

xCELLigence® Immunotherapy Kit Liquid Tumor Killing Assay (anti-CD40)

ASSAY MANUAL

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I. List of Kit Components

Product	Catalog Number
Liquid Tumor Killing Assay (anti-CD40) Complete Kit	8100004
E-Plate® View 96 (1 box of 6 Plates)	
Tethering Reagent (anti-CD40)	250 µL
10X Tethering Buffer	10 mL
Cytolysis Reagent	1.5 mL
RTCA Software Pro	
Liquid Tumor Killing Assay (anti-CD40) Tethering Kit	8100005
Tethering Reagent	
	250 µL
10X Tethering Buffer	
	10 mL
Cytolysis Reagent	
	1.5 mL
Liquid Tumor Killing Assay (anti-CD40) Sample Kit	8100006
E-Plate View 96 (2 Plates)	
Tethering Reagent (anti-CD40)	90 µL
10X Tethering Buffer	10 mL
Cytolysis Reagent	1.5 mL

*RTCA Software Pro – one month trial version can be downloaded for free by providing the serial number of an existing xCELLigence instrument

II. Storage Conditions

Tethering Reagent and Cytolysis Reagent should be stored undiluted at 4°C. **Do not freeze.**

The 10X Tethering Buffer and E-Plates can be stored at 25°C.

III. Additional Materials & Reagents Required

The following are required but are not supplied with this kit:

- Tissue culture media
- Tissue culture flasks/plates
- PBS without calcium and magnesium
- Sterile tissue culture grade water
- An xCELLigence instrument
- Liquid tumor target cells
- Immune effector cells
- Small molecule drugs and/or biological effector molecules (antibodies, etc.)

IV. Introduction

ACEA's xCELLigence Real-Time Cell Analysis (RTCA®) instruments utilize gold biosensors embedded in the bottom of microtiter plate wells to non-invasively monitor the status of adherent cells using the principle of cellular impedance. In short, adherent cells act as insulators – impeding the flow of an alternating microampere electric current between biosensors. This impedance signal is measured automatically, at a frequency defined by the user (every 10 seconds, once per hour, etc.), and provides an extremely sensitive readout of cell number, cell size/shape, and cell-substrate attachment strength. (For a more detailed explanation of cellular impedance, please see: www.aceabio.com/product/rtca-mp/).

Over the past decade xCELLigence has been used extensively to study immune cell-mediated killing of adherent cancer cells. This xCELLigence Immunotherapy Kit expands the repertoire of xCELLigence to include monitoring immune cell-mediated killing of non-adherent liquid cancers (B cells in particular). Below, the xCELLigence RTCA assay principle and work-flow are first described using adherent target cells as an example. After a general overview of how the xCELLigence RTCA assay has been adapted for monitoring liquid tumor cell killing, a detailed protocol is provided.

xCELLigence Immune Cell Killing Assay Principle

In contrast to adherent cancer cell targets, immune effector cells are non-adherent and therefore produce a minimal impedance signal in and of themselves. Because of this, when adherent cancer cells are treated with various effectors (NK cells, T cells, CAR-T cells, oncolytic viruses, checkpoint inhibitors, bispecific antibodies, BiTEs, etc.) it is possible to selectively monitor the kinetics of cancer cell destruction in real-time. The major distinguishing features of this technology include enhanced sensitivity, the preclusion of labels, simple workflow and, importantly, continuous kinetic measurement of cancer cell health/behavior. The xCELLigence immunotherapy workflow is outlined in **Figure 1**.

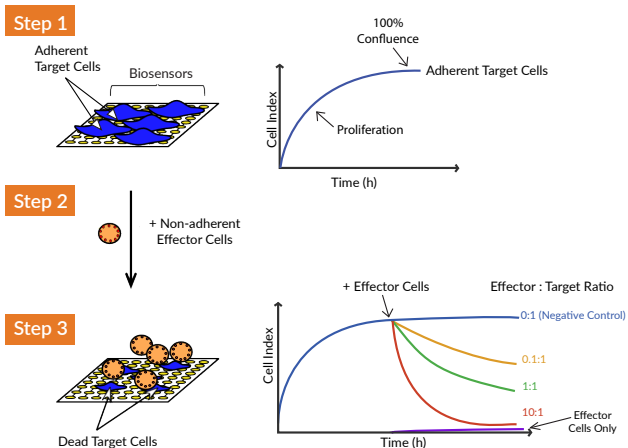


Figure 1. Monitoring immune cell-mediated killing of adherent cancer cells in real-time using xCELLigence.

Step 1: Adherent target cells (i.e. tumor cells) are first seeded in the wells of an electronic microtiter plate (E-Plate). Adhesion of cells to the gold biosensors impedes the flow of electric current between biosensors. This impedance value, plotted as a unitless parameter called “Cell Index”, increases as cells proliferate and then plateaus as cells approach 100% confluence.

Step 2: When added subsequently, non-adherent effector cells (i.e. immune cells) in suspension do not cause impedance changes in and of themselves (due to lack of adherence to the gold biosensors).

Step 3: If effector cells induce the destruction of the target adherent tumor cells, the corresponding cytolytic activity can be sensitively and precisely detected. The continuous acquisition of impedance data for each well of an E-Plate enables the generation of real-time killing curves for multiple conditions simultaneously.

Using xCELLigence to Monitor Liquid Tumor Cell Killing

With dozens of peer-reviewed studies published over the past decade, the utility of xCELLigence RTCA for probing the efficacy of immunotherapies targeting solid/adherent cancers is now firmly established. However, ~10% of all cancers are liquid in nature, and therefore non-adherent, and cannot be directly monitored by the standard impedance assay. Because they are readily accessible within the bloodstream and aren't confounded by the microenvironment complexities/heterogeneities associated with solid tumors, liquid cancers (B cell cancers in particular) are prominent immunotherapy targets at present. To help accelerate research in this area, ACEA developed this xCELLigence Immunotherapy Kit focused on liquid tumor cell killing. For this purpose, the wells of ACEA's electronic microtiter plate are precoated with an anti-CD40 Tethering Reagent, enabling CD40+ liquid tumor cells to be immobilized on the plate bottom (**Figure 2A**). Whereas Tethering Reagent immobilized liquid tumor cells generate a robust impedance signal and proliferate to the point of confluence (resulting in a plateaued impedance signal), the growth of untethered cells is essentially undetectable (**Figure 2B**). Importantly, with or without anti-CD40 coating of the wells, effector cells such as the NK-92 cells used here produce minimal signal on their own (**Figure 2B**). Addition of NK-92 cells on top of immobilized liquid tumor cells results in target cell death in a dose dependent manner (**Figure 2C**). The tethering and killing behaviors seen in **Figures 2B and 2C** have been observed in all three of the B cell lymphoma cell lines tested (Daudi, Raji, and Ramos), for multiple effector cell types (NK, T, CAR-T), and for combination therapies (CAR-T and checkpoint inhibitors, etc.).

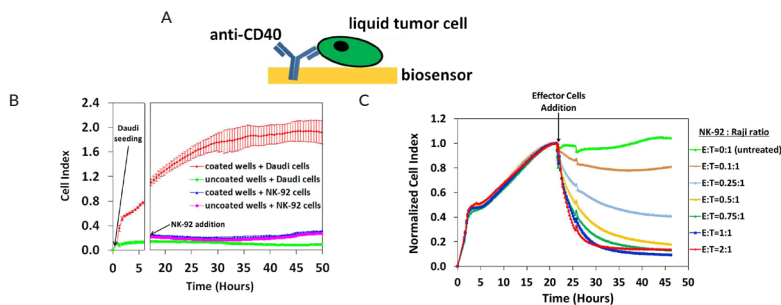


Figure 2. Using the xCELLigence Immunotherapy Kit to monitor liquid tumor cells killing. (A) Precoating the wells of ACEA's electronic microtiter plates with anti-CD40 enables liquid tumor cells to proliferate on, and be detected by, the biosensors. (B) Controls showing the selective proliferation of Daudi B lymphoblasts on biosensors coated with anti-CD40. As expected, with or without anti-CD40 coating non-adherent NK-92 effector cells produce minimal signal. Error bars are standard deviation. (C) Raji target cells were seeded at 60,000 cells/well. The efficiency with which target cells are killed is dependent on the number of effector cells added per well.

Assay Protocol

Note: Users are encouraged to use this protocol as a guideline. It is recommended that, on a case by case basis, the effector cells and target cells be carefully titrated and cell numbers be determined empirically for the application of interest.

1. Overview

The below protocol has specifically been optimized for using the xCELLigence RTCA SP or MP instruments to monitor effector cell mediated killing of B lymphoblasts (Daudi, Raji, and Ramos cell lines were used in the optimization of this protocol) which have been immobilized on the well bottoms of an E-Plate View 96 using the Tethering Reagent (anti-human CD40 antibody) and Tethering Buffer provided with this kit. Assay conditions may require additional optimization if different cell lines or antibodies are used.

2. Reagents & Equipment

The following reagents and equipment were used in developing/optimizing this protocol:

Reagents

- **Cells:** Daudi Burkitt's lymphoma B lymphoblasts (Cat# CCL-213™), Raji Burkitt's lymphoma B lymphoblasts (Cat# CCL-86™), and NK-92 non-Hodgkin's lymphoma cytotoxic NK cells (Cat# CRL-2407™) were purchased from ATCC.
- **Cell Culture Medium:** RPMI 1640 + 10% FBS + 1% penicillin/streptomycin
- **Interleukin 2 (IL-2):** Peprotech (Cat.# AF-200-02). Working concentration is 200 units/mL for NK-92 cells.

Equipment

- **E-Plate View 96:** Because the well size in ACEA's E-Plate View 96 is identical to the well size in the E-Plate 16, the media volumes and cell numbers used in the below protocol are compatible with usage of the lower throughput E-Plate 16. ACEA's other plate types, the E-Plate L8 and E-Plate 384, are also compatible with this liquid tumor killing assay, but cell numbers/media volumes would need to be optimized for the different well dimensions in these plates.
- 6 • **xCELLigence RTCA SP or MP instruments:** While all of the xCELLigence RTCA instruments can be used for this liquid tumor killing assay, the cell numbers and

media volumes listed in this protocol are optimized for the dimensions of the wells in the E-Plate View 96 – which is only compatible with the xCELLigence RTCA SP and MP instruments. For a full description of the seven different xCELLigence instruments please visit: www.aceabio.com/products/xcelligence-rtca/

3. Workflow Summary

Allowing an initial day for target cells to attach to and proliferate in the E-Plate View 96 wells, this assay protocol has been developed for continuous monitoring of cell killing over the course of 4 subsequent days.

- **Day 1:**

- a. Coat E-Plate View 96 with diluted Tethering Reagent for 3 hours. (Alternatively, the coated E-Plate View 96 can be kept at 4°C with Tethering Reagent Solution for up to one week. Make sure the coated wells do not dry out.) Wash, add medium, and take background measurement.
- b. Prepare target cells and add to the coated wells of the E-Plate View 96.
- c. Leave E-Plate View 96 at room temperature for at least 30 minutes to let cells settle.
- d. Load E-Plate View 96 into xCELLigence RTCA instrument and start data acquisition to monitor target cell attachment and proliferation.

- **Day 2:**

- e. Prepare effector cells.
- f. Pause xCELLigence data acquisition; remove E-Plate View 96 from instrument and place inside hood; remove nascent media from wells; add effector cells to E-Plate View 96.
- g. Load E-Plate View 96 back into xCELLigence RTCA instrument and start data acquisition to monitor effector cell-mediated killing of immobilized target cells.

- **Days 2-5:**

- h. Continue data acquisition for as long as desired.
- i. Analyze data.

4. Detailed Protocol

Coating E-Plate View 96 with Tethering Reagent (3 hours)

1. Tethering Buffer is provided as 10 mL of a 10x solution. Dilute this to 1x by adding 90 mL of sterile tissue culture grade water to the bottle.
2. The Tethering Reagent (anti-CD40) is provided at a concentration of 500 µg/mL. Using 1x Tethering Buffer, dilute the Tethering Reagent 125-fold to achieve a final concentration of 4 µg/mL.
3. Coat the E-Plate View 96 by adding 50 µL of the 4 µg/mL Tethering Reagent solution to each well.
4. Incubate at room temperature for 3 hours.
 - » **Critical:** The extent of Tethering Reagent adhesion to well bottoms may vary as a function of incubation temperature and duration. To ensure reproducibility between experiments be sure to maintain consistency in the parameters of this coating step.

Washing and Background Measurement (5 minutes)

5. Remove Tethering Reagent coating solution and discard.
6. Gently wash wells twice with 200 µL of PBS.
7. To each well add 50 µL of complete medium (or whatever medium will be used during the assay).
8. Place E-Plate View 96 back into instrument and let equilibrate at 37°C for one hour before taking background measurement.

Target Cell Seeding (45 minutes)

9. Determine target cell concentration and seed 40,000-60,000 cells in a volume of 100 µL/well. With the medium already present in the wells (from the background measurement step) this gives a final volume of 150 µL/well.
 - » **Critical:** The number of cells used in this killing assay will ultimately depend on the cell type being used. Conducting preliminary experiments to determine the optimal cell number is recommended.
10. Leave E-Plate View 96 at room temperature for 30 minutes to facilitate uniform immobilization of target cells on plate bottom.
 - » **Critical:** Failure to perform this step can result in large well to well variation in the cell seeding density/pattern (which can affect the impedance signal)

for the following reason: Immediate warming to 37°C can cause convection currents to form within the well, pushing cells to the well perimeter and resulting in an uneven distribution of cells on the impedance biosensors. This well-known phenomenon is not unique to ACEA's E-Plates; it occurs in all microtiter plates.

Monitoring Target Cell Adhesion and Proliferation (24 hours)

11. Place E-Plate View 96 back into xCELLigence instrument located inside incubator and initiate data acquisition. The purpose here is simply to monitor target cell adhesion and proliferation prior to initiating cell killing. Accordingly, measuring impedance every 15 minutes for 24 hours is appropriate.

Effector Cell Addition (15 minutes)

12. Effector cells (NK-92 cells are used as an example here) are typically grown in the presence of interleukin 2 (IL-2) at a concentration of 200 units/mL (NK-92 cells). To avoid IL-2-mediated stimulation of target cells during the killing assay, pellet effector cells and resuspend in media lacking IL-2. Determine effector cell concentration and prepare serial dilutions.
13. Pause data acquisition, remove E-Plate View 96 from instrument and move into hood.
14. Gently aspirate 100 µL of nascent medium from wells. This leaves 50 µL of medium in the wells.
15. Add 100 µL of the NK-92 dilutions per well, being careful to not disturb or remove the immobilized target cells. Effector:Target cell ratios will be defined by the density of the effector cells in the serial dilutions.
16. Leave E-Plate View 96 at room temperature for 30 minutes to facilitate uniform distribution of effector cells on top of immobilized target cells.

Cytolysis Positive Control (10 minutes)

17. For a positive control demonstrating rapid and complete target cell killing, perform the same steps used for effector cell addition, but instead of effect cells add 100 µL of Cytolysis Reagent that has been diluted 10-fold using culture medium. This detergent based reagent will cause the Cell Index to drop to ~0 within minutes.

Monitoring the Killing of Immobilized Target Cells (assay duration defined by user)

18. Place E-Plate View 96 back into xCELLigence instrument located inside incubator and initiate data acquisition. The purpose here is to monitor effector cell-mediated killing of the target cells immobilized on the bottom of the E-Plate View 96 wells. Impedance measurements every 15 minutes are sufficient for this purpose. The total duration of data acquisition should be programed to be longer than deemed necessary; data acquisition can be terminated whenever appropriate.

Data Analysis

The full functionality of the RTCA data analysis and representation software is reviewed in detail in the RTCA Software Pro manual.

Troubleshooting

Potential problems and corresponding solutions are listed below:

1. There is large variation between replicate wells:
 - It is important to perform the full 30 minute room temperature incubation after seeding both target cells and effector cells. This ensures even distribution of the cells across the biosensors.
 - To ensure similar cell seeding densities between wells, make sure cells are well suspended prior to seeding into the E-Plate View 96.
 - The reverse pipetting technique is effective for preventing bubble formation and assuring consistent volume dispensing.
2. No signal is detected in wells expected to have a signal from target cell immobilization:
 - Verify that target cells have indeed been immobilized on the E-Plate View 96 well bottoms by replacing nascent medium (which may contain un-captured cells) with fresh medium and then examining wells under a microscope.
 - Make sure to use the Tethering Reagent (anti-CD40) and Tethering Buffer provided with this kit at the recommended dilutions. These have been optimized extensively.
 - Using flow cytometry, verify CD40 expression on the surface of the target cells.

For life science research only.
Not for use in diagnostic procedures.

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