Protocol

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# Agilent xCELLigence Immunotherapy IMT Assay (anti-CD29) Tethering Kit and Sample Kit

Recommended Use: Monitoring liquid tumor cell killing

For Research Use Only. Not for use in diagnostic procedures.

## Kit components

**Table 1.** List of kit components.

| Product | Part Number |
|---|---|
| IMT Assay (anti-CD29) Tethering Kit (up to 6 Plates) | 8100008 |
| 250x Tethering Reagent (anti-CD29), 125 µL | |
| 10x Tethering Buffer, 10 mL | |
| Cytolysis Reagent, 1.5 mL | |
| IMT Assay (anti-CD29) Sample Kit (up to 2 Plates) | 8100009 |
| 250x Tethering Reagent (anti-CD29), 45 µL | |
| 10x Tethering Buffer, 10 mL | |
| Cytolysis Reagent, 1.5 mL | |

## 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.

## Additional materials and reagents required

The following are required but are not supplied with these kits:

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

## Introduction

Agilent xCELLigence real-time cell analysis (RTCA) instruments use gold biosensors embedded in the bottom of microtiter plate wells to noninvasively monitor the status of adherent cells using the principle of cellular impedance. Adherent cells act as insulators by impeding the flow of an alternating microampere electric current between the biosensors. This impedance signal is measured automatically, at a frequency defined by the user (every 10 seconds, once per hour, etc.), and provides a sensitive readout of cell number, cell size/shape, and cell-substrate attachment strength.

Over the past decade xCELLigence has been used to study immune cell-mediated killing of adherent cancer cells. This IMT Assay Kit expands the repertoire of xCELLigence to include monitoring immune cell-mediated killing of non-adherent liquid cancers. Below, the xCELLigence RTCA assay principle and workflow are first described using adherent target cells as an example. Following a general overview of how the xCELLigence RTCA assay has been adapted for monitoring liquid tumor killing, a detailed protocol is provided.

### xCELLigence IMT assay principle

In contrast to adherent cancer cell targets, immune effector cells are nonadherent and therefore produce a minimal impedance signal. When adherent cancer cells are treated with various effectors (NK cells, T cells, CAR-Ts, 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 continuous kinetic measurement of cancer cell health/behavior. The xCELLigence immunotherapy workflow is outlined in Figure 1.

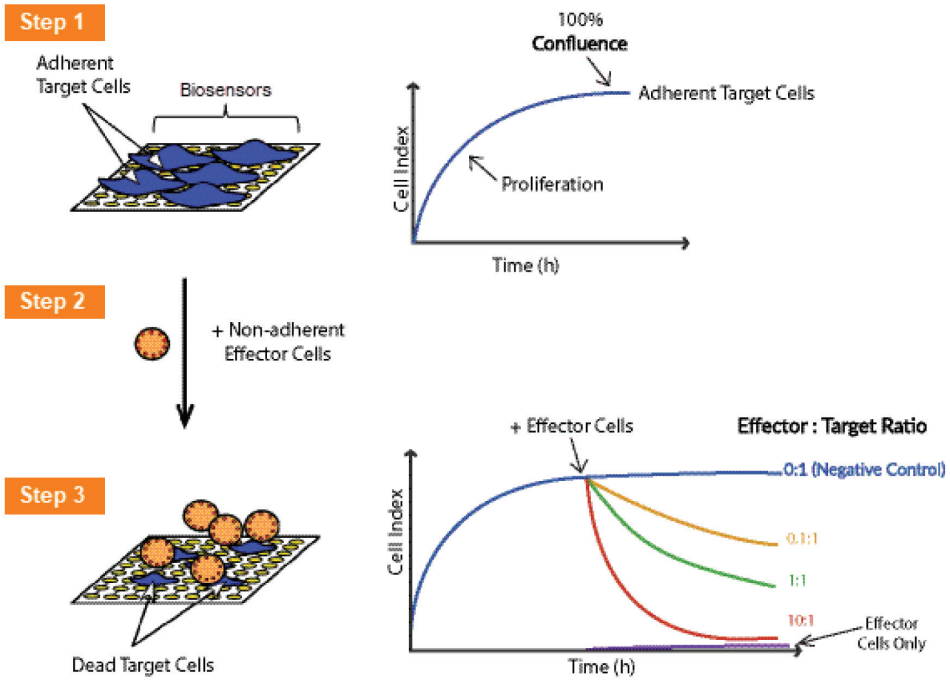


**Figure 1.** Monitoring immune cell-mediated killing of adherent cancer cells in real time using Agilent xCELLigence.

**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 them. 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, nonadherent effector cells (i.e. immune cells) in suspension do not cause changes in impedance (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

The dozens of peer-reviewed studies published over the past decade have established the utility of xCELLigence RTCA for probing the efficacy of immunotherapies targeting solid/adherent cancers. However, ~10% of all cancers are liquid in nature, and therefore nonadherent, and cannot be directly monitored by the standard impedance assay. Liquid cancers (leukemic cell cancers in particular) are prominent immunotherapy targets at present because they are readily accessible within the bloodstream and are not confounded by the microenvironment complexities/heterogeneities associated with solid tumors and they express surface proteins that are either absent or present in low abundance on other cell types. To help accelerate research in this area, Agilent developed this xCELLigence IMT Assay Kit focused on liquid tumor cell killing. For this purpose, the wells of Agilent E-Plates are precoated with anti-CD29 Tethering Reagent, enabling CD29+ leukemic cells (K562) to be immobilized on the plate bottom (Figure 2A). Addition of NK-92 cells on top of immobilized K562 cells results in target cell death in a dose-dependent manner (Figure 2B). Because NK-92 cells express CD29, they have the potential to be tethered by the anti-CD29 Tethering Reagent used in this assay, which can lead to the effector cells contributing substantially to the impedance signal. While the xCELLigence RTCA Software Pro can subtract out this NK-92 signal, to selectively monitor the signal of the target cells, the user should be aware and optimize their assay accordingly.

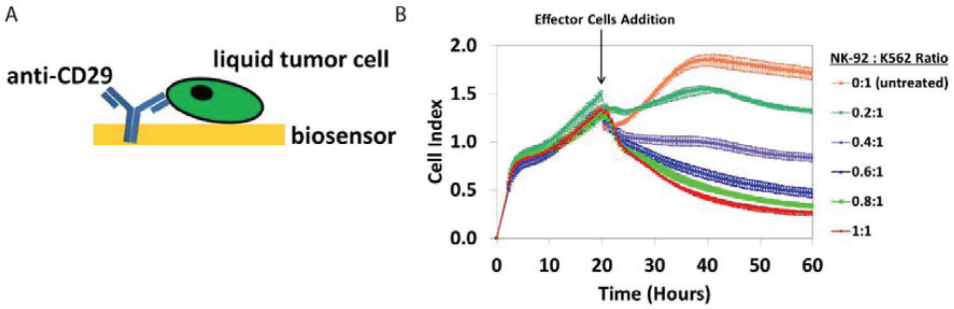


**Figure 2.** The Agilent xCELLigence IMT Assay Kit for monitoring K562 cell killing. (A) Precoating the wells of the Agilent E-Plates with anti-CD29 enables K562 cells to proliferate on and be detected by the biosensors. (B) K562 cells were seeded at 60,000 cells/well. When left untreated, the immobilized K562 cells proliferated to the point of confluence. Upon addition of increasing quantities of NK-92 effector cells, the impedance signal decreased in a dose-dependent manner.

## Precautions

See the Safety Data Sheet

## Protocol

### Overview

The below protocol has specifically been optimized for using the xCELLigence RTCA SP or MP instruments to monitor effector cell mediated killing of leukemic cells (K562 cells 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 CD29 antibody) and Tethering Buffer provided with this kit. Assay conditions may require additional optimization if different cell lines or antibodies are used.

### Reagents and equipment

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

### Reagents

- **Cells:** K562 Myelogenous leukemia cells (part number CCL-243) and NK-92 non-Hodgkin's lymphoma cytotoxic NK cells (part number CRL-2407) were purchased from ATCC.
- **Cell Culture Medium:** RPMI 1640 + 10% FBS + 1% penicillin/streptomycin
- **Interleukin 2 (IL-2):** Peprotech (part number AF-200-02). Working concentration is 200 units/mL for NK-92 cells.

### Equipment

- **E-Plate View 96:** The media volumes and cell numbers used in the protocol below are compatible with usage of the lower throughput E-Plate 16 because the well size in the Agilent E-Plate View 96 is identical to the well size in the E-Plate 16. the Agilent E-Plate 384 is also compatible with this IMT 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 IMT 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.

### 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

1. Coat an E-Plate View 96 with diluted Tethering Reagent for 3 hours at room temperature. Alternatively, coat the E-Plate View 96 with diluted Tethering Reagent at 4 °C overnight. Wash, add medium, and take a background measurement.
2. Prepare target cells and add to the coated wells of the E-Plate View 96.
3. Incubate the E-Plate View 96 at room temperature for at least 30 minutes to let the cells settle.
4. Load the E-Plate View 96 into the xCELLigence RTCA instrument and start data acquisition to monitor target cell attachment and proliferation.

### Day 2

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

### Days 2 to 5

8. Continue data acquisition for as long as desired.
9. Analyze the data.

## Detailed protocol

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

1. Tethering Buffer is provided as 10 mL of a 10x solution. Dilute to 1x by adding 90 mL of sterile tissue culture grade water to the bottle.
2. The Tethering Reagent (anti-CD29) is provided at a concentration of 500 µg/mL. Using 1x Tethering Buffer, dilute the Tethering Reagent 250-fold to a final concentration of 2 µg/mL.
3. Coat the E-Plate View 96 by adding 50 µL of the 2 µg/mL Tethering Reagent to each well.
4. Incubate at room temperature for 3 hours. Alternatively, incubate the E-Plate View 96 at 4 °C overnight.

   **CAUTION:** 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.
6. Gently wash wells twice with 200 µL of PBS.
7. To each well, add 50 µL of complete medium (or the medium to be used during the assay).
8. Place the E-Plate View 96 back into the instrument and let it equilibrate at 37 °C for 10 minutes before taking a background measurement.

### Target cell seeding (45 minutes)

9. Determine target cell concentration and seed 40,000 to 60,000 cells in a volume of 100 µL/well. With the medium present in the wells (from the background measurement step), this gives a final volume of 150 µL/well.

   **CAUTION:** The number of cells used in this IMT assay will ultimately depend on the cell type being used. Conducting preliminary experiments to determine the optimal cell number is highly recommended.

   **CAUTION:** Because K562 cells are highly undifferentiated, with the potential to spontaneously differentiate into progenitors of the erythrocytic, granulocytic, and monocytic series, extra care must be taken when culturing these cells before their use in an xCELLigence assay. K562 cells should be always be maintained at a density below $1 \times 10^6$ cells/mL
10. Incubate the E-Plate View 96 at room temperature for 30 minutes to facilitate uniform immobilization of target cells on plate bottom.

    **CAUTION:** 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 Agilent 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 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 recommended.

### Effector cell addition (15 minutes)

12. Effector cells (NK-92 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 IMT assay, pellet effector cells and resuspend in media without IL-2. Determine effector cell concentration and prepare serial dilutions.
13. Press the skip button to fast-forward through the remaining sweeps in the currently running step of data acquisition; do not use the pause button. Remove E-Plate View 96 from instrument and place it in the hood.
14. Gently aspirate 50 µL of medium from each well, leaving 100 µL in each well.
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. Incubate the 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 in place 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 the killing of immobilized target cells (assay duration defined by the user)

18. Place the E-Plate View 96 back into xCELLigence instrument and initiate data acquisition. The purpose here is to monitor the killing of the target cells immobilized on the bottom of the E-Plate View 96 wells. Impedance measurements every 15 minutes are recommended. 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:

### 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.
- Ensure cells are well suspended prior to seeding into the E-Plate View 96 for consistent cell seeding densities between wells.
- The reverse pipetting technique is effective for preventing bubble formation and assuring consistent volume dispensing.

### No signal is detected in wells expected to have a signal from target cell immobilization

- Verify that target cells have been immobilized on the E-Plate View 96 well bottoms by replacing cultured medium (which may contain uncaptured cells) with fresh medium and then examining wells under a microscope.
- Ensure the use the Tethering Reagent (anti-CD29) and Tethering Buffer provided with this kit at the recommended dilutions. Reagents have been optimized for this kit.
- Verify CD29 expression on the surface of the target cells using flow cytometry.

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RA44349.2476041667



Printed in the USA, July 14, 2021
5994-3237EN

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