Application Note

Cell Analysis

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Evaluating Functional Potency of Immunotherapies Targeting Liquid Tumors

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Abstract

This application note describes use of a tethering approach to immobilize liquid tumor cell lines of various origins on the surface of gold biosensors embedded in the bottom of an electronic microplate (Agilent E-Plate). Using this approach, multiple common leukemic cell lines, such as Raji, were successfully tethered to the E-Plate biosensors, resulting in a robust impedance signal. After the tethered cells attained a certain level of growth and confluence, effector cells were added at different effector:target (E:T) ratios, resulting in a dose-proportional decrease in Cell Index (CI). For the assay to be accurate, it is important that the tethering reagent is selective for the target cells, precluding any impedance signal derived from the effector cells.

Introduction

A growing understanding of the molecular interactions between immune effector cells and target tumor cells, coupled with refined gene therapy approaches, is giving rise to novel cancer immunotherapeutics with remarkable efficacy against both solid and liquid tumors. The most successful immunotherapies are those targeting blood-borne tumors. Chimeric Antigen Receptor (CAR) T cell therapy has been one of the most prominent breakthroughs in cancer immunotherapy for relapsed and refractory hematopoietic malignancies. With the FDA approval of CD19-directed CAR T for acute lymphoblastic leukemia, non-Hodgkin lymphoma, diffuse large B cell lymphoma, and the designation of Breakthrough Therapy for B cell maturation antigen (BCMA) directed CAR Ts for multiple myeloma, this technology has generated great excitement in the scientific community and it has resulted in numerous basic, applied, and preclinical studies worldwide. Effector cell potency assays are essential for assessing the functional activity of therapeutic products prior to clinical use. Among these, impedance-based technologies, such as Agilent xCELLigence real-time cell analysis (RTCA), have been well established for evaluating effector cell-mediated cytotoxicity against target cancer cells.[1-9] In this application note, Agilent aimed to adapt the Agilent xCELLigence Real-Time Cell Analysis (RTCA) potency assay for in vitro assessment of immunotherapies targeting tumor cell lines originating from liquid tumors.

Potency assay principle targeting hematopoietic tumors

The wells of the Agilent E-Plate are precoated with a tethering reagent specific for a cell surface marker expressed on the liquid cancer cell, enabling the suspension cells to be immobilized and adhere to the plate bottom embedded with biosensors (Figure 1). As tethered cells proliferate, the electric current flowing between the biosensors is impeded. The magnitude of this impedance reflects the collective effects of changes in cell number, size and attachment quality. Addition of immune effectors (such as NK and CAR T cells, oncolytic viruses, checkpoint inhibitors, and bispecific antibodies) results in time- and density-dependent target cell destruction, and the corresponding cytolytic activity is sensitively and continuously detected. This strategy allows assessment of the functional potency of immunotherapies targeting specific cancers of hematopoietic origin, with enhanced reproducibility and throughput using a simple workflow. Table 1 illustrates widely studied liquid cancer cell lines that have been immunophenotyped and validated for selective tethering approaches and the xCELLigence RTCA potency assay.

Protocol: Liquid tumor immunotherapy potency assay

This application note describes the experimental setup for assessing effector-mediated cytolysis of various liquid tumor cell lines. While the natural killer cell line, NK-92, is used as an example, other effector cells, such as CAR T cells, can also be used. Peripheral blood mononuclear cells (PBMCs) are another option; however, since PBMCs represent a heterogeneous population, certain immune cell types may be captured by specific tethering reagents. Optimal effector-to-target (E:T) ratios should be empirically determined prior to the assay. This protocol allows the identification of target cell killing kinetics as well as the optimal time point for cytotoxicity with different effector:target ratios.

This protocol has been developed for continuous monitoring of cell killing over two days, allowing an initial day for target cells to attach to and proliferate in the biosensor plates. Current tethering kits cover a subset of liquid tumor models that express the corresponding antigens. As is known, the effectiveness of tethering depends on both the reagent's affinity for the antigen and the antigen expression level on the target cells. Robust attachment of target cells to the biosensor is essential for generating reliable impedance signals, and antigen expression can be influenced by various factors, such as cell health and culture conditions. Like other cell-based assays, it is critical to optimize key handling parameters. These include the appropriate cell passage range, seeding density, and assay medium. Such optimization is especially important for cell types not listed in Table 1. Moreover, as impedance is an exceptionally sensitive readout, maintaining consistent cell conditions and using a standardized passage range is strongly recommended to ensure reproducibility across experiments.

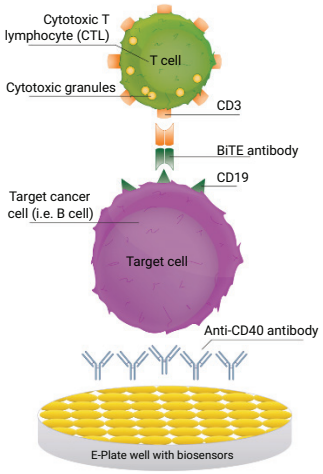

Figure 1. Precoating the wells of an Agilent E-Plate with a tethering reagent enables liquid tumor cells to proliferate on, and be detected by, biosensors.

Table 1. Widely studied model liquid cancer cell lines that have been immunophenotyped and validated for selective tethering approaches and the Agilent xCELLigence RTCA potency assay.

Cancer Type	Validated Cell Line	Recommended Cell Passage	Seeding Density (Cells/Well)	Selective Tethering Mechanism	E-Plate Type	Assay Time (Days)
Acute Lymphoblastic Leukemia (ALL)	NALM6	≤ 15 passages*	35 ± 5 x 10^3	CD9	E-Plate 96, E-Plate VIEW 96	≤ 3**
Chronic Myelogenous Leukemia (CML)	K562	≤ 15 passages*	20 ± 5 x 10^3	CD71	E-Plate 96 PET, E-Plate 96, E-Plate VIEW 96	≤ 3**
Non-Hodgkin Lymphoma (NHL)	Raji	≤ 15 passages*	35 ± 5 x 10^3	CD19	E-Plate 96, E-Plate VIEW 96	≤ 3**
			35 ± 5 x 10^3	CD40	E-Plate 96 PET, E-Plate 96, E-Plate VIEW 96	
Multiple Myeloma (MM)	RPMI 8226	≤ 15 passages*	25 ± 5 x 10^3	CD9	E-Plate 96 PET, E-Plate 96, E-Plate VIEW 96	≤ 3**

*Cell passages up to 15 post-purchase have been validated. Use of cells at higher passages will require additional assay optimization.
**If an extended assay duration (>3 days) is desired, further optimization will be required.

Day zero:
Immobilization of liquid tumor cells

a. Coat E-Plate 96 with diluted tethering reagent for three hours. Wash, add medium, and take the background impedance (CI) measurement.

b. Prepare liquid tumor target cells, and add to the coated wells.

c. To allow the cells to settle, leave the plate at room temperature for at least 30 minutes.

d. Load the plate into an xCELLigence RTCA instrument, and start data acquisition to monitor target cell attachment and proliferation.

Day one:
Addition of immunotherapeutic effectors

e. Prepare NK-92 effector cells.

f. Pause xCELLigence data acquisition; remove the plate from the instrument and place inside a laminar flow hood. Add effector cells at different E:T ratios to the target cells. If it is necessary to remove some of the existing medium, using a pipette rather than a vacuum aspirator is recommended.

g. Place the plate back into the xCELLigence RTCA instrument, and start data acquisition to monitor effector cell-mediated killing of immobilized target cells. Collecting data at 15-minute intervals is recommended; however, the exact data collection frequency can be determined by the user.

Days one to three:
Assessment of cancer cell destruction

h. Continue data acquisition for as long as desired.

i. Analyze data using the Immunotherapy module of the RTCA Software Pro.

   – If an extended assay duration, longer than three days, is desired, further assay optimization is required

Table 2. Representative data for NK-92-mediated cytolysis of various target cell lines.

Cancer Type	Cell Line	E:T Ratio	Percent Cytolysis (Hours)			KT_{50} (Hours)
			4 Hours	24 Hours	48 Hours	
Acute Lymphoblastic Leukemia (ALL)	NALM6	1:1	9	86	87	17.5
Chronic Myelogenous Leukemia (CML)	K562	1:1	28	70	74	9.5
Non-Hodgkin Lymphoma (NHL)	Raji	1:1	58	88	84	3.5
Multiple Myeloma (MM)	RPMI 8226	1:1	28	75	81	17.2

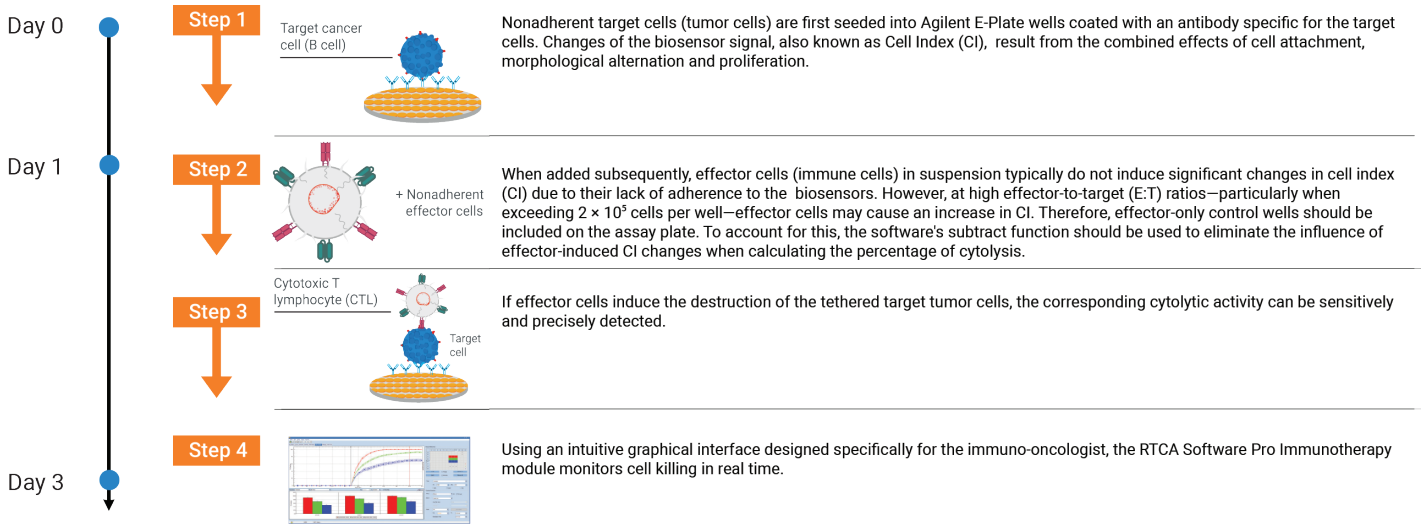

Figure 2. Monitoring immune cell-mediated killing of immobilized liquid cancer cells in real-time using Agilent xCELLigence biosensor E-Plates.

Results and discussion

Real-time leukemia and multiple myeloma killing by NK-92 cells

To evaluate the potency of a cytotoxic NK-92 cell treatment of liquid cancers as a function of time using the xCELLigence RTCA potency assay, target tumor cells were immobilized onto the plate bottom by the indicated selective tethering mechanisms. Various doses of NK-92 effector cells were added to the tumor cells to achieve different E:T ratios. The cytolysis of target cells was detected by the biosensors, and reported as a decrease of impedance (CI), which was monitored by the xCELLigence RTCA instrument over the next one to two days. Figures 3A and 3B illustrate the liquid tumor cell attachment through cell surface marker-specific tethering on the biosensor-embedded wells. As expected, the efficiency with which the tethered target cells are killed depended on the ratio between NK-92 effector cells and the target cancer cells. Target cells alone (E:T=0:1) are used as negative controls for cytolysis.

While the CI decrease after effector addition directly correlates with cell viability, it can be readily converted to percent cytolysis through mathematical calculations that take into account the signal from the target cells alone control. As shown in Figures 3C and 3D, percent cytolysis increases in a time and E:T ratio dependent manner. For the two liquid tumor cell lines shown in Figure 3, at several E:T ratios tested, the percentage of cytolysis reaches a plateau after 40 to 45 hours that is less than 100%, indicating incomplete lysis by NK-92. We speculate that at low E:T ratios, the effector cells are a limiting factor and cannot kill all the target cells, which continue to proliferate. It is possible that after a certain period of coculturing, the two cell populations eventually reach an equilibrium in cell number, reflected by the plateauing of the signal. Figures 3E and 3F show the extent of NK-92-mediated cytolysis of target cells at different E:T ratio at either four or 24 hours after NK-92 addition.

One major advantage of continuous impedance-based monitoring is that the time dependency of cytolysis is captured at a high frequency of measurements defined by the user (as frequently as every 30 seconds. However, a 15-minute interval is typically sufficient to reliably measure cytolytic effects of treatments), which can be challenging with traditional end-point approaches. So, kinetic parameters that encompass such temporal information can be effectively derived. One example is the KT_{50} parameter that represents the time required to achieve 50% cytolysis at a given E:T ratio. A lower KT_{50} value signifies a more efficient cytolytic kinetic (representative data, Table 2). As expected, at a constant E:T ratio of 1:1, there is a wide range of NK-92 killing efficiency against broad spectrum of liquid cancer types. While the percent of cytolysis parameter shows the potency of a specific E:T ratio at a given time point, the KT_{50} parameter provides the temporal dimension and insights for the rate of cell killing based on the target cell type.

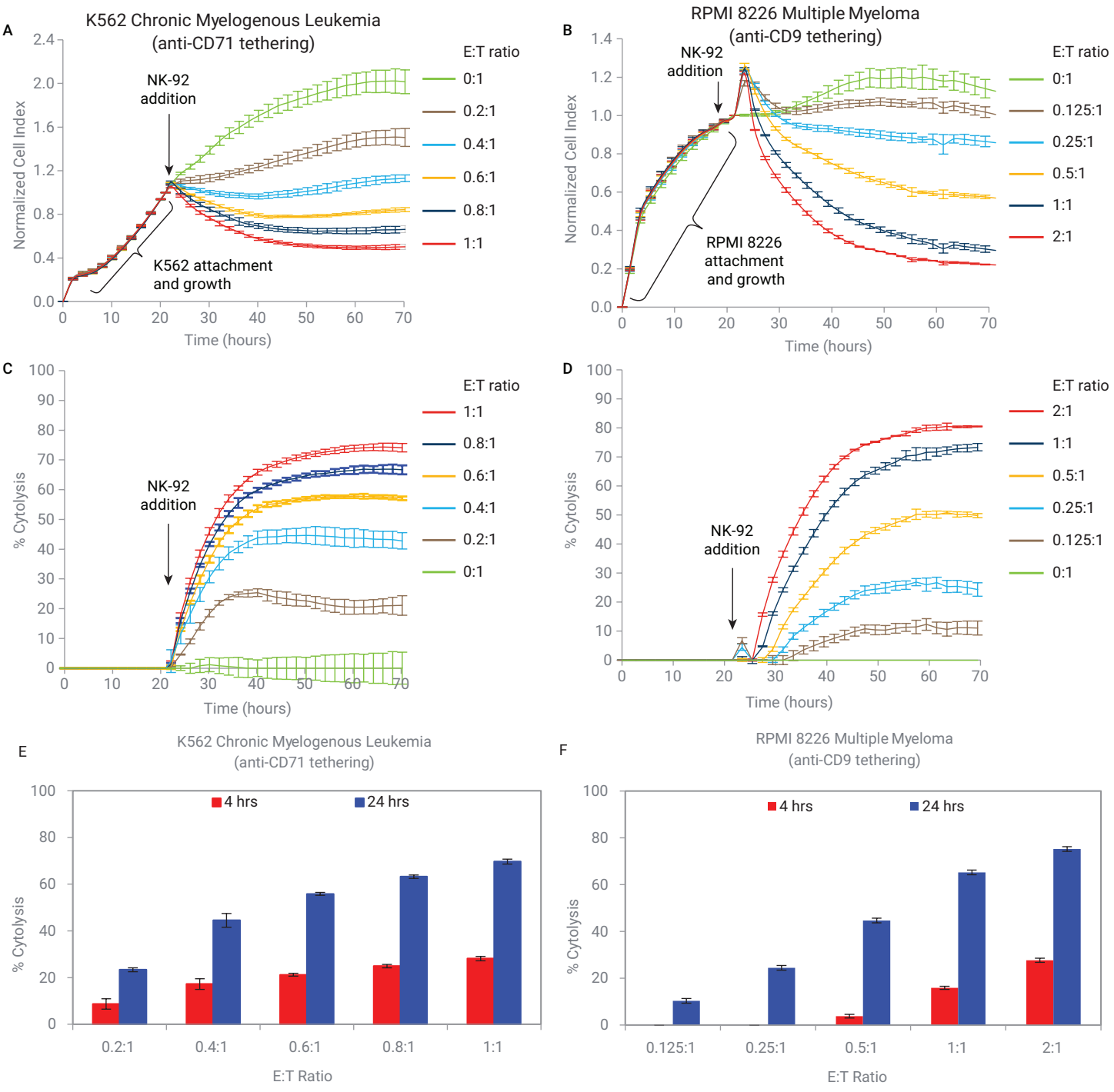

Figure 3. An example of a typical liquid tumor potency assay. (A, C, E) K562 cells, tethered by anti-CD71 antibody, and (B, D, F) RPMI 8226 cells, tethered by anti-CD9 antibody, were seeded at 30,000 and 60,000 cells/well, respectively. (A, B) When left untreated, the immobilized K562 and RPMI 8226 cells proliferate to the point of confluence. However, upon addition of increasing quantities of effector NK-92 cells, the impedance signal decreases in a dose-dependent manner. Samples have been internally normalized for the CI value measured before NK-92 addition (Normalized CI). (C, D) The CI plot is converted to a percent cytolysis plot by the Agilent xCELLigence immunotherapy software. (E, F) Percent cytolysis measured at four and 24 hours after NK-92 addition for different E:T ratios.

Conclusion

We have demonstrated the utility of the Agilent xCELLigence Real-Time Cell Analysis instrument to evaluate the potency of an immunotherapeutic against a broad spectrum of liquid tumors while monitoring the cytolysis kinetics of liquid cancers at physiologically relevant E:T ratios. This protocol involves less work than traditional assays. Target liquid tumor cells were seeded and tethered into a precoated E-Plate, after which effector cells were added, and the kinetics of cancer cell destruction was noninvasively monitored over the course of days. Data acquisition was continuous and automatic. The quantitative and real-time nature of the impedance data made it easy to compare the potency between immunotherapy treatments and dosages.

Using this surface-tethering approach, effector cells as well as biological molecules such as Bispecific T cell Engagers (BiTEs), and blocking antibodies (for example, against the immune checkpoint inhibitor PD-1) can easily be assayed.[4] The xCELLigence platform is well suited for liquid cancer potency assessments, providing quantitative evaluation with high reproducibility and a simplified workflow.

Suggested reading

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Products used in this application

Agilent products

Agilent xCELLigence Real-Time Cell Analyzers

Agilent xCELLigence RTCA E-Plates

Agilent xCELLigence RTCA Reagents, Kits, and Accessories

Agilent xCELLigence RTCA Software

www.agilent.com/lifesciences/rtcaanalyzers

Published in the USA, June 27, 2025
5994-1066EN

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