

xCELLigence® Immunotherapy Kit - B Cell Killing Assay

ASSAY MANUAL

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I. List of Kit Components

This kit is available in three different configurations:

Contents	Complete Kit (cat.# 8100004)	Tethering Kit + Software (cat.# 8100006)	Tethering Kit (cat.# 8100005)
E-Plate® View 96 (12 plates total; 2 boxes of 6 plates) (cat.# 06472451001)	✓		
Tethering Reagent (480 µL) (cat.# 8710238)	✓	✓	✓
10x Tethering Buffer (10 mL) (cat.# 871B617)	✓	✓	✓
Cytolysis Reagent (1.5 mL) (cat.# 8710239)	✓	✓	✓
Assay Manual	✓	✓	✓
Software Manual	✓	✓	✓
xCELLigence® Immunotherapy Software 1.0	✓	✓	

II. Storage Conditions

Plates and reagents should be stored as follows:

Reagent	Storage
E-Plate® View 96	25°C
Tethering Reagent	4°C
10x Tethering Buffer	25°C
Cytolysis Reagent	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
- Target B 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 microelectrodes 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 electrodes. 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® applications 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 B cell killing, a detailed protocol is provided.

V. xCELLigence® Immune Cell Killing Assay Principle

In contrast to adherent cancer target cells, 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, CARTs, oncolytic virus, 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.

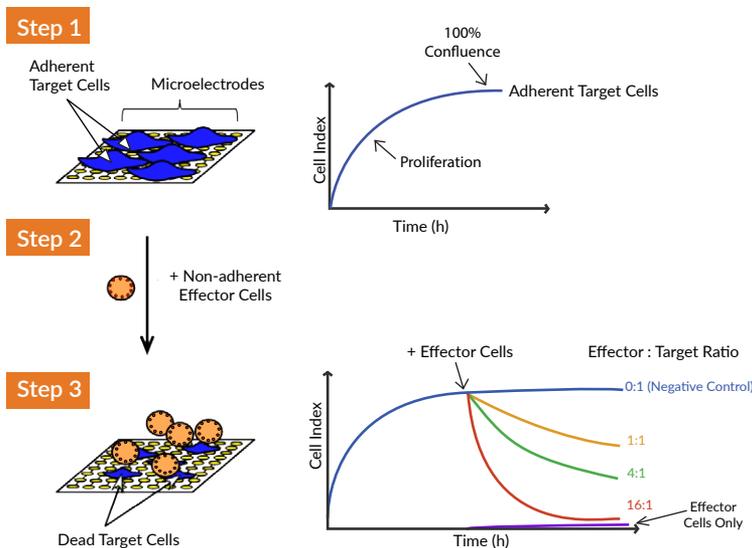


Figure 1. Monitoring immune cell-mediated killing of adherent cancer cells in real-time using xCELLigence®.

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 microelectrodes impedes the flow of electric current between electrodes. 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 microelectrodes).

Step 3: If effector cells induce the destruction of the target adherent tumor cells, this 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.

VI. Using xCELLigence® to Monitor B 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, are therefore non-adherent, and cannot be directly monitored by the standard impedance assay. Moreover, 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 B cell killing. For this purpose, the wells of ACEA's electronic microtiter plate are precoated with an anti-CD40 antibody, enabling B cells to be immobilized on the plate bottom (Figure 2A). Whereas antibody immobilized B cells generate a robust impedance signal and proliferate to the point of confluence (resulting in a plateaued impedance signal), the growth of untethered B 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 B cells results in target cell death in a dose dependent manner (Figure 2C). The tethering and killing behaviors seen in Figures 2 B and C 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, CART), and for combination therapies (CART + checkpoint inhibitors, etc.).

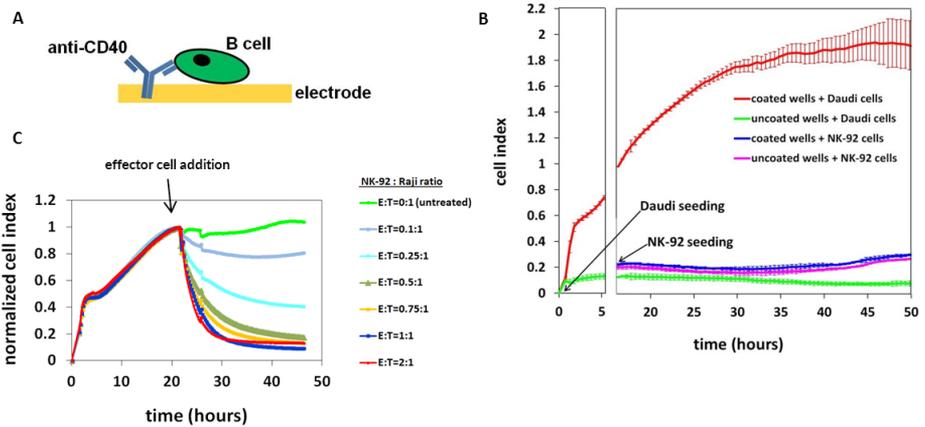


Figure 2. The xCELLigence® Immunotherapy Kit for monitoring B cell killing. (A) Precoating the wells of ACEA's electronic microtiter plates with anti-CD40 enables B cells to proliferate on, and be detected by, these sensors. (B) Controls showing the selective proliferation of Daudi B lymphoblasts on electrodes 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) The efficiency with which Raji B lymphoblasts are killed is dependent on the number of NK-92 cells added per well.

VII. Assay Protocol

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-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 B lymphoblasts (Burkitt's lymphoma; cat.# CCL-213™), NK-92 non-Hodgkin's lymphoma cytotoxic NK cells (cat.# CRL-2407™), and TALL-104 acute lymphoblastic leukemia cytotoxic T lymphoblast cells (cat.# CRL-11386™) 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 100 or 200 units/mL for TALL-104 and NK-92 cells, respectively.

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 B cell killing assay, but cell numbers/media volumes would need to be optimized for the different well dimensions in these plates.
- **xCELLigence® RTCA SP or MP instruments:** While all of the xCELLigence® RTCA instruments can be used for this B cell 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/

VII. Assay Protocol

3. Workflow Summary

Allowing an initial day for target B 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; wash; add medium; equilibrate in 37°C incubator for 1 hour; take background measurement.
- b. Prepare B 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 B cell attachment and proliferation.

- **Day 2:**

- a. Prepare T cells (or other effector cells).
- b. Pause xCELLigence® data acquisition; remove E-Plate® View 96 from instrument and place inside hood; remove nascent media from wells; add T cells (or other effector cells) to E-Plate® View 96.
- c. Load E-Plate® View 96 back into xCELLigence® RTCA instrument and start data acquisition to monitor effector cell-mediated killing of immobilized B cells.

- **Days 2-5:**

- a. Continue data acquisition for as long as desired.
- b. Analyze data.

VII. Assay Protocol

4. Detailed Protocol

Coating E-Plate® View 96 with Anti-CD40 (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 (i.e. anti-CD40 antibody) is provided at a concentration of 500 µg/mL. Using 1x Tethering Buffer, dilute the antibody 125-fold to achieve a final concentration of 4 µg/mL.
3. Coat the wells of the E-Plate® View 96 with anti-CD40 antibody by adding 50 µL of the 4 µg/mL solution.
4. Incubate at room temperature for 3 hours.
 - » **Critical:** The extent of antibody 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)

1. Remove antibody coating solution and discard.
2. Gently wash wells twice with 200 µL of PBS.
3. To each well add 50 µL of complete medium (or whatever medium will be used during the assay).
4. 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)

1. Determine B cell concentration and seed 80,000 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.
2. Leave E-Plate® View 96 at room temperature for 30 minutes to facilitate uniform immobilization of B 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 electrodes. This well known phenomenon is not unique to ACEA's E-Plates®; it occurs in all microtiter plates.

VII. Assay Protocol

Monitoring Target Cell Adhesion and Proliferation (24 hours)

1. Place E-Plate® View 96 back into xCELLigence® instrument located inside incubator and initiate data acquisition. The purpose here is simply to monitor B 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)

1. Effector cells (NK-92 and TALL-104 are used as examples here) are typically grown in the presence of interleukin 2 (IL-2) at a concentration of 100 units/mL (TALL-104 cells) or 200 units/mL (NK-92 cells). To avoid IL-2-mediated stimulation of B cells during the killing assay, pellet effector cells and resuspend in media lacking IL-2. Determine effector cell concentration and prepare serial dilutions.
2. Pause data acquisition, remove E-Plate® View 96 from instrument and move into hood.
3. Gently aspirate 100 µL of nascent medium from wells. This leaves 50 µL of medium in the wells.
4. Add 100 µL of the NK-92 or TALL-104 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.
5. Leave E-Plate® View 96 at room temperature for 30 minutes to facilitate uniform distribution of effector cells on top of adherent target cells.

Cytolysis Positive Control (10 minutes)

1. For a positive control demonstrating rapid and complete B cell killing, perform the same steps used for effector cell addition, but instead of effector 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 T Cell-Mediated Killing of Immobilized B Cells (assay duration defined by user)

1. Place E-Plate® View 96 back into xCELLigence® instrument located inside incubator and initiate data acquisition. The purpose here is to monitor T cell-mediated killing of the B 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 xCELLigence Immunotherapy Software 1.0 manual.

VIII. 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 B cells and effector cells. This ensures even distribution of the cells across the electrodes.
 - 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 B cell immobilization:
 - Verify that B 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 antibody) 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 your target B cells.

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

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