capability to perform both brightfield and fluorescent imaging, along with powerful quantitative analysis tools, enables detailed monitoring of T-cell activation and proliferation.

Agilent BioTek Gen5 software for imaging and microscopy readily identifies activation-induced formation and expansion of T-cell clusters from acquired images (Figure 10A). Key metrics for immune cell activation – including cell cluster confluence percentage, cluster count and area – are automatically calculated and reported throughout the time course (Figure 10B and C). Captured high-resolution images and generated movies provide a detailed record of cell morphology for deeper evaluation.

This method is particularly beneficial for studying the effects of various stimulants, such as IL-2 and CD3/CD28, on T-cell behavior. Additionally, the precise monitoring of CAR T-cells and their response to specific antigens like EpCAM provides valuable data for immunotherapy research. Overall, the combination of flexible imaging capabilities, environmental controls and robust data analysis tools makes this approach highly effective for studying T-cell dynamics and advancing immunotherapy research.

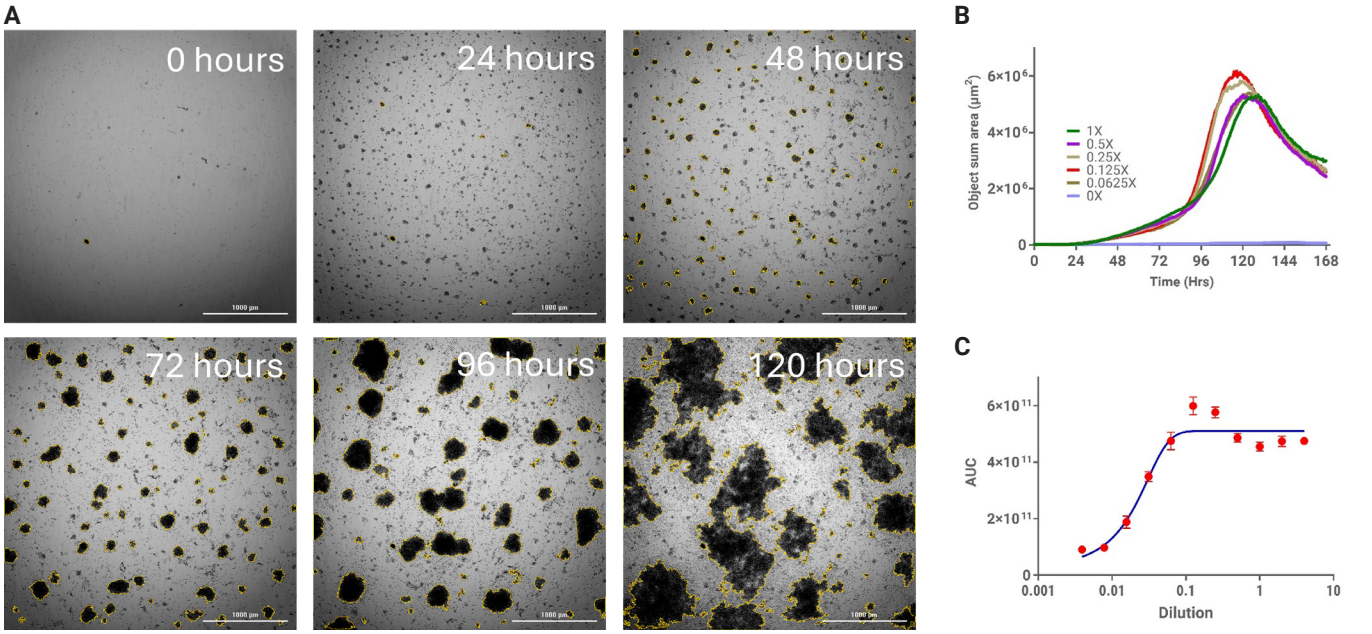


Figure 10. Quantification of T-cell clustering using automated label-free imaging and analysis. Naïve T-cell lymphocytes were incubated with a CD3/CD28 stimulation cocktail at a range of concentrations to identify optimal conditions for activation. (A) Representative brightfield images were acquired over a multi-day activation period using the Cytation 5 with the BioSpa live-cell analysis system and 4x objective lens. Automated object identification and masking (yellow outlines) for quantification was conducted using Gen5 image analysis software. (B) Kinetic profiles reporting total cluster area over time for each concentration of activation cocktail. (C) Area under the curve (AUC) values calculated from the kinetic profiles are used to identify optimal concentration of activation cocktail based on peak response. Data represents the mean and standard deviation of eight determinations.

Automated kinetic imaging solution for quantifying cell potency

Recent advancements have highlighted the benefits of automated imaging to assess the cytotoxic effects of immune cells on cancer cell targets. The Agilent Cytation cell imaging multimode reader and Lionheart FX automated microscope enable researchers to kinetically monitor CAR T-induced killing of target cancer cells in real time, providing detailed kinetic profiles of CAR T-cell dynamics.

This automated imaging-based approach allows for continuous monitoring of cell cultures without manual intervention, reducing the risk of contamination and variability. The high-resolution imaging and precise quantification of target cell killing using either direct cell counts or target cell signal enable accurate assessment of CAR T-cell efficacy (Figure 11). Wide field of view imaging enables a large sample area to be readily captured and analyzed with a single image, including whole-well imaging within 384-well microplates. Additionally, the instrument's ability to perform kinetic analyses and calculate dose responses incorporating area under the curve (AUC) values offers a comprehensive understanding of the cytotoxic effects over time, enhancing the reliability and reproducibility of results (Figure 12).

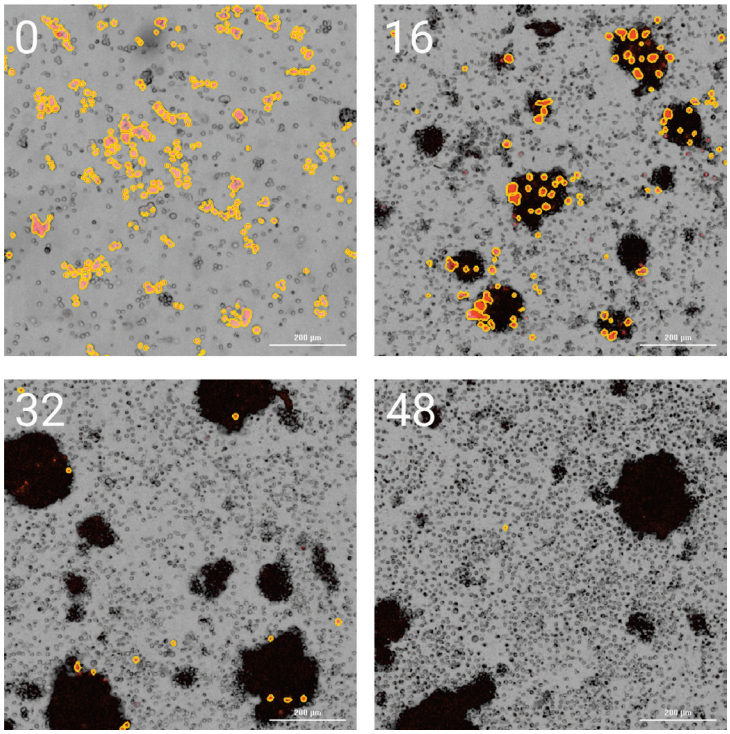


Figure 11. Fluorescent and brightfield images of T47D-mKate2 cells exposed to activated CAR T-cells. Images were captured with a 4x objective from the same well in the TRITC and brightfield channels every hour for 48 hours. Images reflect a digitally zoomed region of the same well, with object masks (yellow outline) applied around mKate2-positive viable target cells.

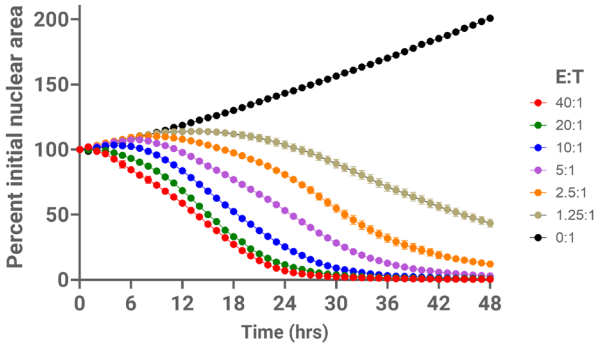


Figure 12. Kinetic profiles of T47D-mKate2 cell area in response to different ratios of activated CAR T-cells to target cells (E:T). Dilutions of activated CAR T-cells were added to T47D-mKate2 cells. Target cell area measurements were made every hour for 48 hours from kinetic fluorescent images. Data is expressed as the percentage of the initial determination. Each data point represents the mean and standard deviation of 4 data points. The untreated control target cell population expanded throughout the timecourse, while target cells treated with CAR T-cells exhibited significant cell-induced death in relation to E:T ratio.

Integrated automated multiplate incubator for high-throughput analysis

The Agilent BioSpa live-cell analysis system combines the full capabilities of the Cytation imaging platform with the BioSpa 8 automated multi-plate incubator. The BioSpa system supports cell potency assays in up to 8 microplates simultaneously, providing real-time control and continuous monitoring of temperature, CO_2/O_2 and humidity levels. This ensures optimal conditions for cell cultures and minimizes manual intervention while delivering reproducible results across multiplate applications.

3D assay formats for measuring cell potency

Innovative 3D culture methods developed over the last decade better mimic *in vivo* conditions, including cell-to-cell interactions, nutrient gradients and extracellular matrices. The use of 3D immuno-oncology models is driven by two primary objectives: to develop more predictive high-throughput cellular models upstream of clinical trials and to reduce the use of animal models. Widely adopted 3D culture techniques include hydrogel-based methods and ultralow attachment (ULA) coatings in microplate wells to allow cells to self-aggregate.

Combining 3D cancer cell models with immune effector cells provides an informative system for characterizing cell-induced cytotoxicity and inhibition of growth. Microscopy is effective at probing these 3D model systems, providing critical insights into sample characteristics, such as size, migration and invasion behavior, and cell viability. The Lionheart FX and Cytation automated live-cell imaging systems, equipped with brightfield and widefield fluorescence imaging modes, along with powerful Gen5 image analysis tools, provide flexible platforms for a broad range of 3D immuno-oncology applications. Low-magnification brightfield imaging enables label-free kinetic measurements of 3D sample size and quantifying invasion into the surrounding matrix. The addition of non-perturbing cellular markers for viability and cytotoxicity enables quantification of immune cell killing, via widefield fluorescence imaging (Figure 13A), with relative fluorescence intensity reported (Figure 13B).

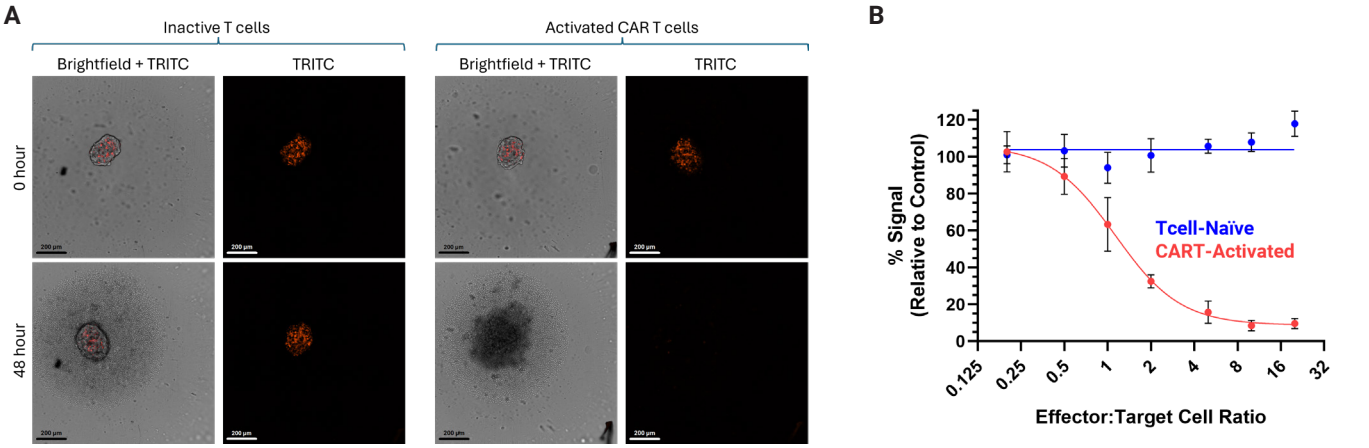


Figure 13. Quantification of T-cell-induced killing in 3D model system. **(A)** Automated widefield fluorescence imaging and analysis was used to quantify cell potency of either inactive T-cells or activated CAR T-cells. **(B)** Fluorescence signal (mKate2) in the TRITC channel was measured for each condition where decreases in integrated signal is interpreted as target cell death.

High resolution confocal imaging reveals deeper insight into 3D cell models and immune cell engagement

While brightfield and widefield fluorescence imaging provide straightforward solutions for many 3D applications, these techniques are typically limited at higher magnifications due to the thick nature of the samples and high background signal. The improved optical sectioning ability achieved with confocal fluorescence imaging provides a powerful approach for 3D immuno-oncology studies in which greater cellular detail is required. The Cytation C10 confocal imaging reader presents a versatile system for conducting 3D immuno-oncology studies, combining spinning disk confocal and widefield microscopy with multimode microplate reading. The confocal mode can reveal detailed insight into the spatial arrangement of cellular characteristics within a 3D sample. The system can be used to assess the ability of immune effector cells to infiltrate solid tumor models and conduct cell killing (Figure 14). Incorporating the BioSpa live-cell analysis system expands the capabilities of the Cytation imaging multimode readers to support long-term kinetic cytotoxicity assays using 3D technologies and immune cells.

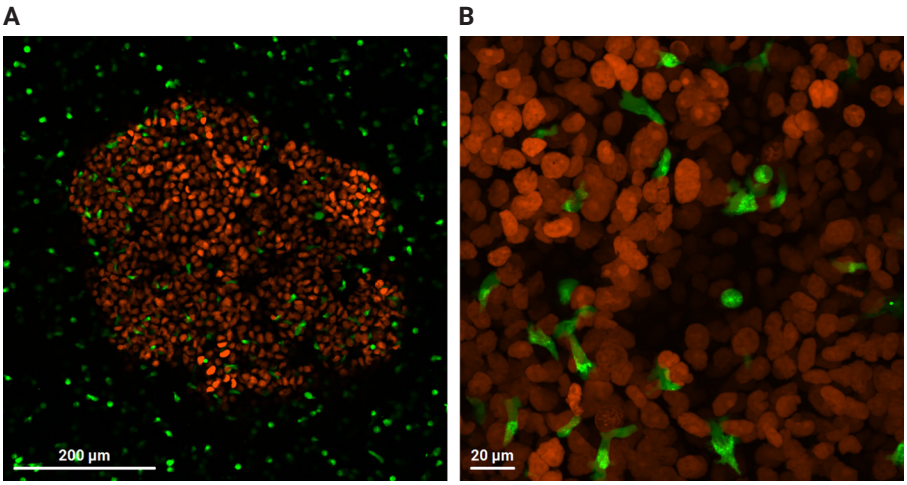


Figure 14. High resolution confocal imaging provides detailed spatial insight into T-cell invasion and engagement within tumoroid targets. T47D-mKate2 spheroids were embedded within a collagen matrix in the well of a 96-well plate (one spheroid per well). Activated EpCAM-CAR-T lymphocytes labeled with CellTracker-Green were added to the overlaid culture medium, allowing them to migrate through the matrix towards the spheroid. Images were acquired 48 hours after T-cell addition. (A) Optical section (~25 µm deep within sample) captured with the Cytation C10 and 20x objective reveals CAR T-cells (green) surrounding T47D-mKate2 spheroid target cells (red). (B) Image captured of the same sample with the Cytation C10 60x water immersion objective reveals CAR T-cell invasion and engagement with target cells ~60 µm within T47D tumoroid.

Enhancing T-cell characterization with automated ELISpot assays: Benefits of advanced imaging and liquid handling systems

ELISpot (enzyme-linked immunospot) assays are highly sensitive techniques used to detect and quantify cytokine secretion at the single-cell level. In these assays, T-cells are cultured on a membrane coated with antibodies specific to the cytokine of interest. When T-cells secrete the cytokine, it binds to the antibodies on the membrane. A secondary antibody, linked to an enzyme, is then added, which binds to the captured cytokine. Upon adding a substrate, the enzyme catalyzes a reaction that produces a visible spot on the membrane, corresponding to a single cytokine-secreting cell. The number of spots can be counted manually or using automated imaging systems, providing a direct measure of the frequency of cytokine-secreting T-cells. ELISpot assays are particularly useful for studying antigen-specific T-cell responses, vaccine efficacy and immune monitoring in clinical trials, offering a detailed and quantitative assessment of T-cell function.

The integration of automated imaging approaches and liquid handling systems significantly enhances the efficiency and accuracy of ELISpot assays. The Agilent BioTek Cytation 7 cell imaging multimode reader features an upright color camera that rapidly captures whole-well images of ELISpot samples, which are then analyzed using an automated Gen5 protocol to quantitate immune response to antigenic stimuli (Figure 15). This method supports all standard ELISpot assay formats, including two-color ELISpot kits relying on two capture antibodies with independent color developers to quantify the number of cells secreting two distinct cytokines.

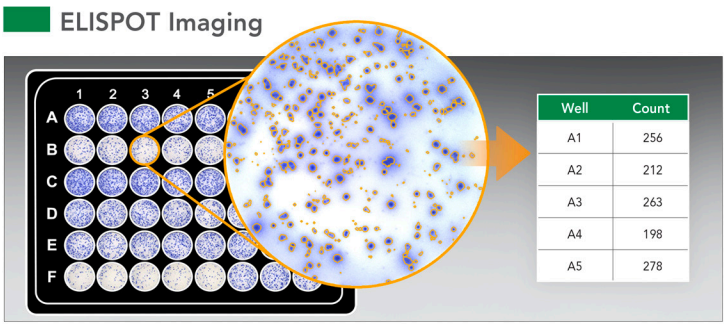


Figure 15. Agilent BioTek solution for conducting ELISpot assays utilizing automated imaging and analysis for robust quantification of cytokine secretion.

This automated approach reduces variability and increases reproducibility by standardizing the imaging process, ensuring consistent results across different experiments and laboratories. Additionally, automated imaging can handle large sample volumes, accelerating the data acquisition process and enabling the analysis of multiple samples simultaneously. This high-throughput capability is particularly beneficial in large-scale studies where the rapid and accurate assessment of immune responses is critical.

Automated liquid handling systems further streamline the ELISpot assay workflow by automating the addition of reagents, washing steps and sample handling. These systems minimize human error and variability, ensuring precise and consistent reagent delivery and reducing the risk of contamination. Automation also improves the efficiency of the assay by reducing the time and labor required for manual pipetting and sample preparation. This allows researchers to focus on data analysis and interpretation rather than on repetitive manual tasks.

Instrument models

The **Agilent BioTek Cytation C10 Confocal Imaging Reader** automates 3D live-cell imaging and analysis. With its spinning disk confocal imaging, the Cytation C10 delivers deeper understanding of 3D cellular characteristics important to many immuno-oncology workflows, with Agilent BioTek Gen5 software providing the deeper analysis needed for 3D samples. Widefield imaging in brightfield and fluorescence enable label-free and fluorescence-tagged assays to support many cell and gene therapy applications.

Where live-cell imaging workflows benefit from higher throughput, the Agilent **BioTek BioSpa Live-Cell Analysis System** enables environmentally-controlled kinetic processes for up to eight microplates or other labware, on a single platform. The system is an integration of the BioSpa 8 automated incubator and a Cytation imaging reader, to support multi-vessel imaging and analysis operations across a broad magnification range suitable for 2D and 3D live-cell workflows.

The **Lionheart FX Automated Microscope** supports live-cell imaging for a range of applications in immuno-oncology research. Widefield fluorescence and brightfield channels, environmental controls and Gen5 software for image analysis enable imaging studies in 2D and 3D cell models.

BioTek microplate reader-based approaches

Advanced ELISA techniques and automation solutions

ELISA (enzyme-linked immunosorbent assay) is a widely used technique for detecting and quantifying specific proteins such as cytokines, which are critical markers of T-cell activation and function. In T-cell characterization studies, ELISA assays are employed to measure the concentration of cytokines secreted by activated T-cells, providing insights into the immune response. A typical ELISA format consists of a microplate coated with antibodies specific to the target cytokine, which bind to the cytokine in the sample. A secondary antibody linked to an enzyme is added, and upon adding a substrate, a detectable signal proportional to the cytokine amount is produced.

Agilent BioTek microplate readers offer several benefits for conducting ELISA assays. They provide high sensitivity and accuracy, ensuring reliable detection of low-abundance analytes. The robust hardware and advanced optical design deliver consistent performance across a wide range of applications. Additionally, these detectors are equipped with user-friendly software that simplifies data analysis and reporting. The integration of automated liquid handling systems significantly enhances the efficiency and accuracy of ELISA assays. Automated systems such as the Agilent BioTek 406 FX washer dispenser streamline the ELISA workflow by automating the addition of reagents, washing steps and sample handling. Automated liquid handling reduces human error and variability, ensuring consistent and reproducible results while increasing throughput for simultaneous processing of multiple samples. Furthermore, combining the Agilent BenchCel, BioTek microplate reader and BioTek plate washer into a single benchtop system provides an integrated solution for processing and reading many ELISA microplates for high-throughput applications (Figure 16).

Figure 16. Automated ELISA workstation. Agilent BenchCel microplate handler, the BioTek 406 FX washer dispenser and Synergy Neo2 multimode reader.

Quantification of T-cell number and viability

Microplate readers and data analysis software provide a range of straightforward and simple approaches for quantifying T-cell activation, viability and proliferation that support high-throughput applications. One common approach is the use of luminescence-based assays, such as CellTiter-Glo, which measures ATP levels as an indicator of cell viability. This method involves lysing the cells to release ATP, which then reacts with a luminescent substrate to produce a measurable light signal. The intensity of the luminescence correlates with the number of viable cells, allowing researchers to quantify T-cell proliferation in response to different stimuli. Additionally, the effect of various concentrations of stimulants like IL-2 on T-cell growth can be monitored by measuring luminescence, providing insights into the optimal conditions for T-cell activation and expansion (Figure 17).

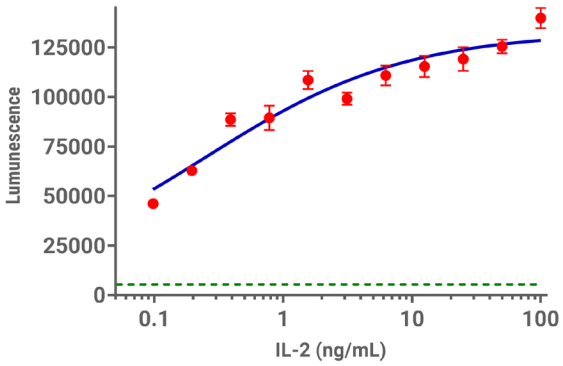


Figure 17. Effect of IL-2 on CAR T-cell growth. Freshly thawed CAR T-cells were incubated with various concentrations of IL-2 for 5 days, after which a portion was transferred to a fresh plate and the luminescence determined. Dashed line indicates luminescence signal of CAR T-cells treated with media only. Data represents the mean and standard deviation of 8 replicates.

Microplate reader-based reporter assays for characterizing gene expression

Reporter assays have long been used to investigate regulatory mechanisms of gene expression in response to stimuli. Reporter constructs rely on the insertion of a genetic regulatory element or promoter region upstream of a reporter gene, followed by the insertion of the construct – generally a plasmid – into the target cell or organism. The reporter gene is designed to generate a readily detectable signal of the expression state of the gene of interest (i.e., if the gene of interest is expressed, an associated increase in the reporter gene product is detected). Two commonly used types of reporter gene products are fluorescent proteins and luciferase, with fluorescence intensity and luminescence intensity as the readout, respectively.

Luciferase reporter assays are a powerful tool for characterizing T-cell activation and function. These assays involve the use of luciferase enzymes, which produce a luminescent signal when they catalyze a substrate (Figure 18). In T-cell studies, luciferase genes are often placed under the control of promoters that are activated by specific transcription factors, such as NFAT (nuclear factor of activated T-cells) or IL-2, which are upregulated during T-cell activation. When T-cells are stimulated, the transcription factors activate the promoter, leading to the expression of luciferase and the production of a luminescent signal. This signal can be quantitatively measured using Agilent BioTek multimode microplate readers, providing a sensitive and real-time readout of T-cell activation. The high sensitivity and dynamic range of luciferase assays make them ideal for screening compounds that modulate T-cell activity and for studying the molecular mechanisms underlying T-cell responses.

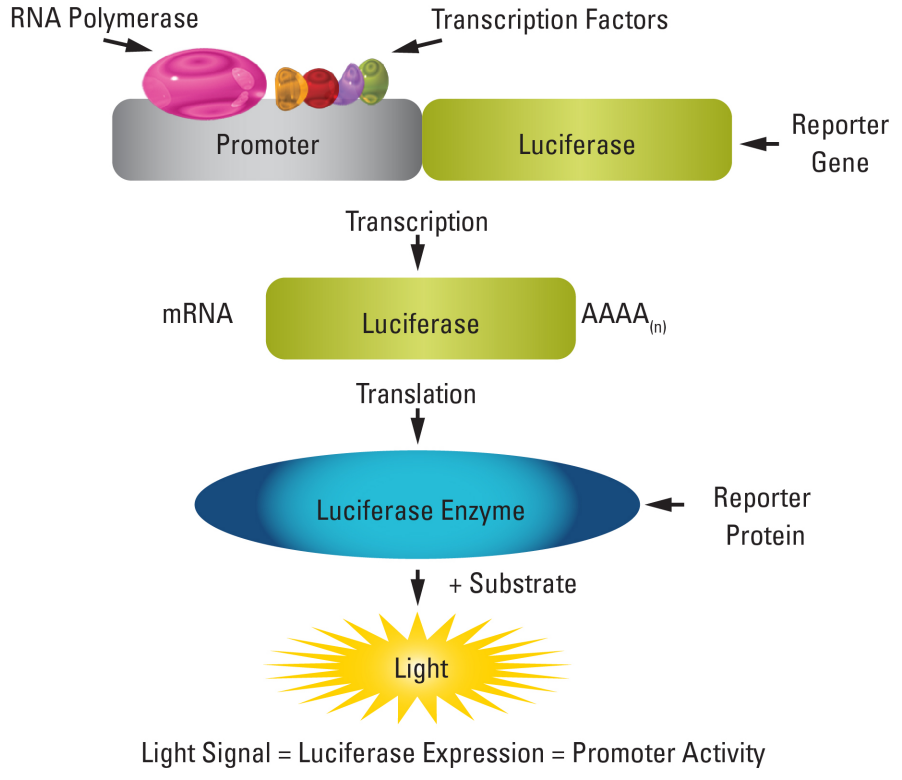


Figure 18. Schematic representation of a luciferase reporter assay.

Fluorescent reporter assays are another widely used method for T-cell characterization. These assays utilize fluorescent proteins, such as GFP (green fluorescent protein) or RFP (red fluorescent protein), which emit fluorescence when excited by light of a specific wavelength. Like luciferase assays, the genes encoding these fluorescent proteins are placed under the control of activation-specific promoters. Upon T-cell activation, the promoters drive the expression of the fluorescent proteins, resulting in a measurable increase in fluorescence. Agilent BioTek microplate readers are used to detect and quantify the fluorescence, allowing for the analysis of multiple parameters simultaneously, such as the expression of surface markers and intracellular cytokines. This capability provides a comprehensive view of T-cell activation and differentiation, making fluorescent reporter assays a valuable tool for immunological research and therapeutic development.

Instrument models

The modular design of the Agilent BioTek **Synergy Neo2 hybrid multimode reader** has quadruple-monochromator optics and filter-based optics for fluorescence and luminescence detection, plus UV-Vis absorbance. The variable bandwidth monochromators provide sensitivity and specificity to support reporter gene assays. A fiber-free bottom optical path ensures high signal from a cell monolayer in the microplate well.

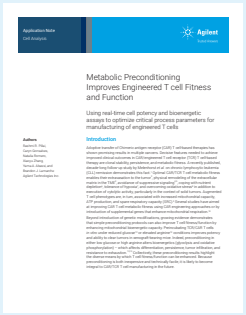
The Agilent BioTek **Synergy H1 multimode reader** offers both monochromator-based and filter-based optics for fluorescence and luminescence detection, along with UV-Vis absorbance detection. The fluorescence and luminescence sensitivity make it suitable for reporter assays and many others. The Synergy H1 is configurable to address current and future workflow requirements.

Monochromator-based UV-Vis absorbance, temperature control and shaking make the Agilent BioTek **Epoch 2 microplate spectrophotometer** a workhorse for assays in 6- to 384-well microplates, measuring absorbance from 200 to 999 nm. Agilent BioTek Gen6 software controls and provides data analysis for the Epoch 2, enabling basic end point and kinetic measurements at single, dual or multiwavelength. ELISA methods for cytokine detection are efficiently performed with the Epoch 2.

xCELLigence real-time cell analysis

The ultimate objective of adoptive cell-based therapies for cancer treatment is the selective, targeted recognition and persistent annihilation of cancerous cells. However, before achieving cytolysis of cancer cells, CAR T-cells have to overcome several barriers. Once *ex vivo*-expanded CAR T-cells are infused into the patient, they must engraft, expand and traffic to the site of the tumor. Trafficking of CAR T-cells designed against solid tumors is especially challenging as it poses both a physically and energetically intensive process. CAR T-cells not only have to extravasate to the site of the TME but also overcome immunosuppressive conditions such as acidic pH, limiting nutrients and hypoxia before they can encounter tumor cells.

The xCELLigence real-time cell analysis (RTCA) system uses non-invasive impedance-based biosensors to evaluate cellular status precisely in real time. The xCELLigence RTCA systems can be used to analyze trafficking, activation, clustering, mediated immune cell killing and persistence of CAR T-cells. This technology can also be used to assess the on-target-off-tumor activity of CAR T-cells, which is critical for the development of safe CAR T-cell therapies. The accompanying xCELLigence RTCA Software Pro supports users and their organizations in achieving the requirements of each section of 21 CFR Part 11 and the related sections of EU Annex 11.



Application note:

Metabolic Preconditioning Improves Engineered T cell Fitness and Function

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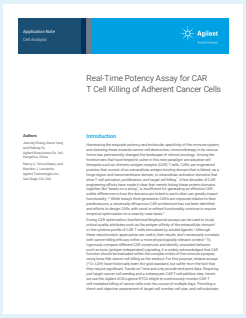

Application note:

Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells

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How xCELLigence RTCA technology works

The xCELLigence RTCA uses a highly sensitive, real-time cellular impedance assay to noninvasively measure cell properties using gold microelectrodes fused to the bottom surface of an E-Plate. The presence of adherent cells at the electrode–solution interface impedes electron flow. The magnitude of this impedance is dependent on the number of cells, the size and shape of the cells, cell barrier function formation and the cell-substrate attachment quality (Figure 19). Neither the gold microelectrode surfaces nor the applied electric potential (22 mV) influence cell health or behavior. The xCELLigence RTCA workflow allows users to obtain highly sensitive, quantitative and reproducible data with minimal handling time. Simply add cells to the E-Plates and start monitoring cell number, proliferation rate, cell size, shape and cell-substrate attachment in real time. Cellular impedance of electron flow caused by adherent cells is reported using a unitless parameter called cell index.

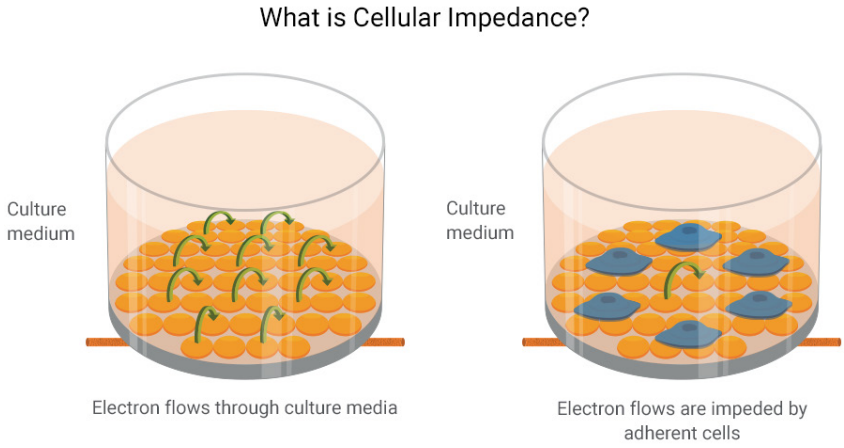
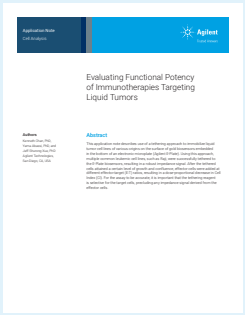


Figure 19. Cellular impedance explained.

Cell and gene therapy assays

The xCELLigence RTCA system offers several critical assays for cell therapy development including immune cell killing, migration, activation and clustering.



Application note:

Evaluating Functional Potency of Immunotherapies Targeting Liquid Tumors

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Immune cell-mediated target cell killing assay

Immune cell-mediated killing assays are instrumental to evaluate the potency of immune cell-based therapies. The RTCA technology supports FDA recommendations for verifying the potency of cell therapy products as a part of the release process.6 The xCELLigence RTCA system provides a highly sensitive, label-free and real-time assay of immune cell-mediated killing of target cancer cells (Figure 20). Importantly, the system provides kinetic-based parameters (e.g., rate of cytolysis and KT50 values) which are more informative than endpoint assays.

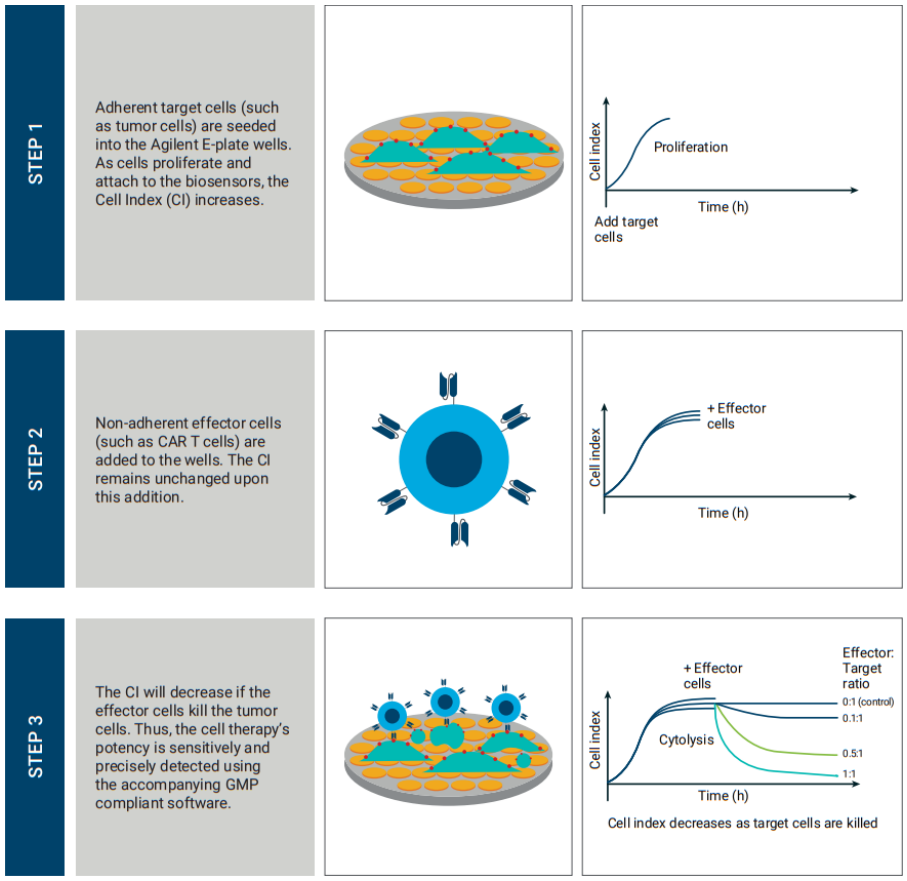
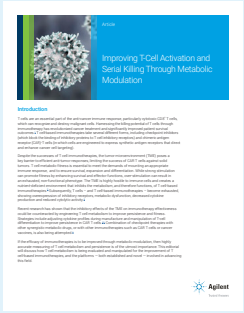


Figure 20. The xCELLigence RTCA assay. Cells are seeded, immune effector cells are added to the E-Plates and then immune cell killing kinetics are monitored under physiological conditions.



Article:

Improving T-Cell Activation and Serial Killing Through Metabolic Modulation

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Serial killing assay

Serial killing, or persistent killing, is the process by which a single immune cell engages with and kills multiple cancer cells successively. After killing one cancer cell, the immune cell can detach and move on to recognize and kill another cancer cell. This capacity for serial killing reflects the persistence of immune cells, indicating how long they can remain functional and continue killing targets. A more persistent immune cell population can sustain its activity for extended periods, which is correlated with prolonged clinical remission and improved survival in recipients. Thus, the serial killing assay is a valuable tool for assessing and optimizing immune cell design, helping to better predict the performance of immunotherapies *in vivo*.

The xCELLigence RTCA systems have been successfully utilized for serial killing assay, demonstrating the ability of EpCAM CAR T-cells to kill an EpCAM antigen-expressing cell line, T47D, progressively during seven rounds of killing (Figure 21). The assay provides a unique value for long-term and non-invasive serial killing by CAR T-cells.

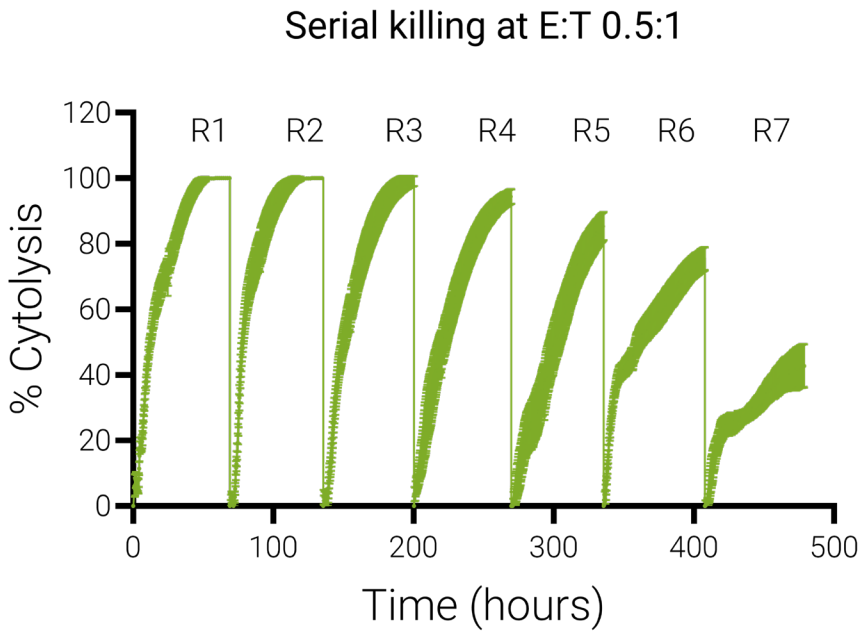

Figure 21. % cytolysis of EpCAM CAR T-cells against EpCAM-expressing cell line, T47D cells, assessed over seven rounds of killing. An effector-to-target (E:T) ratio of 0.5:1 was used in each round of killing. After 60–72 hours of co-culture of CAR T-cells and T47D cells, the CAR T-cells were collected, centrifuged and transferred to the next 96-well E-Plate containing fresh T47D cells for the subsequent round of killing measurement. The data are presented as mean ± SD.

Application note:

A Novel Real-Time Co-Culture Assay Using xCelligence RTCA eSight for Immune Cell Invasion and Cytotoxicity

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Immune cell migration and extravasation assay

The TME of solid tumors poses significant challenges to immune response. Moreover, recapitulating the characteristics of TME in assays can be critical for identifying and refining cellular immunotherapies. For example, the engineered cytotoxic natural killer (NK) and CD8+ T-cells have to extensively infiltrate the solid tumors to execute effector functions.

Agilent established extravasation assay using the xCELLigence RTCA system to allow immune cells to invade through the extracellular matrix (ECM) layer that is in direct contact with target tumor cells, thus replicating conditions for both invasion and cytotoxicity in tissues or solid tumors (Figure 22). This assay removes the need for processing the ECM to collect embedded cells at various time points for endpoint cytotoxicity assays.

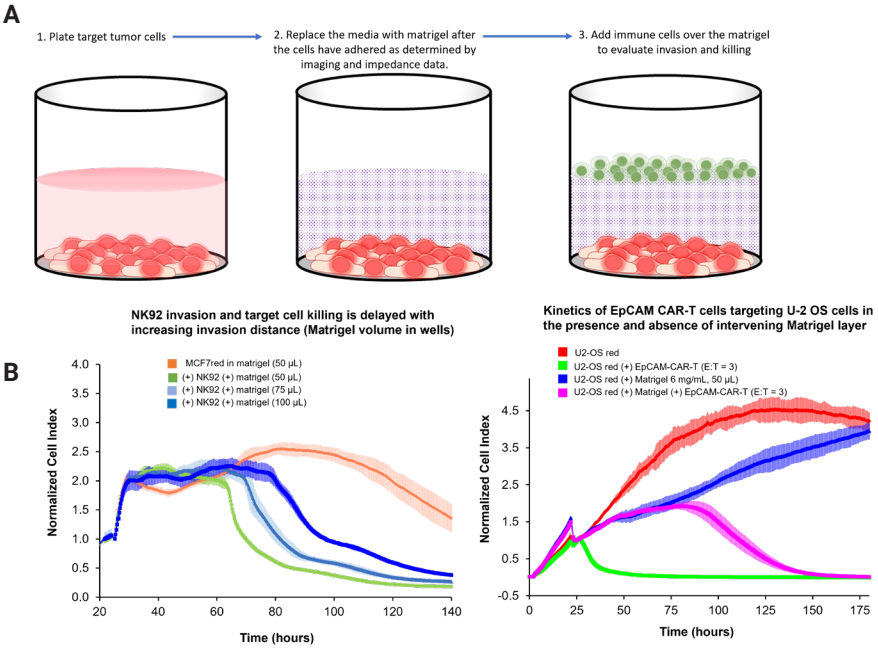
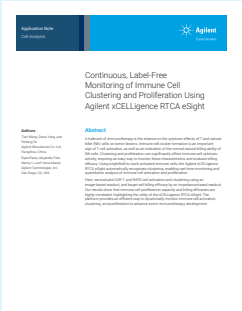


Figure 22. (A) Outline of invasion-cytotoxicity assay step. (B) NK-92 and EpCAM CAR-T-cells invasion and target cell killing in the presence and absence of Matrigel.



Application note:

Continuous, Label-Free Monitoring of Immune Cell Clustering and Proliferation Using Agilent xCELLigence RTCA eSight

Download Here

Immune cell activation and clustering

Immune cell cluster formation is an important sign of T-cell activation, as well as an indication of the normal natural killing ability of NK cells. Clustering and proliferation can significantly affect immune cell cytotoxic activity.

The Agilent xCELLigence RTCA eSight can be used to automatically recognize clustering (Figure 23). Using brightfield, it is possible to track activated immune cells, enabling real-time monitoring and quantitative analysis of immune cell activation and proliferation.

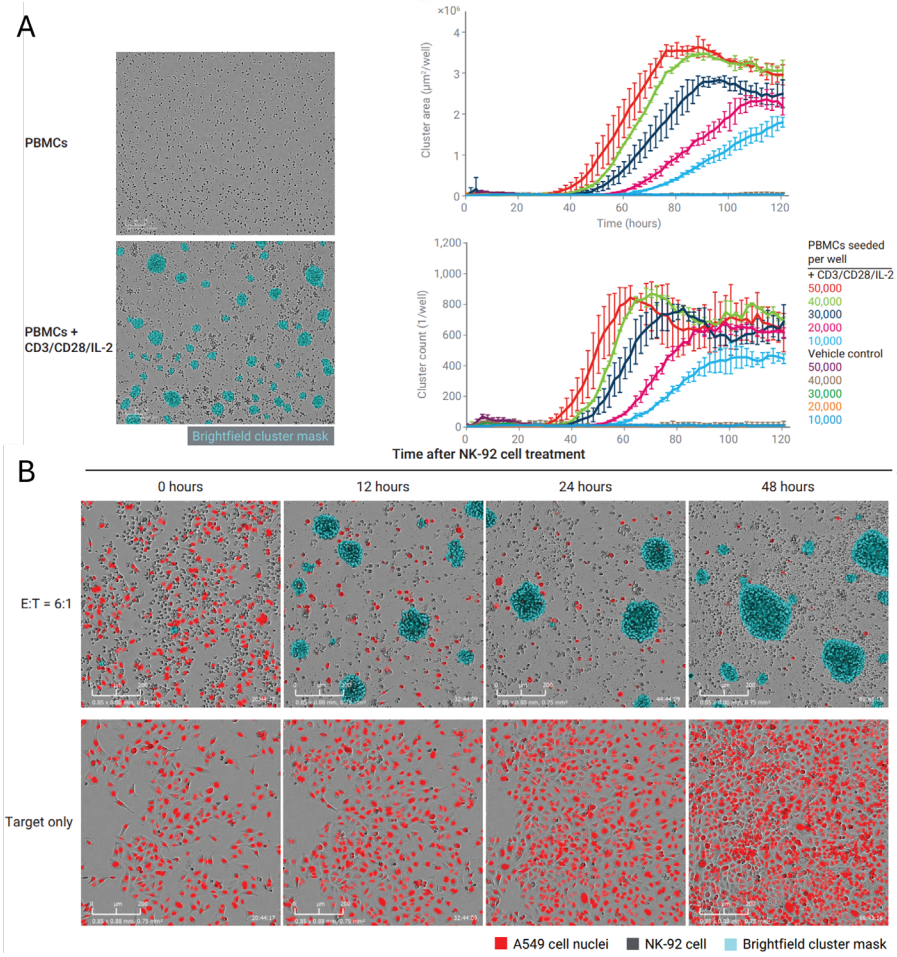
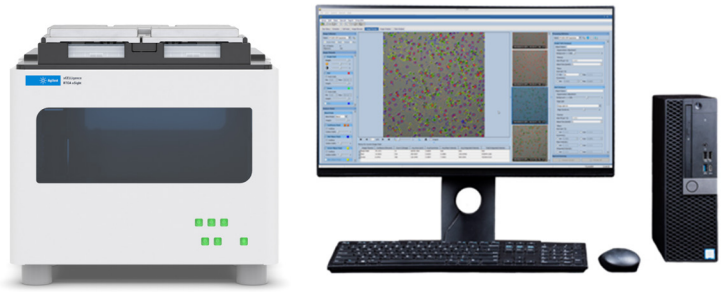


Figure 23. (A) Monitoring in vitro T-cell activation using the Immune Cell Clustering module with images at 72 hours after cell seeding with or without stimulation, and plots of cluster area and count as a function of time, respectively. **(B)** The cluster recognition of NK-92 cells and cluster area increase over time.

Find selected publications on this topic in the appendix.

Instrument models



The **xCELLigence RTCA eSight** enables comprehensive insight into cell health, cell behavior, cell function and cell biology processes using live, simultaneous and real-time biosensor impedance-based and image-based measurements. The eSight system combines the label-free xCELLigence RTCA technology with live-cell imaging in three colors (red, green and blue). This combination allows for informative live-cell analysis and increased insight into cell health.

The **xCELLigence RTCA MP** model can host up to six 96-well electronic microplates (E-Plate 96), which can be run in parallel or independently of one another.

The **xCELLigence RTCA HT** model can run a 384-well electronic microplate (E-Plate 384), which is ideal for high-throughput screening experiments. Integrate up to 4 instruments controlled by a single control unit for a total of 1,536 wells to meet high throughput sample screening needs. The RTCA HT model can be used as a single instrument on a standard lab bench or integrated into a multi-instrument high-throughput workflow with automated liquid handling.

The **xCELLigence RTCA HT-BioTek BioSpa8** integrates the xCELLigence RTCA HT instrument with the BioTek BioSpa 8 automated incubator to expand the screening throughput of your RTCA HT instrument to eight 384-well plates.

The **xCELLigence RTCA DP** enables three separate electronic 16-well plates to be controlled and monitored in parallel or independently of one another. This flexible plate batch processing allows maximum productivity for multiple users.

The **xCELLigence RTCA SP** model can run a 96-well electronic microplate (E-Plate 96), which is ideal for cell health characterization, immune cell-mediated killing, viral cytopathic effects, cytotoxicity, cell adhesion, cell barrier function (TEER), cell signaling (e.g., GPCR) and other related applications.

The **xCELLigence RTCA S16** instrument uses biosensors to continuously monitor live-cell proliferation, morphology change and attachment quality in a label-free and real-time manner.

Related kits

The **xCELLigence Immunotherapy Kits** can be used with the real-time cell analysis systems for a non-invasive solution to a broad range of liquid cancer immunotherapies and suspension tumor cell killing applications. Determine the potency of immune cells against liquid tumors *in vitro*.

Process development and CMC

Cell-based therapies have unique chemistry, manufacturing and controls (CMC) requirements that need to be considered as early as possible during their development life cycle. Given the cellular nature of the therapy, it is pertinent to implement a CMC strategy that informs on quality, potency and safety of the therapy throughout the manufacturing process. For CAR T-cells in the early stages of clinical development, a few specifications are finalized, and some tests are still under development. Cellular characterization data collected during early studies can inform release criteria used in later development to ensure product and process consistency. Thus, characterization studies are crucial to support product development and comparability assessments. The current guidelines from the FDA recommend starting with a matrix of characterization assays early in the development of CAR T-cells (Figure 24). As the therapy progresses to the CMC stage, the corresponding characterization assays must be further qualified and validated. In addition to assays informing on product safety, purity and identity, the product potency also needs to be addressed.[6] Potency assays should be developed and based on mechanism of action of the product, which can continue to be refined as more clinical data becomes available.

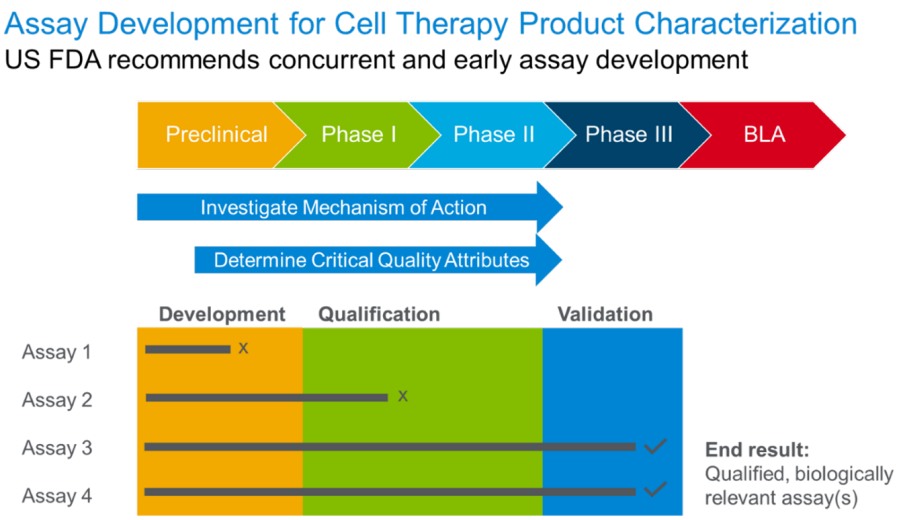


Figure 24. Concurrent and early assay development recommended by the FDA. *Source: ASH 2023 US FDA presentation "The Regulatory Perspective: Cell Manufacturing Considerations".*

To ensure product consistency during CAR T manufacturing, it is important to evaluate and optimize critical process parameters (CPPs) that may impact product quality and potency.

Potency assays

One of the most important CQA of CAR T-cell therapy is its potency. Based on current FDA guidelines, the CAR T-cell drug product must be tested for potency. These guidelines recommend that potency assays should be used in several ways throughout the product life cycle, including lot release testing, stability evaluation, in-use studies, delivery device compatibility studies and manufacturing process studies.[6]

Several approaches are currently used in the field to evaluate the potency of a CAR T drug product. These include immune cell killing assays and cytokine secretion assays. Agilent Technologies offers various sensitive and robust potency solutions which can be implemented in various stages of CAR T development and product release (Table 4).

Table 4. Agilent solutions for potency assays.

Assay type	Approach	Instrument
Immune cell killing	Impedance	xCELLigence RTCA systems (MP, HT, HT-BioTek BioSpa 8, DP, SP, S16)
	Impedance and imaging	xCELLigence RTCA eSight
	Real-time imaging	Cytation
	Reporter assays	Cytation
Cytokine secretion	Flow cytometry	NovoCyte
	ELISA	Cytation

Agilent Technologies offers several innovative solutions that address the critical needs of CMC and CAR T process development that can be implemented as part of deep characterization and evaluation of the potency of the CAR T-cell therapy. These solutions were discussed in the previous section. Table 5 highlights how these solutions can be used to address CMC requirements.

Table 5. Agilent solutions to address CMC requirements.

Agilent solution	Key assays for assessment of CQAs, CPPs and characterization assays	21 CFR 11 compliant software
 **NovoCyte flow cytometers** These high-performance benchtop instruments offer top-notch capabilities, high-quality data and an easy-to-use platform. They have contributed to the characterization of CAR T-cells, monitoring CAR T-cell fate, immunophenotyping cell subpopulation, assessing transduction efficiency, evaluating purity during preclinical testing, clinical manufacturing and long-term tracking.	– Immune cell proliferation – Viability – Transduction efficiency – Immunophenotyping – Immune cell activation/exhaustion – Bead-based cytokine assays (potency) – Immune cell killing (potency) – Characterization of cellular starting material	Yes
 **Cytation cell imaging multimode readers** A versatile platform with advanced imaging capabilities and powerful integrated image analysis tools. Combining widefield and confocal microscopy with conventional microplate reading, the Cytation C10 provides a unique platform for enhanced assay flexibility that delivers detailed insights into complex immuno-oncology models.	– Immune cell proliferation – Viability – Reporter assays (potency) – Cell killing assays (potency)	Yes
 **Synergy multimode readers** They combine flexibility, speed and precision to support CAR T research across a broad range of well-established cell- and biochemical-based assay formats, including high-throughput screening applications.	– Reporter assays – Viability assays – Cytotoxicity assays – ELISA assays (potency)	Yes
 **xCELLigence RTCA system** This platform offers a label-free, real-time measurement of the immune-cell-mediated killing of cancer cells that is robust, specific, accurate and reproducible. This satisfies the needs of analytical and in-process development in cell therapy manufacturing.	– Immune cell killing assays (potency) – Proliferation assays – Migration assays – T-cell activation assays	RTCA Software Pro: Yes RTCA eSight Software: In development
 **Seahorse XF analyzer** A recognized leading technology for the study of immune cell metabolism. It detects rapid and transient metabolic changes in real time, revealing unique insights into the critical drivers behind immune cell function.	– T-cell metabolic fitness enabled by the XF T-cell metabolic profiling kits – Immune cell activation assays monitor/modulate T-cell activation in real time	In development

Conclusion

By harnessing the power of the immune system, adoptive cell therapies offer a promising approach for the treatment of numerous diseases including cancer, autoimmune and infectious diseases. While we have seen that autologous CAR T-cell therapy provides an unparalleled adaptive response to hematological tumors, major roadblocks still hinder the immunotherapeutic development and commercialization for solid tumors.

Researchers and manufacturers must ensure reproducibility, efficacy, potency and metabolic fitness before treatments reach the clinic. CQAs and CPPs should be recognized and established to ensure safety and efficacy of the cell therapy products, requiring a matrix of characterization assays implemented early in discovery stage.

Agilent's comprehensive cell analysis portfolio plays an instrumental role in monitoring and optimizing the CQAs and CPPs of CAR T-cell products from discovery, through process development, to manufacturing. These tools not only facilitate the identification and characterization of immune cells but also provide valuable insights into the metabolic fitness, activation status and cytotoxic capabilities of engineered T-cells.

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Appendix: Selected publications

NovoCyte flow cytometers

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