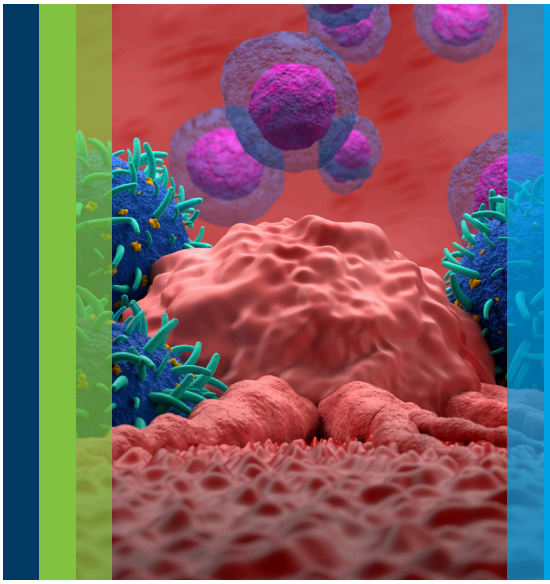

Article

Enhancing Immune Cell Cytotoxicity and Persistence Insights Using Real-Time Cell Analysis

Introduction

Cell therapies – the transfer of autologous or allogenic cells for therapeutic means – have evolved into a highly promising area of drug development for a wide range of applications, including regenerative medicine and immunotherapy. As of 2023, 65 non-genetically modified cell therapies, and 10 genetically modified cell therapies have already been approved globally and are available for clinical use.[1] With more than 2,000 further therapies currently in development, cell therapy has the potential to dramatically transform the medical landscape, providing treatments and reducing patient suffering.

As advanced therapy medicinal products (ATMPs), cell therapies are required to meet defined safety, purity, and potency requirements as part of their process development and approval criteria. In order to meet potency requirements, cell therapies must demonstrate the ability to safely exert a defined biological effect.[2,3] However, as cell therapeutics are "living drugs" consisting of live effector cells, the mechanism of action (MoA) can be complex, multi-faceted, and difficult to determine using one single assay or marker.[4] As a result, potency assays must be robust and reliable, and capture the full spectrum of effector cell activities.

This editorial will discuss the potency assays available for cellular immunotherapies, the regulatory criteria they must meet, and the challenges faced in measuring immune cell cytotoxicity. It will also explore the solutions available to overcome these challenges and how real-time cell analysis platforms are advancing the field of immunotherapy.

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Optimizing the development of cellular therapies

Since the approval of the chimeric antigen receptor (CAR) T-cell therapy tisagenlecleucel (Kymriah) to treat B-cell acute lymphoblastic leukemia in 2017, immune cell-based adoptive cell therapies (ACTs), have seen extensive progress.[5] CAR T-cell therapies are often autologous, using the patient's own genetically edited immune cells to circumvent the immunosuppressive properties of tumors and destroy cancer cells.[6] There are currently six CAR T-cell therapies approved for clinical use in the United States, all of which are produced by a similar method (Figure 1).

Although CAR T-cell therapy has been highly successful in treating hematological malignancies, there has been limited success in CAR T-cell therapies for solid tumors.[7,8] Efforts can be inhibited by a lack of tumor-specific antigens, limited infiltration into solid tumor tissue, and the highly immunosuppressive tumor microenvironment (TME). In addition, the need for exact major histocompatibility complex (MHC)-matched donor cells can limit harvesting potential and delay treatment.[5] CAR T-cell therapies can also be associated with cytokine release syndrome (CRS) – a severe toxicity event induced by the powerful CAR T cell-directed immune response. Although this can be managed with immunosuppressants, it can be a considerable concern for recipients.[9] To overcome these limitations, CAR natural killer (NK) and macrophage cell therapies are currently in development. CAR NK cells can directly recognize tumor antigens for a rapid cytotoxic response, without the need for MHC antigen presentation.[5] The less specific nature of the NK cell response limits graft versus host disease in allogenic settings, and increases the feasibility of "off-the-shelf" manufacturing at a fraction of the price of CAR T cells.[10] CRS can also be avoided in CAR NK-cell therapies, as the NK cell cytokine release profile is less inflammatory than that of T cells.[11] Pre-clinical studies of CAR macrophage therapies have shown improved infiltration of solid tumors, boosting of anti-tumor T cell responses, and significantly improved survival in mice.[12]

In recent years, cellular bioenergetics has been shown to be key in understanding the fate, function, and fitness of immune cells, including genetically modified T cells. The metabolic poise of immune cells is a critical determinant that is intrinsically linked to anti-tumor efficacy, especially within the immunosuppressive tumor microenvironment.[13,14]

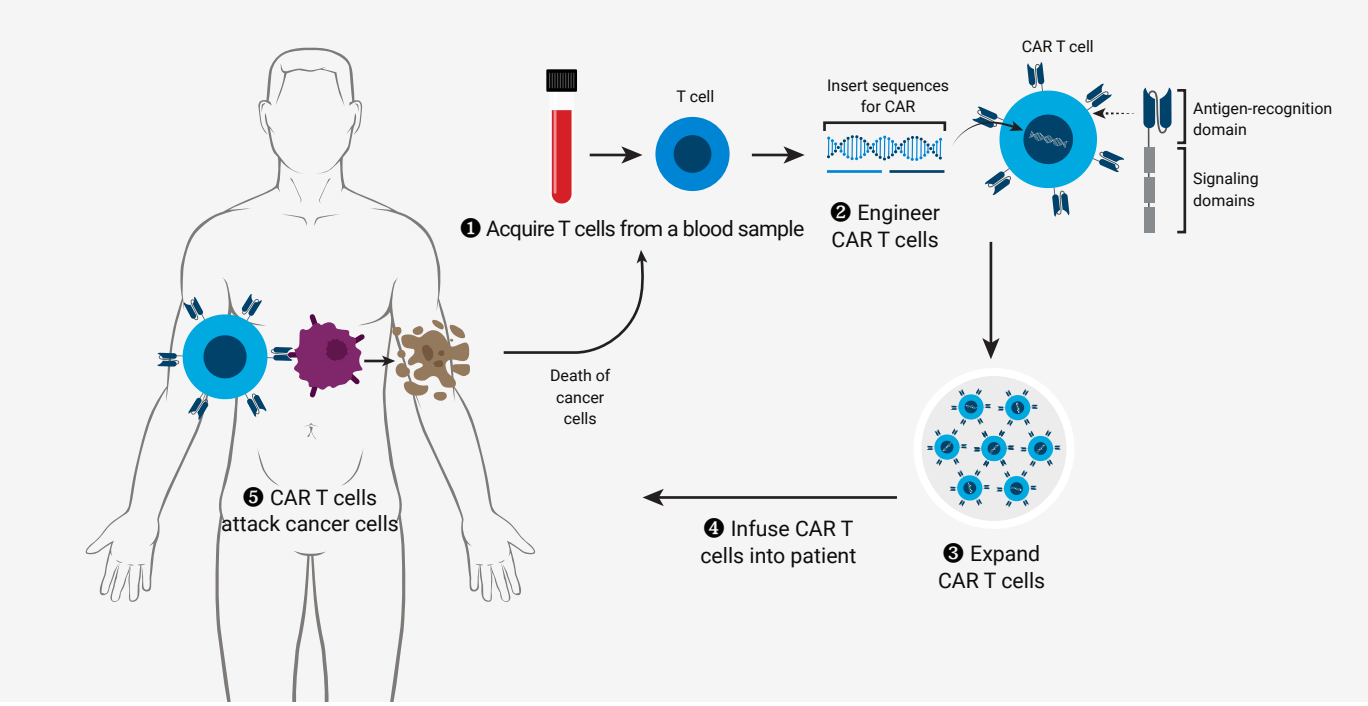


Figure 1. Steps involved in producing CAR T-cell therapies. T lymphocytes are harvested and purified from the patient or donor's blood. The T cells are then activated, either by antigen-presenting cells also purified from the patient, or anti-CD3 antibodies coupled with growth factors such as interleukin-2.[15] CARs designed to target specific tumor antigens are integrated into the genomes of the activated T cells via delivery methods such as mRNA transfection and lentiviral transduction.[15] Once the CAR T-cell populations are expanded and infused back into the patient, the genetically modified CARs are able to specifically recognize tumor cells, and destroy them using classic cytotoxic T lymphocyte methods such as the release of perforin and granzymes.[16]

Therefore, 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.

Despite the limitations and ongoing improvement of CAR cell therapies, the overall impact has been incredibly beneficial. Clinical studies show high efficacy and increased survival and remission rates, supported by the persistence of CAR T cells.[17,18] However, as a form of "living drug", the complexity and inherent heterogeneity of these therapies can result in challenging regulatory processes. This heterogeneity generates difficulty in bringing products to market and delays further benefits to patients. Therefore, robust, reliable, and easily scaled manufacturing and quality control processes are essential.

The criteria for potency testing

As part of their complex approvals process, ACTs are required to demonstrate consistent and reliable potency. Regulatory bodies such as the United States Food and Drug Administration (FDA) and the European Union's European Medicines Agency (EMA), provide guidance on the structure and data requirements of potency assays, as well as current good manufacturing practice (cGMP) guidelines to produce approved cell therapies.[3,19] The exact requirements vary between regulating bodies, but the overall purpose of a potency assay is consistent. These assays must determine the specific ability of the therapeutic to effect the defined MoA, as assessed by appropriate and rigorous clinical data or laboratory testing.[3] To this end, potency assays are expected to fulfill certain criteria. For example, recent draft guidance published by the FDA highlighted the desirable characteristics of potency assays, including: mitigation of risk to product potency, precision, accuracy, specificity, robustness, minimization of assay redundancy, and minimization the use of animals. (Figure 2).[20]

In order to develop a potency assay for a cell therapy, the MoA must first be characterized. However, as cell therapies involve complex, living cells, these MoAs are also likely to be complex and multifactorial. Cell products are also inherently variable and sensitive to their environment. Processing and storage can affect biological activity and cell properties, with potentially significant effects on safety or function. As a result, multiple potency assays (known as an "assay matrix") may be required to fully characterize the biological mechanisms at all stages of process development, manufacture, and release.[21,22] Where possible, these assays should comprise a functional evaluation of the product's MoA, such as a tumor cell killing assay for an ACT. However, if a functional assay is unsuitable or unavailable, measurement of a surrogate marker indicating biological activity can be used.[2]

Once the MoA is defined and a relevant and robust assay is

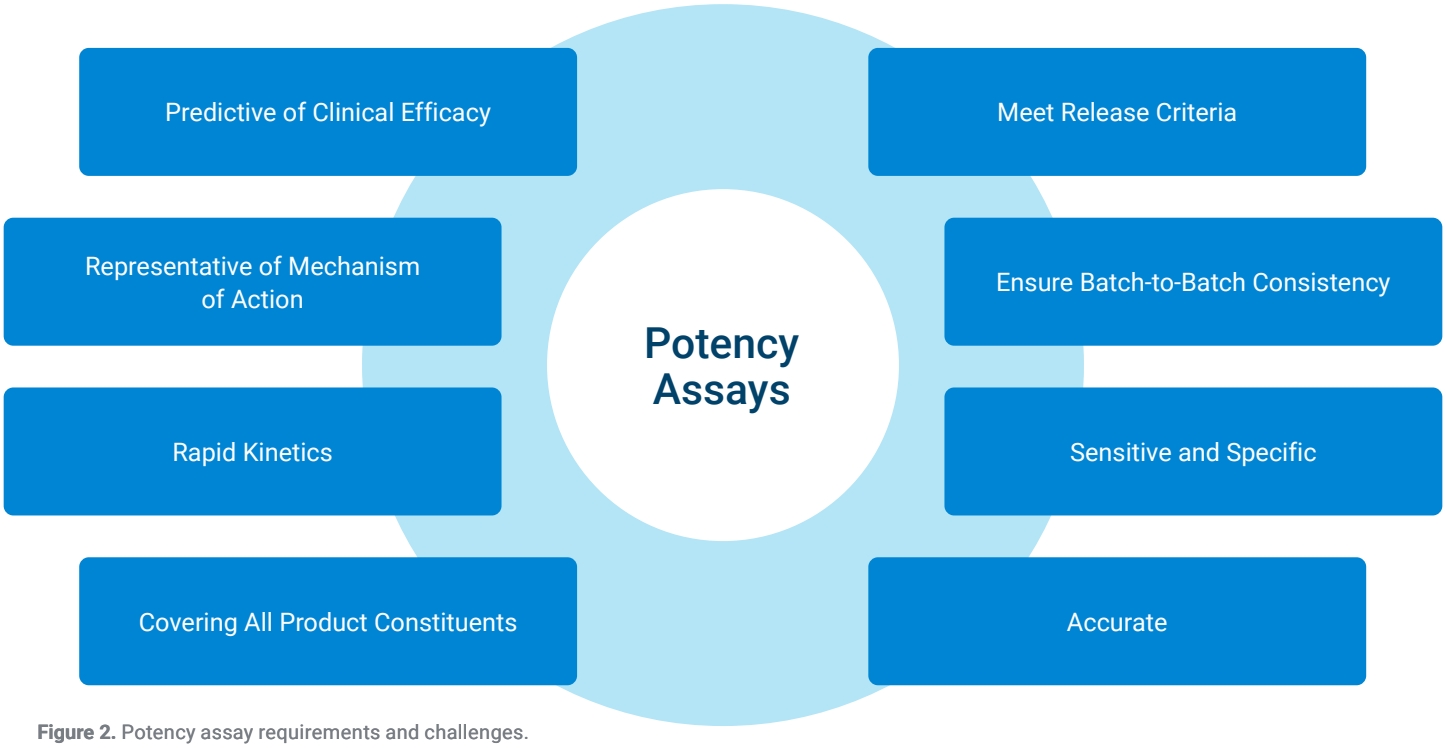


Figure 2. Potency assay requirements and challenges.

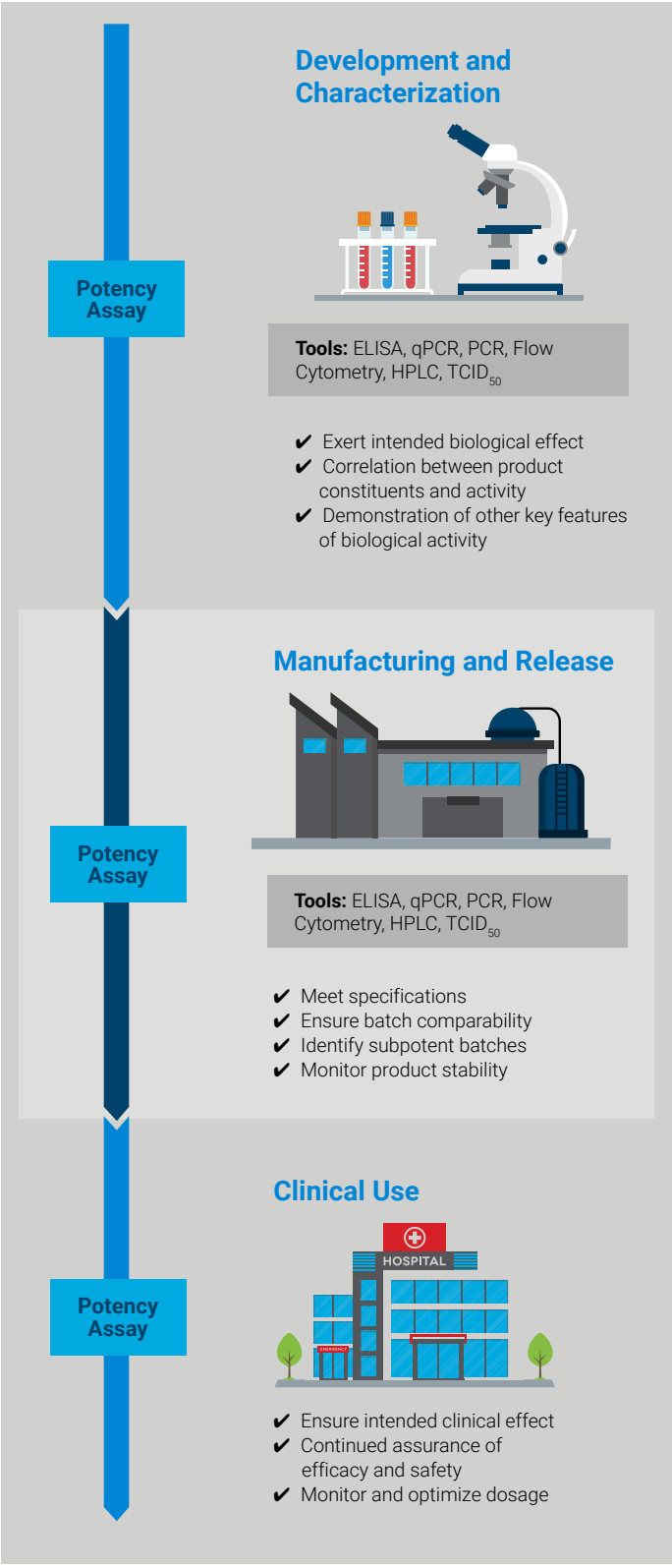


Figure 3. Significance of potency assays for cell therapies during product development.

selected, assay validation is performed and critical quality attributes (CQAs) are identified. Appropriate reference standards and controls must be selected, along with predefined pass/fail release criteria.[23] It is a mandatory requirement that potency testing be carried out throughout the process development and during manufacturing to ensure batch consistency, product stability, and efficacy (Figure 3).[3,24,25] Clinical efficacy and safety of cell therapies are closely linked to the potency of engineered immune cells, and even small deviations in potency may result in lack of effect or adverse events in highly sensitive patients.[4] Clinical studies can be relatively insensitive to minor changes in potency due to patient variability, therefore potency testing must also continue throughout commercial production. These potency assays must be tolerant of the inherent heterogeneity of cellular products but also sensitive to meaningful changes in potency to ensure consistency and safety.[3,26,27]

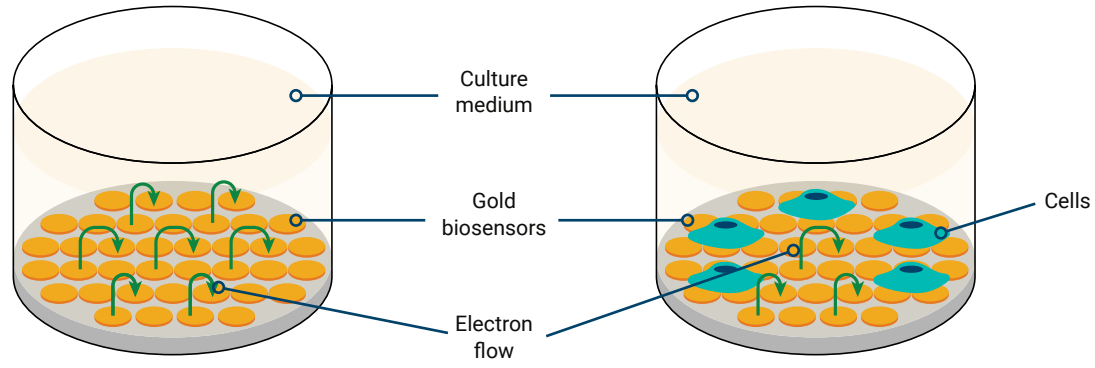
The challenges of developing robust potency assays

As cellular immunotherapies are so unique, appropriate potency assays can vary significantly from product to product. It is essential that these potency assays capture the dynamic interactions between the therapeutic and the target cell. For CAR products, potency assays are predominantly cytotoxicity-based, aligning with the main mechanism of action of both T cells and NK cells. In some cases, surrogate markers of cytotoxic activity are employed, such as the induction of degranulation markers (CD107a or granzyme B), or of inflammatory and pro-apoptotic cytokines (most commonly IFN-γ, TNFα or IL-2).[28] The four most common *in vitro* cytotoxicity assays are cellular impedance, bioluminescence, and flow cytometry. The chromium release assay has, in the past, been considered the standard of functional potency testing for cellular immunotherapies. In this assay, target cells are labeled with radioactive chromium (^{51}Cr) and incubated with the activated CAR cells. Following effector-mediated killing, ^{51}Cr is released into the culture medium, proportionally to the number of cells destroyed.[29] Other cytotoxicity assays include the bioluminescence luciferase assay (in which cytotoxicity is assessed by the relative reduction in luminescence emitted by target cells expressing a luciferase transgene) and flow cytometry (whereby specific target cell populations are enumerated using fluorescently-labeled antibodies).[30,31]

Label-based assays, such as chromium release and bioluminescence assays, rely on target cells stably expressing a transgene, or being labeled by the radioactive isotope. This

can be time- and labor-consuming and is not necessarily suitable for every type of cell. Moreover, the nature of these label-based assays requires multiple manual procedures, which can easily increase variability. In addition, the use of radioactivity in the chromium release assay requires a level of infrastructure and licensing that not all laboratories may possess, the radioactivity involved can be harmful to lab personnel, and cells can leak chromium non-specifically.

Despite the range of potency tests available for cell therapies, numerous challenges remain in developing robust, accurate assays with a rapid turnaround. The autologous nature of many cell therapies such as CAR T cells poses multiple challenges in potency assay development. Limited availability of cells for testing means that assays must be developed to use as little material as possible. In addition, the high levels of heterogeneity between patients make quantification of CQAs difficult due to a lack of standardized reference material.[23] Several of these common cytotoxicity assays, such as the chromium release assay and bioluminescence assay, are endpoint in nature. Therefore, complex kinetic data modeling of the dynamic interactions of target and effector cells can



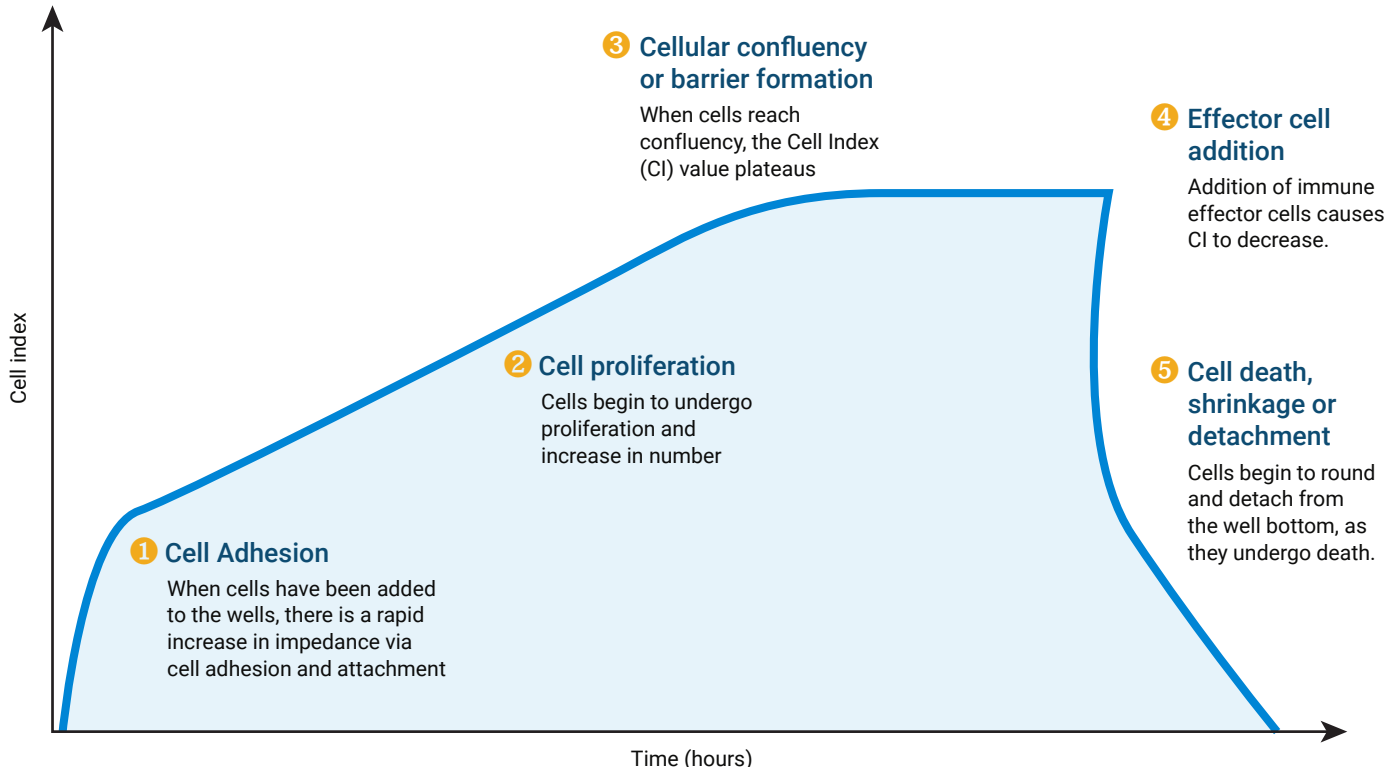


Figure 4. The mode of action of a cellular impedance assay.

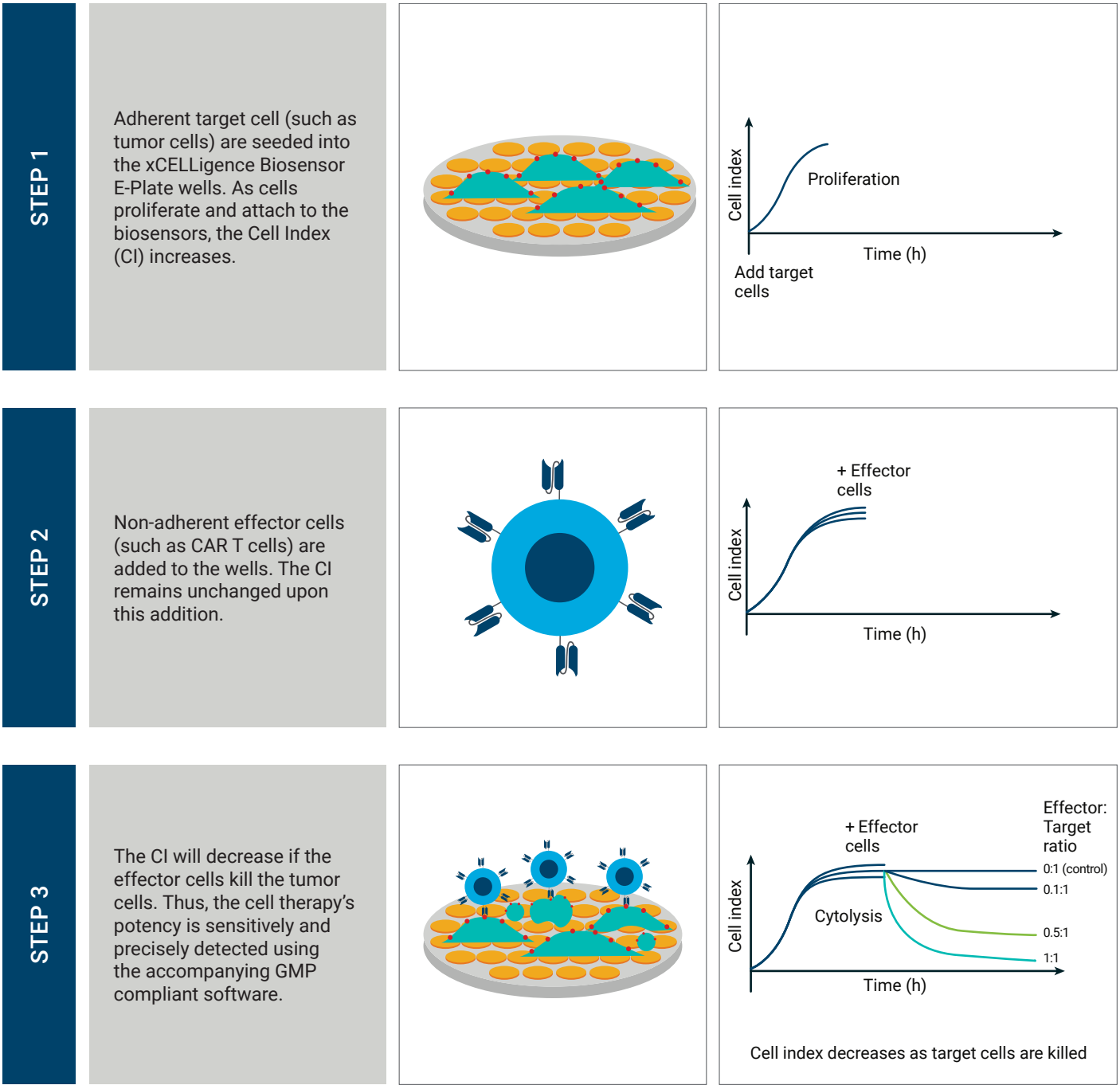


Figure 5. The xCELLigence RCTA workflow utilizes impedance to monitor cell killing.

be lost, as can information on effector cell persistence. Rates and efficiency of effector cell killing can vary over the course of the experiment, making endpoint dependent assays less reliable than real-time analysis, as endpoint assays can only provide a single value. In addition, these assays can be time-consuming with complex setups, effectively restricting their throughput capabilities and their utility for certain autologous therapies that require rapid turnarounds.

While other potency assay options can surmount some of the challenges of complex endpoint assays, they too have their own limitations. For example, flow cytometry assays offer both direct measurement of potency (e.g., measuring CD19 levels as a direct evaluation of malignant B cell killing by CAR T cells) and the simultaneous analysis of various surrogate markers (e.g., CD107a as a marker of cytotoxic T cell degranulation).[32,33] However, some studies suggest that these

surrogate markers of *in vitro* cytotoxicity do not necessarily show a significant correlation with clinical efficacy.[34] *In vivo* models of potency can offer a more accurate picture of clinical efficacy, but animal studies are lengthy, expensive, and require large numbers of animals, again limiting their utility for potency assay development. With many of the conventional potency assay options for cellular immunotherapies beset by multiple challenges and limitations, there is a clear need for a label-free, non-invasive, and sensitive solution that is able to produce results in real-time.

The Real-Time Cell Analysis solution

Cellular impedance assays have gained traction in recent decades as a label-free, non-radioactive alternative. This assay measures the flow of an electric current between microelectrodes embedded in the base of a biosensor plate (Figure 4). Impedance of this current is correlated with the number of cells in the well, in addition to their viability, adhesion, and morphological characteristics.[35,36] Cell impedance is measured in real time as "cell index" (CI), a unitless scale that changes in proportion to the number of cells over time.[37]

These real-time cellular assays are increasingly being used to determine potency in cellular immunotherapeutics. They can overcome the challenges raised by other popular potency methods. Cellular impedance assays do not require radiation and are both label-free and non-invasive. The real-time element allows the analysis of both cytotoxicity and cellular persistence. In addition, when compared directly to chromium release assays, cellular impedance methods show increased sensitivity to cellular lysis, making them an ideal solution for the potency testing of cellular immunotherapies such as ACTs.[38,39]

The Agilent xCELLigence Real-Time Cell Analysis (RTCA) platform offers a robust, simple, and automated solution for cellular potency assays. The system measures quantitative real-time kinetics with extremely high sensitivity and allows for the study of diverse populations of effector and target cells. xCELLigence RTCA technology can monitor cell number, size, morphology, and attachment through cellular impedance in both single plate and high-throughput scenarios. The ability to house these instruments inside standard tissue culture incubators or hypoxia chambers, enables fine-tuned control of environmental conditions for live cell analysis. In addition, the combination of cellular impedance with live cell imaging in the xCELLigence RTCA eSight allows for increased confidence in results and comprehensive insight into cellular health and potency.

Each well of the specialized xCELLigence Biosensor E-Plates is coated with gold biosensors that form an interdigitating array covering approximately 70-80% of the surface of the wells. This allows for large populations of cells to be measured simultaneously, with top-notch sensitivity. For measuring potency of cellular immunotherapies such as CAR T cells, the workflow is more convenient and simpler than other label-based assays (Figure 5). Adherent target cells (i.e., tumor cells) are cultured in the E-Plate wells, and the CI increases as they proliferate. As confluence approaches 100%, non-adherent effector cells (e.g., CAR T cells) are added. Immune effector cells are non-adherent and therefore typically produce a minimal impedance signal. Cytotoxic action by the effector cells will decrease CI, which can be evaluated in real time with cGMP and 21CFR Part 11-compliant software, enabling an accurate, immediate assessment of therapeutic potency.

xCELLigence RTCA solutions are already being used extensively for cancer research around the globe, with proven effectiveness in a variety of immunotherapy applications, including CAR cell therapeutics, antibody-mediated cytotoxicity, and oncolytic viruses.[40] xCELLigence impedance assays for CAR T cell potency against liquid cancers have been tested and validated, confirming their potential applications for this type of immunotherapeutic.[41] Non-adherent liquid tumor cells (e.g., leukemic cancer cells) stay in suspension and thus require the use of a tethering kit for impedence readout. Agilent's xCELLigence Immunotherapy Kits provide a tethering reagent (for example anti-CD29 or anti-CD19) to enable non-adherent liquid cancer cells to be immobilized for the assay.

Development of CAR T therapies against solid tumors has also benefited from xCELLigence technology, as the RTCA technology has been used to validate the potency of advanced CAR T cells against mesothelioma and pancreatic cancer models.[42] In addition, these assays have also shown that CAR T cell cytotoxicity against solid tumors is unaffected by cryopreservation, which is key in developing therapies that can be stably stored.[43] In the developing field of CAR NK cells, xCELLigence-based potency assays have proven the cytotoxic efficacy of genetically modified NK cells and shown the ability of CAR NK cells to work synergistically with current therapies.[44,45]

The benefits of xCELLigence impedance assays are not limited to cellular cancer therapies. Investigation of potency assays for monoclonal antibody immunotherapeutics have shown that impedance-based techniques outperformed standard release and membrane permeability assays, with far more sensitive and accurate results.[46] In addition, the 2022 "Recent Issues in Bioanalysis" workshop with thought leaders in industry and regulatory bodies, highlighted

the xCELLigence RTCA system as the optimum move-forward platform for characterization and clinical testing of oncolytic viruses, over the traditional plaque assay.[47] On the regulatory compliance side, xCELLigence RTCA Software Pro also supports the requirements of FDA 21 CFR Part 11 to ensure the authenticity and integrity of electronic data. These high levels of data security protect the confidentiality of research information, and aid in the generation of highly accurate, reliable results, which are essential to progress through the pre-clinical and clinical stages of biotherapeutic developement.

Conclusion

As the field of cellular immunotherapies continues to develop, the need for robust and reproducible potency assays becomes increasingly urgent. For immunotherapies, cellular impedance assays using the Agilent xCELLigence RTCA platform can overcome the limitations of traditional endpoint assays and provide real-time, label-free, rapid, and highly sensitive insights into cell potency, and thus cell killing. As such, the xCELLigence RTCA system can help advance the development and manufacturing of various cellular immunotherapeutics. Lastly, it can ensure compliance with regulatory guidelines, facilitate significant time and cost savings, and thus support the development of novel therapeutics.

Learn more about xCELLigence cell analyzers

Learn more about how immune cell metabolism is changing the immuno-oncology discovery game

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RA45320.6538773148

Printed in the USA, February 15, 2024
5994-7162EN

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