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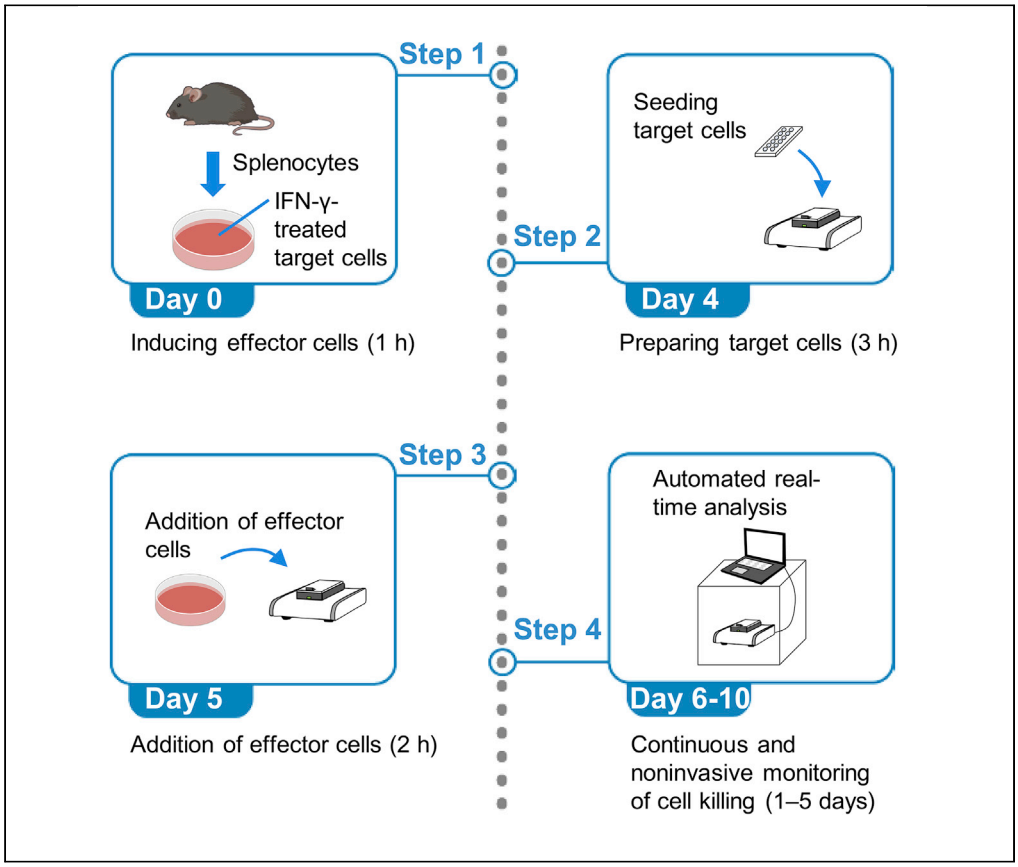
Protocol

A protocol for quantifying lymphocyte-mediated cytotoxicity using an impedance-based real-time cell analyzer



Current standard assays to analyze lymphocyte-mediated antitumor cytotoxicity employ radioisotopic or fluorescent labels. However, such assays are not suitable for real-time analysis. Here we describe a protocol that facilitates the analysis of lymphocyte-mediated toxicity using a label-free, impedance-based real-time cell analyzer. This analyzer measures cellular electrical impedance, expressed as the cell index value, noninvasively and continuously. In contrast with label-dependent assays, this protocol simultaneously generates real-time killing curves useful for quantifying lymphocyte-mediated cytotoxicity in real time.

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Highlights

A label-independent assay assesses lymphocyte-mediated cytotoxicity

Cellular electrical impedance expressed as the cell index value

Continuous and noninvasive measurements of changes in cell number

Generates real-time killing curves, in contrast to label-dependent assays

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A protocol for quantifying lymphocyte-mediated cytotoxicity using an impedance-based real-time cell analyzer

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SUMMARY

Current standard assays to analyze lymphocyte-mediated antitumor cytotoxicity employ radioisotopic or fluorescent labels. However, such assays are not suitable for real-time analysis. Here we describe a protocol that facilitates the analysis of lymphocyte-mediated toxicity using a label-free, impedance-based real-time cell analyzer. This analyzer measures cellular electrical impedance, expressed as the cell index value, noninvasively and continuously. In contrast with label-dependent assays, this protocol simultaneously generates real-time killing curves useful for quantifying lymphocyte-mediated cytotoxicity in real time.

For complete details on the use and execution of this protocol, please refer to Kanemaru et al. (2021).

BEFORE YOU BEGIN

Today, the common standard assays to analyze the cytotoxic effects of lymphocytes against tumor cells employ radioisotopic or fluorescent labels; however, real-time analysis is difficult to achieve using these assays. Here we describe a protocol that facilitates the analysis of lymphocyte-mediated cytotoxicity using a label-independent real-time cell analyzer, the xCELLigence system.

This analyzer employs microtiter plates to noninvasively and continuously measure electrical impedance, reported as the cell index value (Delconte et al., 2016; Fasbender and Watzl, 2018). The continuous acquisition of data from each well simultaneously generates real-time killing curves under multiple conditions (Figure 1). For example, if effector cells induce the destruction of target cells, the corresponding cytolytic activity is detected as a decreased cell index value.

The protocol below describes the specific steps for using the mouse melanogenic melanoma-derived cell line B16-F1 as the target and mouse splenic lymphocytes as the effector. All animal experiments below were performed with approval from the Animal Research Committee of Kumamoto University (Kumamoto, Japan).

> ***Note:*** Because this system detects the destruction of adherent target cells by nonadherent effector cells, this protocol requires the former as targets and the latter as effectors. If you use nonadherent cells as targets, such as leukemia-derived cell lines, consult previously reported approaches to tether target cells to the plate using a specific antibody, such as an anti-CD40 antibody for Raji cells (Cerignoli et al., 2018). If target and effector cells are adherent, we assume that this protocol will be difficult to use in a cytotoxicity assay.

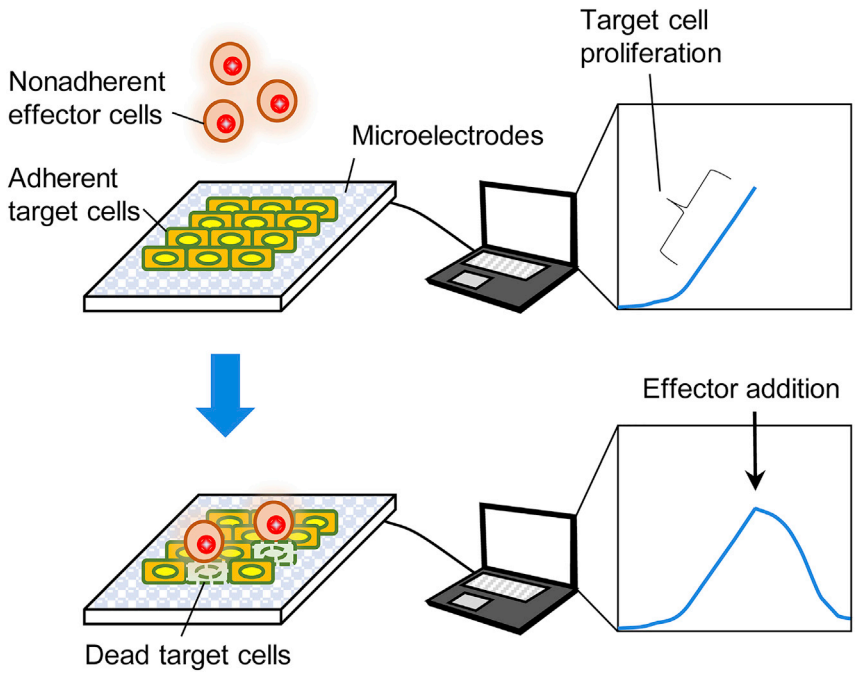

Figure 1. Principal of assessing lymphocyte-mediated cytotoxicity using an impedance-based real-time cell analyzer, the xCELLigence system

The microtiter plates (E-Plates) of the xCELLigence system contain gold biosensors embedded in the bottom of each well. The electrical impedance of each well is reported as the cell index value, which continuously and noninvasively represents changes in cell number. If nonadherent effector cells induce the destruction of the adherent target cells, the corresponding cytolytic activity is detected. The continuous acquisition of data for each well simultaneously generates real-time killing curves under multiple conditions.

Induction of effector cells: Immunization of mice with B16-F1 cells

Timing: 2 weeks

1. Preparation of B16-F1 cells
   a. B16-F1 (Riken RCB2649) cells are cultured in D-MEM (High Glucose) with L-Glutamine, Phenol Red, and Sodium Pyruvate (FUJIFILM Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA).
   b. Cells are collected upon reaching 50%–70% confluence.
      i. Cells are washed with PBS, dispersed in 0.25% Trypsin-EDTA phenol red (Thermo Fisher Scientific) at 37°C for 3 min, and resuspended in fresh culture media.
      ii. After centrifugation at 300 × *g* for 5 min, the cell pellet is resuspended in PBS, and the concentration is adjusted to 10^7 cells/mL.
2. Two WT C57BL/6J female or male mice, aged 8 weeks, are subcutaneously injected with 0.1 mL of the cell suspension (1×10^6 B16-F1 cells/mouse) and maintained in a specific pathogen-free facility for 2 weeks. The mean tumor diameter will reach 6 mm after 2 weeks.

   Note: Approximately 5.0×10^6 effector cells can be obtained from one mouse. In this protocol, two mice are used because $\geq 7.0 \times 10^6$ effector cells are required. The number of mice will vary depending on the required number of effector cells.

Induction of effector cells: Preparation of target cells

Timing: 2 days

3. B16-F1 cells are treated with IFN-γ (100 U/mL, BioLegend, San Diego, CA, USA).
   a. When the culture reaches 30%–50% confluence, the medium is replaced with 100 U/mL of IFN-γ in D-MEM.
   b. Cells are cultured for 48 h.

Note: IFN-γ treatment of target cells enhances the cytotoxic ability of the effector cells (Hildner et al., 2008). Specifically, IFN-γ-treated target cells express higher levels of MHC class I or FAS molecules, leading to more efficient killing of target cells by cytotoxic T lymphocytes compared with that achieved using untreated cells (Lee et al., 2006; Müllbacher et al., 2002; Merritt et al., 2004).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
D-MEM (High Glucose) with L-Glutamine, Phenol Red, and Sodium Pyruvate	FUJIFILM Wako	043-30085
Antibiotic-Antimycotic	Thermo Fisher Scientific	Cat# 15240096
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	Cat# 25200072
recombinant mouse IFN-γ (carrier-free)	BioLegend	Cat# 575306
BioMasherII	Nippi	320 103
microscope slides	Matsunami Glass	S9215
40-μm Cell Strainer	Falcon; Corning	352340
E-Plate 16	Agilent Technologies	5469830001
L-(+)-Lactic acid	Sigma-Aldrich	L6402
Experimental models: Cell lines		
B16-F1 cells	Riken BRC	RCB2649
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Charles River Laboratories Japan	https://www.crj.co.jp/product/rm/detail/b6j
Software and algorithms		
RTCA Software Light	Agilent Technologies	N/A
Other		
xCELLigence S16 system	Agilent Technologies	N/A

STEP-BY-STEP METHOD DETAILS

Induction of effector cells through coculture with IFN-γ-treated B16-F1 cells—Day 0

Timing: 1 h

1. Unfractionated splenocytes (4×10^7) obtained from mice bearing tumors formed by B16-F1 cells 2 weeks after implantation are cocultured with 2×10^6 IFN-γ-treated (100 U/mL) B16-F1 cells (Hildner et al., 2008; Kanemaru et al., 2017).
   a. The spleen is resected from tumor-bearing mice 2 weeks after implantation.
   b. The spleen is disrupted using a Dounce homogenizer (BioMasherII; Nippi, Tokyo, Japan) in 500 μL of PBS.

Note: If this homogenizer is not available, the spleen is disrupted using autoclaved microscope slides. For example, in 3 mL of PBS on a 6-cm dish, the spleen is disrupted between two microscope slides (S9215, Matsunami Glass, Osaka, Japan).

   c. Splenocytes are filtered through 40-μm filters (Falcon; Corning, NY, USA).
   d. Count the cells after washing twice with PBS.

e. Preparation of target cells to induce effector cells. B16-F1 cells treated with IFN-γ for 48 h are washed with PBS, dispersed in 0.25% trypsin at 37°C for 3 min, and resuspended in fresh culture medium. After centrifugation at 300 × *g* for 5 min, the cell pellet is resuspended in PBS and washed twice.
f. B16-F1 cells are resuspended in fresh culture media and counted.

2. Approximately 4 × 10^7 splenocytes and 2 × 10^6 IFN-γ-treated B16-F1 cells are cocultured in 20 mL of fresh culture media in a 15-cm dish and incubated for 5 days.

Note: The proliferation of B16-F1 cells requires high glucose concentrations. Therefore, the same medium (D-MEM [high glucose] containing 10% FBS and 1% Antibiotic-Antimycotic) is used in this protocol. The medium may be changed to one suitable for splenocytes at this step; however, we did not reproduce the results using another medium.

IFN-γ treatment of target cells—Day 2

Timing: 1 h

3. B16-F1 cells are treated with IFN-γ (100 U/mL).
   a. When the culture reaches 30%–50% confluence, the medium is replaced with 100 U/mL IFN-γ in D-MEM.
4. Cells are incubated for 48 h.

Configuration of the xCELLigence system and seeding target cells—Day 4

Timing: 3 h

5. Warm the medium in a 37°C water bath 30 min before starting the experiment.
6. Open the RTCA Software Light (Agilent Technologies, Santa Clara, CA, USA) application and set the schedule of the xCELLigence S16 as follows:

Step	Sweeps	Interval	Comments
1	1	1 min	Measuring medium background
2	100	15 min	Monitoring target cells
3	500	15 min	Monitoring after addition of effector cells

Alternatives: There are several xCELLigence models depending on the required throughput. For example, the maximum throughput of S16 is 16 wells; however, those of DP, SP, and MP are 48, 96, and 576 wells, respectively. The cytotoxicity assay can be performed using these models.

7. Preparing the E-Plate 16 utilized for the xCELLigence S16. Conducting background measurements (step 1).
   a. Transfer 50 μL of warm medium to all wells.
   b. Place the E-Plate 16 into the xCELLigence S16 station.
   c. Start step 1 (1 min and 1 sweep) to measure background.
   d. Remove the E-Plate 16 from the station.

Alternatives: While the xCELLigence S16 model employs RTCA Software Light, the DP, SP, and MP models employ RTCA Software, which calculates %Cytolysis. Furthermore, depending on the model, the type of E-plate can be changed (16 or 96 wells).

8. Add target cells to the E-Plate 16 plate.
   a. B16-F1 cells treated with IFN-γ for 48 h are washed with PBS, dispersed in 0.25% trypsin at 37°C for 3 min, and resuspended in fresh culture medium. After centrifugation at 300 × *g* for 5 min, the cell pellet is resuspended in PBS and washed twice.
   b. Cells are resuspended in fresh culture media and counted.
   c. Add the cell suspension (0.5–2 × 10^4 in 100 μL of culture medium) to each well of the E-Plate 16 (150 μL of total media; cell numbers can be modified depending on the experiment).

Note: We found that this protocol requires 2.5 × 10^3–4 × 10^4 target cells/well.

Example E-plate layout

	1		2	
	B16-F1	Control (untreated) effector cells	B16-F1	Lactate-treated effector cells
A	0.5 × 10^4 B16-F1 cells	0	1.0 × 10^4 B16-F1 cells	0
B	2.0 × 10^4 B16-F1 cells	0	4.0 × 10^4 B16-F1 cells	0
C	0	1.0 × 10^6 effector cells	0	1.0 × 10^6 effector cells
D	0	5.0 × 10^5 effector cells	0	5.0 × 10^5 effector cells
E	0	2.5 × 10^5 effector cells	0	2.5 × 10^5 effector cells
F	2.0 × 10^4 B16-F1 cells	1.0 × 10^6 effector cells	2.0 × 10^4 B16-F1 cells	1.0 × 10^6 effector cells
G	2.0 × 10^4 B16-F1 cells	5.0 × 10^5 effector cells	2.0 × 10^4 B16-F1 cells	5.0 × 10^5 effector cells
H	2.0 × 10^4 B16-F1 cells	2.5 × 10^5 effector cells	2.0 × 10^4 B16-F1 cells	2.5 × 10^5 effector cells

9. Equilibrate the E-Plate at 15°C–25°C for 30 min to allow the cells to settle to the bottom of the well.
10. Incubate the E-Plate at 37°C in a CO_2 incubator for approximately 18–36 h.
    a. Place the E-Plate 16 in the station in a 37°C incubator.
    b. Start Step 2 of RTCA Software Light and incubate to allow cell attachment and proliferation, monitor impedance for 25 h (15 min × 100 sweeps), and take readings every 15 min.

Note: Set the read time to exceed the expected experimental time.

11. Cell growth is dynamically monitored until cells reach the exponential growth phase and form a monolayer (approximately 18–36 h).

⚠ CRITICAL: When the target cells approach 100% confluence, the cell index value plateaus, which can cause cell death. Therefore, the effector cells should be added before this occurs.

Optional: To assess the effects of reagents on effector cells, one may consider the timing of such treatment at this stage. For example, we treat effector cells with 20 mM lactate for 24 h before performing the next step to assess the effect of lactate on the effector cells (Kanemaru et al., 2021). As shown step 12a, after collecting floating effector cells in the cocultures, effector cells are incubated in medium with or without 20 mM lactate (Sigma-Aldrich, St. Louis, MO, USA) in a 15-cm dish. After 24 h, collect and count effector cells and proceed to step 12b.

Addition of effector cells—Day 5

Timing: 2 h

12. Add effector cells to individual wells containing the target cells at different ratios (Effector:Target ratios = 12.5:1, 25:1, and 50:1).

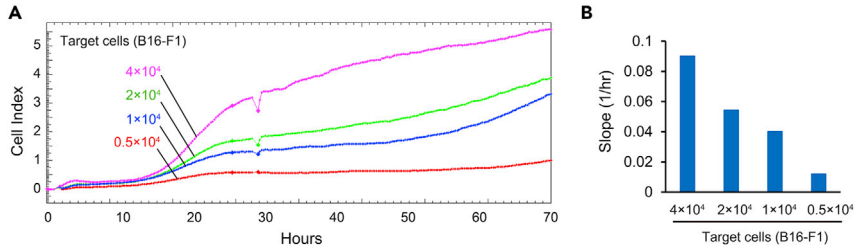

Figure 2. Analysis of the proliferation of target cells using the xCELLigence system

(A and B) Proliferation of the indicated dilutions of target cells (B16-F1). Electrical impedance was measured and reported as the cell index value (A). Rate of change of the cell index (0–70 h) is represented by the slope (B). Similar results were obtained from three independent experiments.

a. Gently collect floating effector cells in the cocultures. Centrifuge at 300 × *g* for 5 min and then count the cells.

⚠ CRITICAL: Please do not use trypsin or collect attached cells, which mainly represent target cells. Use a pipet to gently collect the floating cells (effectors).

b. Use 2.5×10^5, 5×10^5, and 1×10^6 effector cells in 100 μL of medium to prepare suspensions with Effector:Target ratios = 12.5:1, 25:1, and 50:1, respectively.

Alternatives: The Effector:Target ratios can be modified, e.g. 6.25:1 or 100:1.

c. Click the "abort current step" button of the RTCA Software Light to stop step 2 (monitoring target cells).
d. Remove the E-Plate 16 from the station. Using a multichannel pipet, remove 100 μL of medium from each well.

Note: Please ensure that the tip does not touch the bottom of the well and avoid detaching target cells.

e. Add 100 μL (final volume, 150 μL/well) of effector cells prepared in (b) directly to the wells. For background controls that contain only target cells, add 100 μL of fresh medium to each well (final volume, 150 μL/well) (see "example E-plate layout"). For example, in this layout, lanes A and B serve as background controls that contain only target cells.
f. For background controls that only contain effector cells, effector cells are added to wells that do not contain target cells (see "example E-plate layout"). For example, in this layout, lanes C–E are used as such background controls that contain only effector cells.
g. Equilibrate the E-Plate at 15°C–25°C for 30 min to allow the cells to settle to the bottom.

13. Return the E-Plate 16 to the station and click the "Start/Continue" button in the software.
14. The system will automatically continue to take measurements every 15 min (500 sweeps, 125 h). Cell index values are recorded by RTCA Software Light at 15-min intervals.

Note: If you use ≥2 reagents to assess their effect, more controls with or without respective reagents should be considered.

EXPECTED OUTCOMES

The E-plate noninvasively and continuously monitor changes in cell number (Figure 1). The biosensor signal ("cell index") is generated by proliferating target cells (Figure 2). After the addition of effector cells to the culture of target cells, the expected increase in the cell index will be suppressed because of cytolysis of target cells caused by the effector cells (Figure 3).

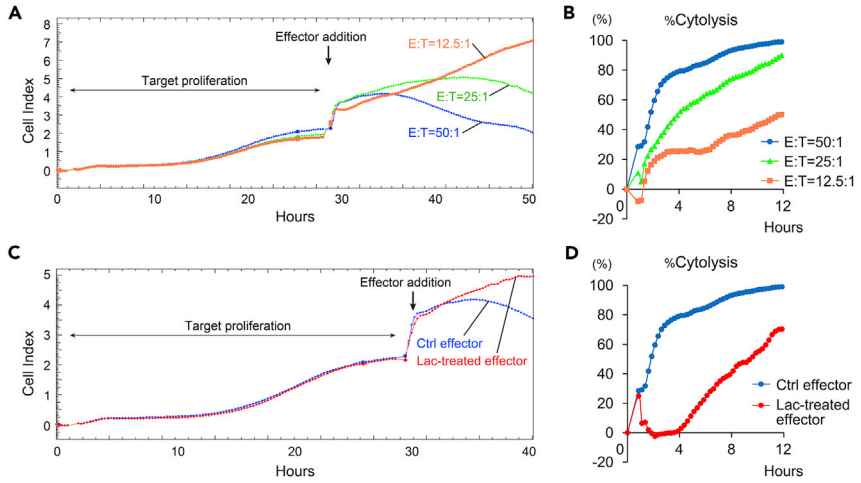

Figure 3. Cytotoxic lymphocyte killing assay using the xCELLigence system

(A and B) After adding effector cells at the indicated effector:target ratios (E:T, lymphocytes:B16-F1 cells), the system continuously measured cytotoxicity every 15 min. Wells contained 2 × 10^4 target cells and 100 × 10^4, 50 × 10^4, or 25 × 10^4 effector cells.

(C and D) The cytotoxicities of lactate (Lac)-treated (20 mM) effector and control effector cells against B16-F1 target cells (E:T = 50:1) were monitored at 15-min intervals. Wells contained 2 × 10^4 target cells and 100 × 10^4 effector cells. %Cytolysis was determined using RTCA Software Light of the xCELLigence system. Three independent experiments produced similar results.

QUANTIFICATION AND STATISTICAL ANALYSIS

%Cytolysis is calculated as follows:

%Cytolysis = (1 – (normalized (*ET* - *E*))/(normalized *T*)) × 100

ET = cell index of the coculture of target cells and effector cells

E = cell index of effector cells

T = cell index of target cells

Example output of the analysis:

1. Subtract the cell index value of effector cells from those of the coculture of target cells and effector cells. For example, *ET1* – *E1* and *ET2* – *E2* are shown in Table 1, and an example of calculated values is shown in Table 2.
2. Normalization of each cell index value. Each cell index value is divided by the 0-time value. For example, *T*/1.7338, (*ET1* – *E1*)/2.448, and (*ET2* – *E2*)/2.1108. An example of the normalized data of Table 2 is shown in Table 3.
3. Calculate %Cytolysis of Table 3 as follows:

For E:T = 50:1, %Cytolysis = (1 – *B*/*A*) × 100

For E:T = 25:1, %Cytolysis = (1 – *C*/*A*) × 100

LIMITATIONS

This protocol has several limitations. First, effector cells were induced by coculture of target cells and splenocytes for 5 days. Therefore, the effector cells may contain cytotoxic T lymphocytes (CTLs) as

Table 1. Example of raw data acquired using the xCELLigence S16 system

	Raw data				
Time (h)	Target (2×10^4)	Effector (100×10^4) *E1*	Effector (50×10^4) *E2*	Effector:Target = 50:1 (100×10^4:2×10^4) *ET1*	Effector:Target = 25:1 (50×10^4:2×10^4) *ET2*
0	1.7338	−0.2221	−0.1622	2.2259	1.9486
0.856945	1.5145	0.7564	0.8778	2.2857	2.5225
1.106945	1.6756	1.0116	1.2774	2.6886	3.2162
1.356945	1.7828	1.6038	1.7329	3.322	3.5367
1.606945	1.8017	2.0974	1.9441	3.5785	3.6496
1.856945	1.8175	2.4632	2.0822	3.7	3.7054
2.106667	1.8141	2.7007	2.1774	3.7357	3.7517
2.356667	1.8166	2.8733	2.2755	3.756	3.7795
2.606389	1.824	3.0149	2.3949	3.7837	3.8189
2.856389	1.8287	3.128	2.5176	3.8294	3.8908
3.106389	1.8389	3.2312	2.6439	3.886	3.9457
3.356389	1.8383	3.3173	2.7641	3.9289	4.0055
3.606389	1.8442	3.3868	2.8746	3.9687	4.0586
3.856389	1.8488	3.4477	2.9627	4.0024	4.0996

well as other immune cells such as regulatory T cells. Therefore, to evaluate specific cytolysis mediated by CTLs, collecting the effector cells may be required. For example, after the induction of effector cells, isolation of $CD8^+$ T cells using a $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) may be useful for purifying the cytotoxic T cells.

Furthermore, this system principally detects the destruction of the adherent target cells. We therefore assume that this protocol cannot be applied if target and effector cells are adherent.

Finally, we did not determine if the cytotoxicity assay applies to target cell lines other than B16-F1 cells. Therefore, this limitation must be addressed through further studies employing different cell lines.

TROUBLESHOOTING

Problem 1

Cell index of target cells does not increase (step 11).

Table 2. Example of calculated data in 15

	Normalized data		
Time (h)	Target (2×10^4) *T*	Effector:Target = 50:1 (100×10^4:2×10^4) *ET1 − E1*	Effector:Target = 25:1 (50×10^4:2×10^4) *ET2 − E2*
0	1.7338	2.448	2.1108
0.856945	1.5145	1.5293	1.6447
1.106945	1.6756	1.677	1.9388
1.356945	1.7828	1.7182	1.8038
1.606945	1.8017	1.4811	1.7055
1.856945	1.8175	1.2368	1.6232
2.106667	1.8141	1.035	1.5743
2.356667	1.8166	0.8827	1.504
2.606389	1.824	0.7688	1.424
2.856389	1.8287	0.7014	1.3732
3.106389	1.8389	0.6548	1.3018
3.356389	1.8383	0.6116	1.2414
3.606389	1.8442	0.5819	1.184
3.856389	1.8488	0.5547	1.1369

Table 3. Example of normalized data of Table 2

	Normalized data		
Time (h)	Target (2×10^4) *A*	Effector:Target = 50:1 (100×10^4:2×10^4) *B*	Effector:Target = 25:1 (50×10^4:2×10^4) *C*
0	1	1	1
0.856945	0.873514823	0.624714052	0.779183248
1.106945	0.966432114	0.68504902	0.918514307
1.356945	1.028261622	0.701879085	0.854557514
1.606945	1.039162533	0.60502451	0.807987493
1.856945	1.048275464	0.505228758	0.768997536
2.106667	1.046314454	0.422794118	0.745830965
2.356667	1.047756373	0.360580065	0.712526056
2.606389	1.052024455	0.314052288	0.674625734
2.856389	1.054735264	0.286519608	0.65055903
3.106389	1.060618295	0.26748366	0.616732992
3.356389	1.060272234	0.249836601	0.588118249
3.606389	1.063675164	0.237704248	0.560924768
3.856389	1.066328296	0.226593137	0.538610953

Potential solution

We determined the increase in the cell index using the B16-F1 cell line as the target. We recommend determining the increase in cell index by serially diluting B16-F1 cells to 0.5×10^4–4×10^4/well. As the culture approaches 100% confluence, the cell index plateaus. We therefore recommend adding the effector cells before this occurs.

Problem 2

Cytolysis is not observed (step 14).

Potential solution

Detached target cells may contaminate the collected effector cells. Please do not trypsinize or collect attached cells on the dish, which are mainly target cells. Please use a pipet to gently collect the floating effector cells. If cytolysis of the effector cells is weak, please increase the ratio of effector to target cells (e.g., E:T=100:1). We confirmed this protocol employing 2.0×10^6/well and 2×10^4/well as the maximum concentrations of effector cells and target cells, respectively.

Problem 3

Insufficient effector cells obtained (step 12).

Potential solution

We found that approximately 5.0×10^6 effector cells can be obtained from one mouse (C57BL/6J, aged 8 weeks). If disruption of the spleen using a Dounce homogenizer is insufficient (step 1 upon induction of effector cells through coculture with IFN-γ-treated B16-F1 cells – Day 0), the final number of effector cells will decrease. If sufficient effector cells cannot be obtained, please increase the number of mice.

Problem 4

The instrument does not recognize the E-plate. An alert is displayed, and measurements do not start (step 7, 10 and 13).

Potential solution

The electrodes on the back of the E-plate are contaminated. Please wipe the electrodes with cleaning wipes dampened with 70% ethanol. The electrodes become contaminated easily because of their positions. Please do not touch the electrodes when carrying the plate.

Problem 5

Results vary using the same conditions (step 14).

Potential solution

Please use an incubator with high humidity to minimize evaporation of media (preferably >90%). If the humidity is insufficient, the results tend to vary, particularly in the wells along the plate's perimeter during long-term measurements.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hisashi Kanemaru (hisashikanemaru@gmail.com).

Materials availability

This study did not generate new and unique reagents.

Data and code availability

All data from the study are presented here or in Kanemaru et al. (2021).

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AUTHOR CONTRIBUTIONS

H.K., Y.M., I.K., and S.F. developed the concept. H.K. and A.K. designed and performed the experiments, and analyzed the data. H.K. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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