Protocol

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Evaluating CAR T Sustained Cytotoxicity Activity Against Solid Tumor Through Serial Killing Assay

Protocol for Agilent xCELLigence RTCA systems

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Introduction

Impedance-based cytotoxicity assays using the Agilent xCELLigence Real-Time Cell Analysis (RTCA) platform are widely used to evaluate CAR T cell functional killing potency in vitro.[1] This label-free, noninvasive technology utilizes a specialized cell culture plate (Agilent xCELLigence RTCA E-Plate) containing interdigitated microelectrode sensors embedded in the bottom of each well, enabling continuous, real-time impedance measurements to monitor adherent tumor cell attachment, proliferation, viability, and morphology. The CAR T cell killing potency assay is performed in an effector-target coculture format, and the impedance signal recorded by the RTCA platforms is reported as an arbitrary parameter known as the Cell Index (CI). In this assay, the CI predominantly reflects adherent tumor cell dynamics, as suspended CAR T cells contribute minimally to the readout. Consequently, CAR T cell-mediated cytotoxicity is detected as a reduction in CI corresponding to tumor cell death.[1]

Beyond initial killing potency, CAR T cell cytolytic activity durability and persistence are critical determinants of therapeutic efficacy. In vivo, CAR T cells encounter repeated tumor challenges and must sustain cytotoxic function through multiple rounds of target engagement while undergoing dynamic changes such as proliferation, differentiation, and potential exhaustion. Short-term or single-endpoint in vitro assays are insufficient to capture these evolving functional states.

To enable longitudinal, real-time monitoring of CAR T cell cytotoxicity across sequential tumor exposures, a repeat-challenge assay has been developed and established using RTCA platforms, including the Agilent xCELLigence RTCA SP, MP, and eSight systems. This approach provides a deeper insight into cytotoxic persistence of immune cell products. While this protocol focuses on real-time cytotoxicity measured by RTCA, comprehensive characterization of rechallenged CAR T cells may also benefit from complementary analyses of immunophenotype and metabolic state using orthogonal technologies such as Agilent NovoCyte flow cytometry and Agilent Seahorse XF analysis. These additional measurements are beyond the scope of the current protocol but are discussed in a separate application note to highlight a multimodal strategy for CAR T cell functional profiling.[2]

Note: The process has been optimized using the EpCAM-expressing breast cell line T47D and EpCAM CAR T cells. If other target cell lines or effectors are used, the assay conditions may require further optimization.

The xCELLigence RTCA DP system, which uses 16-well E-Plates, could be suitable for repeat killing assays if its throughput fits the experiment requirements. The ability of the system to run up to three separate 16-well plates in parallel or independently enhances its productivity and flexibility.

Experimental

Table 1. Reagents and materials used.

Item	Manufacturer	Description	Part Number
T47D	ATCC	Breast cancer cell line	HTB-133
PBS	HyClone	1x DPBS (-Ca, -Mg, -phenol red)	SH30028.02
Trypsin	Gibco	0.05% Trypsin-EDTA (1x), phenol red	25300
Penicillin/ Streptomycin	HyClone	10,000 IU penicillin 10,000 mg/mL streptomycin	30-002-CI
FBS	VWR	Fetal bovine serum (Seradigm)	97068-085
IL-2	StemCell Technologies	Human recombinant IL-2	78036.1
RPMI 1640	Cytiva	RPMI 1640 media	SH3009601
xCELLigence RTCA E-Plate VIEW 96	Agilent Technologies	96-well electronic microplate	300601020

Table 2. Equipment and software used.

Description	Part Number
Agilent xCELLigence RTCA SP – bundle (complete system)	380601030
Agilent xCELLigence RTCA MP – bundle (complete system)	380601040
Agilent xCELLigence RTCA eSight – bundle (complete system)	S2833AA
Separate components	
RTCA Software Pro with IMT module for SP, single license	310100290
RTCA Software Pro with IMT module for MP, single license	310100230
RTCA eSight software with IMT module, single license	S2807-90048

Process overview

This immune cell-mediated serial cytotoxicity assay was established using the T47D breast cancer cell line as a target and CAR T effector cells.

Workflow summary

The day before the cytotoxicity assay, target cells were seeded in an Agilent E-Plate VIEW 96 (E-Plate) at a desired density and incubated at 37 °C/5% CO_2 overnight (18 to 24 hours). The next day, CAR T cells were added to the target cell-containing E-Plate at various effector:target (E:T) ratios (such as, 0.25:1, 0.5:1, 1:1, and 2:1). The CAR T cytolytic activity was continuously monitored for 72 hours. The day before the next round of the assay (approximately 48 hours after effector cell addition), a new target cell E-Plate was prepared by seeding T47D cells and letting them grow overnight. The following day, CAR T cells were collected from the original testing plate, resuspended in fresh media, counted, and added to the new target cell plates at the same E:T ratios. The new round of CAR T cytolysis was monitored for 72 hours. The flow cytometric analysis of the percentage of CAR T cells was performed after each round of the assay as the percentage of CAR changes over time, and the E:T ratios were calculated based on the pure CAR T. This cycle was repeated until a decreasing trend in the killing activity of CAR T was observed (Figure 1).

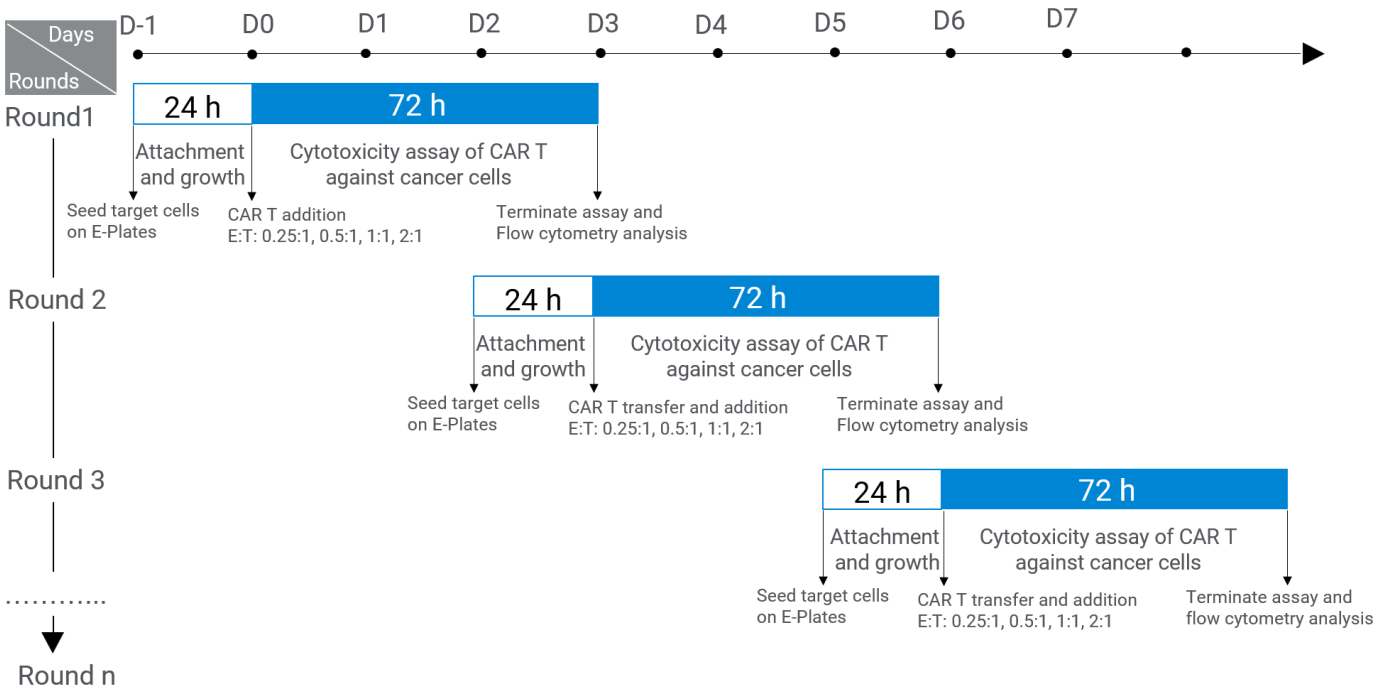

Figure 1. Serial killing assay workflow using Agilent E-Plate VIEW microplate. D represents the day.

Plate layout

In the described experiments, the effector-only and the target-only wells were used as controls and references. To ensure that enough effector cells were available for the next round of killing, as many replicates as possible for the highest E:T ratio, such as 2:1, were included in the plate. For the other test E:T ratios, at least four replicates were incorporated in the plate. As the seeding density of target cells was 8,000 cells/well, the effector-only wells were seeded with 2,000, 4,000, 8,000, and 16,000 CAR T cells to correspond to the number of effector cells at each E:T ratio (Figure 2).

Note: For a standard immune cell cytotoxicity assay, the target cells along with untransduced T cells (UT) or mock CAR T cells should be included in the plate to serve as a reference for nonspecific killing. However, in a serial killing assay, the UT or mock effector cells cannot sustain more than two rounds of killing as they will die off without proper activation. Therefore, target plus UT or mock cell wells are optional for the repeat killing assay.

More than six replicates for target-only wells are recommended, as they are critical for the percentage of cytolysis calculation.

Effector cells from the highest E:T ratio wells were exclusively used the as the source of CAR T cells for each subsequent round of the assay. To ensure sufficient cells for the next run, more replicates for the highest E:T ratio were included in the plate. Due to the relatively low transduction efficiency (28%) of the CAR T product, and the need to determine the percentage of CAR^+ after each killing using a flow cytometer, a large number of effector cells were required. Therefore, 45 replicates were used for an E:T ratio of 2:1. The number of replicates should be determined based on the specific needs of the experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A		E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1	E:T 0.25:1		
B		E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1	E:T 0.5:1		
C		E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1	E:T 1:1		
D		E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1		
E		E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1		
F		E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1		
G		E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1		
H		E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1	E:T 2:1		
	CAR T only	Coculture of effector and target cells									Target only	

Figure 2. Example plate layout for a CAR T cytotoxicity assay against target cells.

Software set up

Table 3. Software set up.

Step	Sweeps	Intervals	Unit	Comments
1	1	1	Minute	Plate background reading
2	100	15	Minute	Overnight monitoring of target cell growth
3	288	15	Minute	Cytolysis monitoring

Detailed instructions

Note: Consistency of quality and performance of target cells is pivotal to reliably evaluate the propensity of effector cell cytotoxicity over multiple challenges. Creating a cell bank of target cells at the same passage for the assay is highly recommended. This approach ensures that each round of the killing potency assessment begins with freshly thawed target cells of the same passage, maintaining consistency throughout the entire serial killing process.

Note: Additional resources can be found in the Reference section at the end of the document.[3-5]

Day 1: Seed target cell to an E-Plate and rest the cryopreserved effector cells overnight

1. Warm target cells growth media (such as RPMI 1640 medium supplemented with 10% FBS, and 1% penicillin/streptomycin) and CAR T cell resting medium (such as Lonza X-VIVO 15 with 5% human serum) in a 37 °C water bath for 30 minutes.
2. E-Plate preparation and background measurement (10 minutes).
    a. Carefully transfer 50 µL of prewarmed media to each well using a multichannel pipette. The reverse pipetting technique is recommended to prevent bubble formation and to ensure consistent volume dispensation.
    b. Place the E-Plate into the RTCA station. Open the RTCA software and enter the experiment and plate layout information. Start step 1, which includes only one sweep to perform the plate background measurement. Once Step 1 is complete, remove the E-Plate from the station and place the plate back in a tissue culture hood for cell seeding.
3. Revive and seed target cells (45 minutes).
    a. Remove a target cell stock vial from the liquid nitrogen tank and thaw in a 37 °C water bath until little ice remains (approximately two minutes).
    b. Spray the vial with 70% ethanol and place it in the tissue culture hood.
    c. Gently transfer cell suspension from the cryovial to a 50 mL centrifuge tube and add 5 mL of warm cell growth medium.
    d. Count viable cells and calculate the volume of additional medium required to adjust the cell suspension to the desired concentration.
    e. Seed the cells by adding 50 µL of cell suspension to each well using a multichannel pipette. A density of 10,000 to 20,000 cells/well works well for most cell types. It is recommended that CI be greater than 0.5 and ideally greater than 1 prior to effector cell addition. It is recommended to perform a cell titration assay prior to cytolysis assays to determine a suitable target cell density.

f. After cell seeding, leave the E-Plate in the hood at room temperature for 30 to 60 minutes, allowing the cells to settle to the bottom of the well evenly.
Critical: Failure to perform this step can result in large well-to-well variation.

g. Transfer the E-Plate to the RTCA station inside a 37 °C incubator and incubate for 18 to 24 hours to allow cell attachment and proliferation.
Critical: It is important to use an incubator with high humidity (preferably > 90%) to minimize evaporation of media (especially for the wells along the perimeter of the plate).

h. Start Step 2 of the RTCA program, monitoring impedance for 30 hours with recording at 15-minute intervals. It is suggested to set the reading time longer than the expected experiment time so that, if any delays occur, no time points will be missed.

4. Revive and rest effector cells (45 minutes).
This step only applies to the initial cytotoxicity assay (round 1) if cryopreserved effector cells are used in the assay.

a. Remove a CAR T stock vial from the liquid nitrogen tank and thaw in a 37 °C water bath until little ice remains (approximately two minutes).

b. Spray the vial with 70% ethanol and place it in the tissue culture hood.

c. Dropwise, add 5 mL of warmed resting media, swirling occasionally. With the remaining media, bring the volume of the cell suspension to 20 mL.

d. Centrifuge the cells 250 *x g* for 10 minutes at room temperature.

e. Resuspend the cell pellet with 5 to 10 mL of resting media. Pipette to homogenize the cell suspension.

f. Count cells and incubate cell suspension of 1 to 5 x 10^6 cells/mL at 37 °C with 5% CO_2 overnight.

Days 0 to 3: Cytotoxicity assay (round 1)

Day 0:

5. The target cell culture medium (RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin) was used for the cytotoxicity assay described in this protocol. However, other media may be used for these assays. Warm the assay medium in a 37 °C water bath for 30 minutes before the assay.

6. Effector cell preparation (30 minutes).
For the initial cytotoxicity assay (round 0):

a. Transfer the overnight rested CAR T cells to a 50 mL conical tube and centrifuge at 400 *x g* for 8 minutes.

b. Resuspend the cell pellet in the assay medium and count the cell number.

c. Prepare a cell suspension at the appropriate density. For example, in our experiments, the seeding density of T47D target cells was 8,000 cells/well. For the highest E:T ratio, 2:1, 16,000 EpCAM CAR T cells/well are needed. Hence, we adjusted the volume to prepare a CAR T cell suspension at a concentration of 1.6 x 10^5 cells/mL.
Critical: The E:T ratio is calculated using the number of pure CAR T cells. Therefore, the transduction efficiency or the percentage of CAR T cells is an essential factor in determining the number of CAR T used at each E:T ratio. The CAR T percentage is determined by a flow cytometer right after the completion of each round of the killing test.

d. Prepare two-fold serial dilutions of CAR T cells used for the remaining test E:T ratios of 1:1, 0.5:1, and 0.25:1.

7. Cell addition to the target cell E-Plate (30 minutes).

a. Pause the experiment and abort the overnight monitoring of the target cell E-Plate.

b. Transfer the target cell plates to the tissue culture hood, then add 100 µL of the effector cell suspension to the plate wells according to the plate layout.

8. Return the plate to the RTCA station and start recording the cytotoxicity of CAR T against target cells at 15-minute intervals for three days.

Day 2: Prepare a target cell E-Plate for the next round of the repeat killing assay

9. Repeat Step 3 above to prepare a target cell E-Plate for the next round of the repeat killing assay.
Critical: If the xCELLigence RTCA SP system, is used for this assay, the monitoring of target cells on the newly prepared E-Plate is skipped due to the station being occupied by the current cytotoxicity assay. However, the CI prior to CAR T addition must be measured. This value is essential for the calculation of percent cytolysis.

Day 3: Initiate the next round of the repeat killing assay (round 2)

10. Effector cell collection from the current round of cytotoxicity assay (60 minutes).
    a. Terminate the cytotoxicity assay by clicking "Pause" in the RTCA software three days after effector cell addition.
    b. Transfer the E-Plate from the RTCA system to the tissue culture hood.
    c. For the next step, work with a single E:T at a time. Gently pipette the media in the E-Plate wells three times to suspend the CAR T cells.
    d. Pool the cell suspensions from the wells treated at E:T ratio of 2:1 into a 15 mL centrifuge tube and centrifuge it at 300 *x g* for 5 minutes.
    e. Resuspend the cell pellet in the assay medium and count the cell number.
    f. Adjust volume to prepare a CAR T cell suspension at the desired cell density.
    g. Prepare dilutions of CAR T cells used for the remaining E:T ratios 1:1, 0.5:1, and 0.25:1.
11. Repeat Steps 7 and 8 for the next round of the CAR T killing assay. Repeat the procedures described above as required. For example, until a declining trend in the killing kinetics and degree is observed (Figure 3).

Troubleshooting

Note: Like any other cell-based assay, the ultimate success of this assay using the xCELLigence RTCA system depends highly on cell quality. This protocol was developed using T47D and EpCAM CAR T cells. The assay conditions may require further optimization if other target cell lines or effectors are used. The assay conditions that may need further optimization include:

- **Assay medium:** If target and effector cells are maintained in very different cell culture media, the following steps should be taken to determine the optimal cytotoxicity assay medium. First, identify which medium among the two supports the proliferation and normal morphology of both cell types. Second, if neither medium seems appropriate, evaluate the impact of mixing equal volumes of target and effector cell media on cell performance. Third, if the mixed medium significantly affects target cell morphology and viability, medium adaptation for the target cell may be necessary. Target cell media can be removed and exchanged with effector cell media a few hours before the start of the cytotoxicity assay in the mixed media.
- **E:T ratio:** Multiple E:T ratios should be included in the assay, ranging from high to low. If complete cytolysis of the targets is achieved within a few hours at the highest E:T ratio or across all test E:T ratios, consider reducing the E:T ratios. The decline in effector cell-killing potency over time may be more easily observed at low E:T ratios.
- **Not enough effector cells for the serial killing assays:** If increasing the number of replicates for the highest E:T ratio still doesn't provide enough effector cells for the next round of cytotoxicity assay, you can include one to two additional effector-target coculture plates at the highest E:T ratio in parallel with the cytotoxicity assay plate. Standard 96-well microplates are used for coculture stock plates to ensure similar culture conditions to the E-Plate.
- **Duration of cytotoxicity assay:** If the cytolysis kinetic is slow, each cytolysis step can be extended to more than three days. The seeding schedule for new target plates should be correspondingly delayed.
- **Effector-alone control:** This control should always be present to ensure that a high number of target cells are not carried over during the transfer of CAR T cells from the current run to the next one. In addition to the fact that a carryover of targets would be reflected in a higher CI, these wells can also be imaged to estimate the amount of target carryover (if any). Additionally, you can check effector cell morphology and visualize viability.
- **Target cell carryover*:*** If target cell carryover occurs during CAR T transfer to the next E-Plate, extra care should be taken in future iterations. Avoid touching the target cell monolayer, use gentle pipetting when collecting effector cell suspension, and consider adjusting centrifuge conditions, such as time and speed, to reduce the carryover.
- **No decrease in CAR T cytotoxic activity over serial killing:** Additional rounds of the killing assay may be necessary for very persistent effector cells. Interestingly, in our experiments, we observed an increase in the cytolytic activity of CAR T cells over the first two rounds of assay due to the activity boost from the initial antigen activation.

To reduce the number of killing rounds, the testing of alternate target cancer cell lines or assay media can be considered.

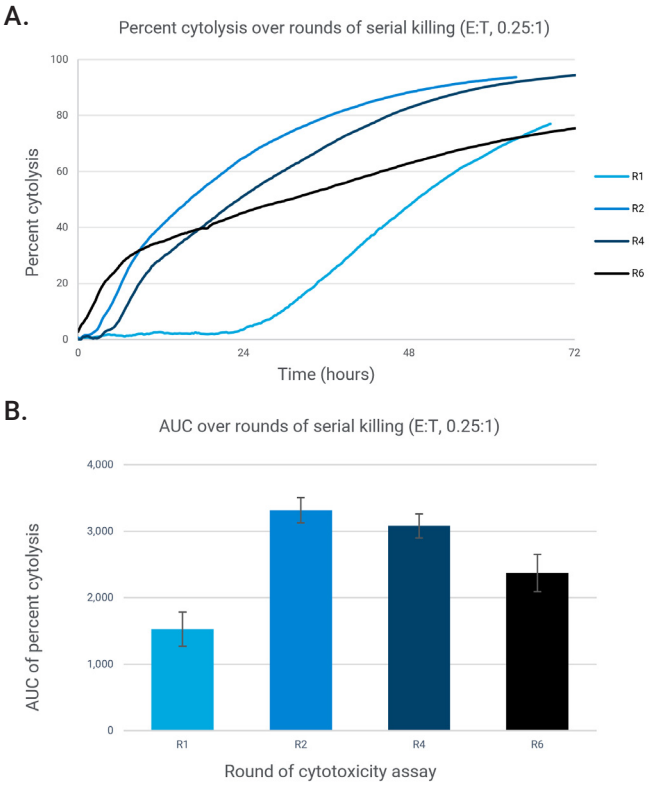

Figure 3. The decline of CAR T cytolytic activity over rounds of serial killing. (A) The overlay of the time courses of percent cytolysis over a 72-hour measurement was obtained from rounds 1, 2, 4, and 6 of the assays, respectively. (B) The area under the curves (AUC) of percent cytolysis obtained from rounds 1, 2, 4, and 6 of the assays. R: round of the assay.

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