Application Note

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Evaluating Functional Potency of Immunotherapies Targeting Tumors of B Cell Origin

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Abstract

This application note describes use of a tethering approach to immobilize liquid tumor cell lines of various origins on to 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 in the tests 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 recent 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. It has resulted in numerous basic, applied, and preclinical studies worldwide. Agilent aimed to adapt the 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 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, electric current flow between the biosensors is impeded. The magnitude of this impedance depends on the cell number, size, and attachment quality. Addition of immune effectors (such as NK cells, CAR-T, oncolytic viruses, checkpoint inhibitors, bispecific antibodies, and so on) 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 much enhanced reproducibility and throughput using a simple workflow. Table 1 illustrates widely studied model liquid cancer cell lines that have been immunophenotyped and validated for selective tethering approaches and the RTCA potency assay.

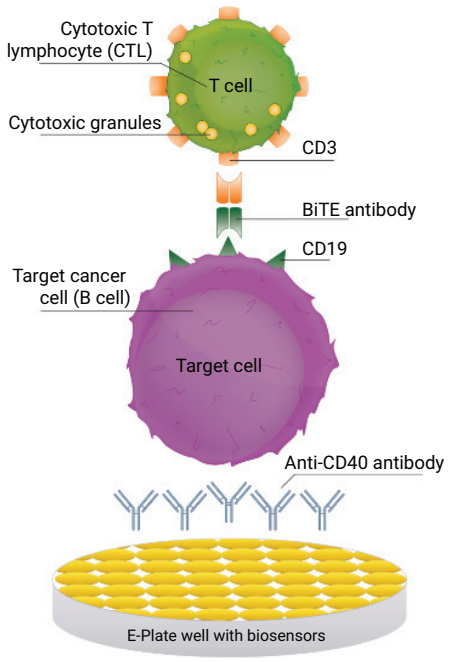

Figure 1. Precoating the wells of an Agilent E-Plate with a tethering reagent enables liquid tumor cells to proliferate on, and be detected by, biosensors.

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

Cancer Type	Validated Cell Line	Selective Tethering Mechanism	Verified Target Expression by Flow Cytometry
Acute Lymphoblastic Leukemia (ALL)	NALM6	CD9	CD3-, CD9+, CD19+, CD20-, CD22+, CD38+, CD138+, CD269+, HLA-DR+
	RS4;11	CD9	CD3- , CD9+, CD14+, CD19+, CD20-, CD22+, CD38+, CD138+, CD269+, CD123+(low)
Chronic Myelogenous Leukemia (CML)	K562	CD29 or CD71	CD3-, CD14-, CD15+, CD19-, CD29+, CD33+, CD71+, CD235a+
Non-Hodgkin Lymphoma (NHL)	Daudi	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
	Raji	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
	Ramos	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
Multiple Myeloma (MM)	RPMI 8226	CD9	CD3-, CD9+, CD19-, CD38+, CD138+, CD269+, HLA-DR+
	MM1R	CD9 and CD71	CD3-, CD9+, CD19-, CD38+, CD71+, CD138+, CD269+, HLA-DR+
Acute Myeloid Leukemia (AML)	HEL 92.1.7	CD29	CD13+, CD29+, CD33+, CD15+, CD123+
Chronic Lymphocytic Leukemia (CLL)	MEC2	CD40	CD3-, CD13+, CD19+, CD20+, CD40+, CD138+

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 NK-92 natural killer cell line is used as an example, any type of effector such as CAR-T or peripheral blood mononuclear cells (PBMCs) can be used. 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 four days, allowing an initial day for target cells to attach to and proliferate in the Agilent E-Plate VIEW 96 wells.

Day one: Immobilization of liquid tumor cells

a. Coat E-Plate VIEW 96 with diluted tethering reagent for three hours. Wash, add medium, and take 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 two: Addition of immunotherapeutic effectors

e. Prepare NK-92 effector cells.

f. Pause xCELLigence data acquisition; remove the plate from instrument and place inside a laminar flow hood. Remove nascent media from wells, but avoid disturbing the attached cells. Use a manual multichannel pipette to remove media rather than a vacuum aspirator. Add effector cells at different E:T ratios to the target cells.

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 30 minute intervals is recommended; however, the exact data collection frequency can be determined by the user.

Days two to five: Assessment of cancer cell destruction

h. Continue data acquisition for as long as desired.

i. Analyze data using RTCA Software Pro.

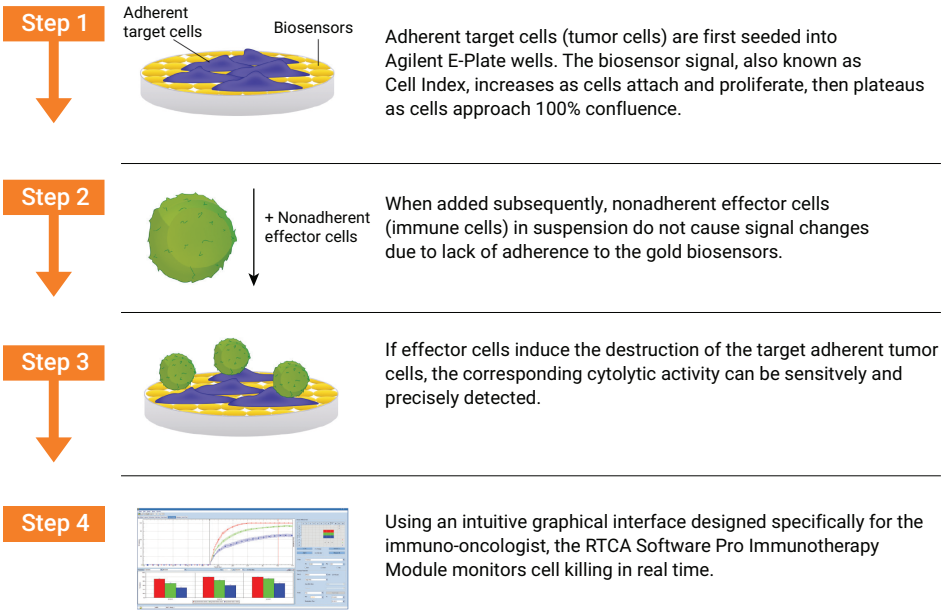

Figure 2. Monitoring immune cell-mediated killing of immobilized liquid cancer cells in real-time using Agilent xCELLigence gold biosensors.

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 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 destruction of target cells were detected by the biosensors, and reported as a decrease of impedance (CI), which was monitored by the 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 NK92. 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.

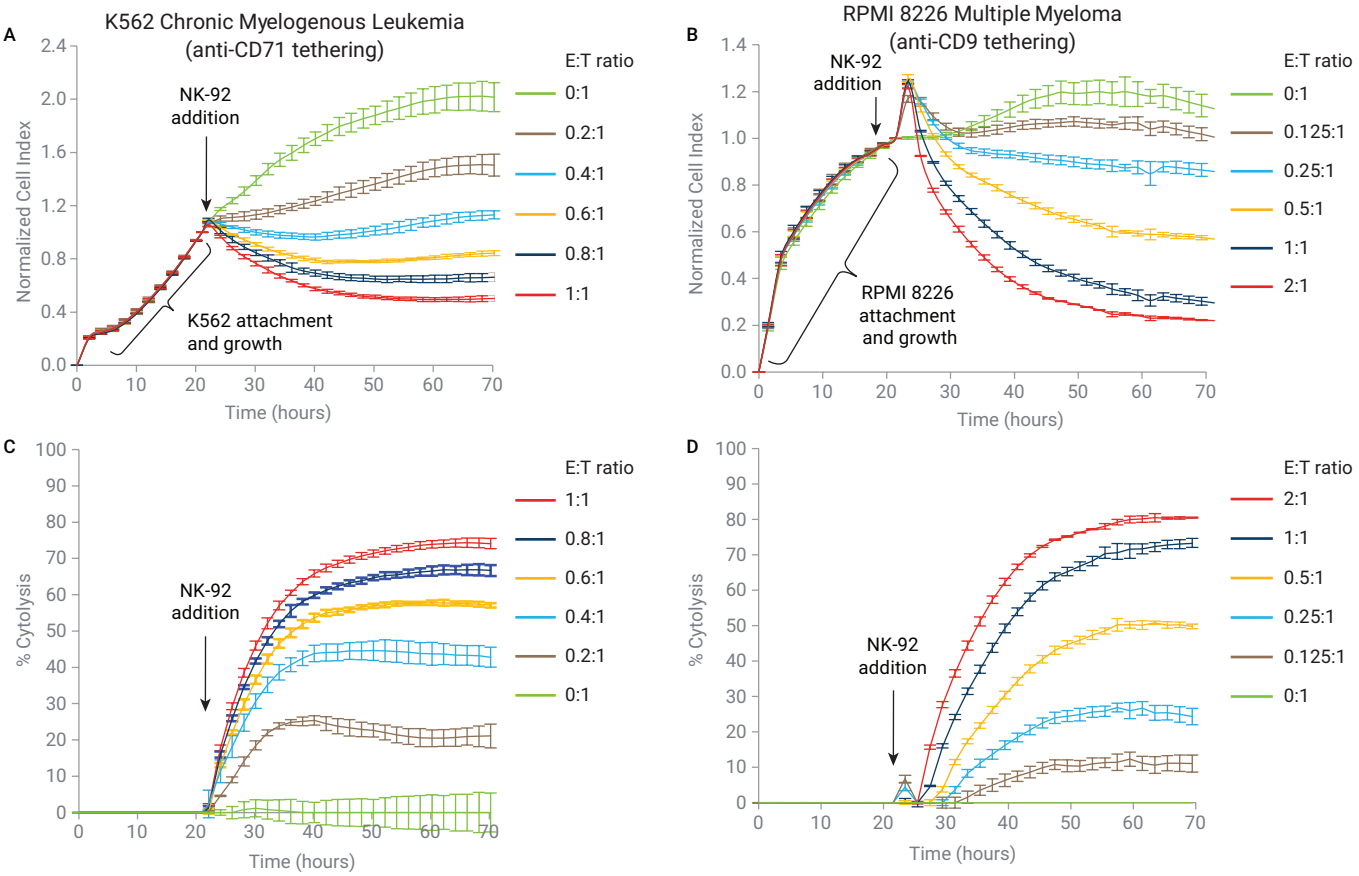

Figure 3. Liquid tumor potency assay. (A, C, E) K562 cells, tethered by anti-CD71 antibody, and (B, D, F) RPMI 8226 cells, tethered by anti-CD9 antibody, were seeded at 30,000 and 60,000 cells/well, respectively. (A, B) When left untreated, the immobilized K562 and RPMI 8226 cells proliferate to the 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 NK92 addition (Normalized CI). (C, D) The CI plot is converted to a %cytolysis plot by the Agilent xCELLigence immunotherapy software. (E, F) %cytolysis measured at four and 24 hours after NK92 addition for different E:T ratios.

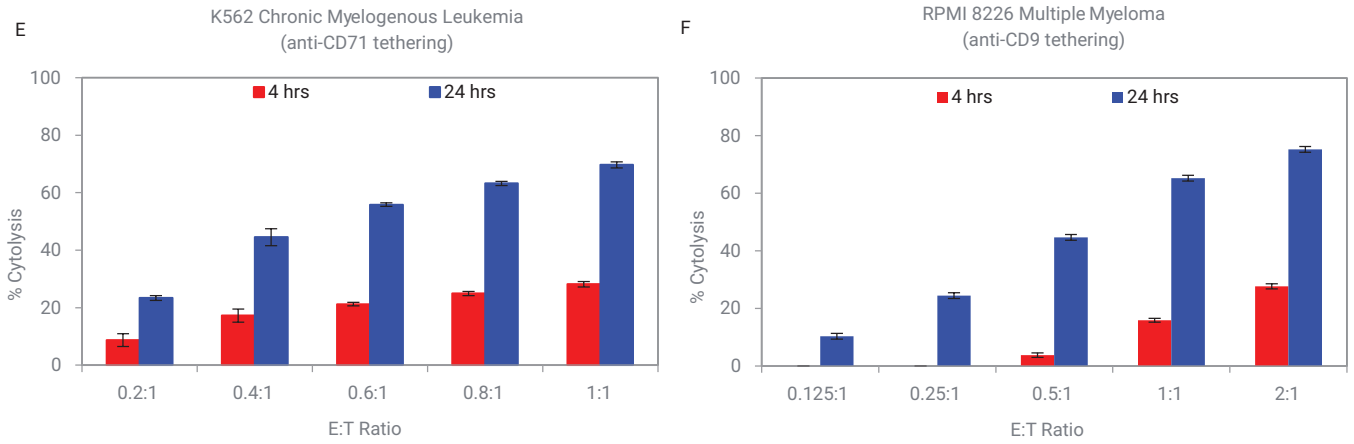

Figure 3 (continued). Liquid tumor potency assay. (A, C, E) K562 cells, tethered by anti-CD71 antibody, and (B, D, F) RPMI 8226 cells, tethered by anti-CD9 antibody, were seeded at 30,000 and 60,000 cells/well, respectively. (A, B) When left untreated, the immobilized K562 and RPMI 8226 cells proliferate to the point of confluence. 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 NK92 addition (Normalized CI). (C, D) The CI plot is converted to a %cytolysis plot by the Agilent xCELLigence immunotherapy software. (E, F) %cytolysis measured at four and 24 hours after NK92 addition for different E:T ratios.

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 (for example, every 10 seconds), 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 NK92 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.

Table 2. Representative data.

Cancer Type	Cell Line	E:T Ratio	%Cytolysis			KT_{50}
			4 Hours	24 Hours	48 Hours	
Acute Lymphoblastic Leukemia (ALL)	NALM6	1:1	9	86	87	17.5
	RS4;11	1:1	5	77	79	15.4
Chronic Myelogenous Leukemia (CML)	K562	1:1	28	70	74	9.5
Non-Hodgkin Lymphoma (NHL)	Daudi	1:1	7	74	82	13
	Raji	1:1	58	88	84	3.5
	Ramos	1:1	0	45	60	29
Multiple Myeloma (MM)	RPMI 8226	1:1	28	75	81	17.2
	MM1R	1:1	63	97	97	3
Acute Myeloid Leukemia (AML)	HEL 92.1.7	1:1	0	60	75	17
Chronic Lymphocytic Leukemia (CLL)	MEC2	1:1	0	53	63	20.9

Conclusion

We have demonstrated the utility of the xCELLigence RTCA to evaluate the potency of an immunotherapy against a broad spectrum of liquid tumors while monitoring the destruction 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, several effector cells (PBMC, NK, CAR-T, as well as biological molecules such as Bi-specific T cell Engagers (BiTEs)), target the EpCAM protein expressed on tumor cells and block antibodies against the immune checkpoint inhibitor PD-1 (Cerignoli *et al.* 2018). The xCELLigence platform is well suited for liquid cancer potency assessments, providing quantitative evaluation with high reproducibility and a simplified workflow for manufactured immuno-oncology therapies.

References

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