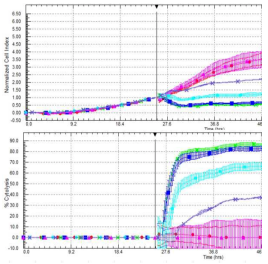
Jun 11, 2024

Impedance (xCELLigence)



In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.5jyl8jqz6g2w/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.5jyl8jqz6g2w/v1

Protocol Citation: Amanda Russell, Philippa R Kennedy 2024. Impedance (xCELLigence). **protocols.io** **https://dx.doi.org/10.17504/protocols.io.5jyl8jqz6g2w/v1**

Protocol status: Working
We use this protocol and it's working

Created: March 06, 2023

Last Modified: June 11, 2024

Protocol Integer ID: 78228

Keywords: cytotoxicity, killing, lymphocyte, natural killer, NK, lysis, attachment, adherent, impedance

Funders Acknowledgement:
Department of Defense
Grant ID: CA200922
Emily Whitehead Foundation

Abstract

This assay is used as a readout of NK cell cytotoxicity of adherent tumor cell lines. Tumor cells are seeded onto a 96-well gold-coated plate. As tumor cells adhere to the plate and proliferate, the flow of electrons is impeded across microelectrodes. Since NK cells minimally impact impedance, tumor cell survival is inferred from this impedance of electron flow. NK cells and drugs are added after 24 hours and a measure of killing is determined by reduced impedance, indicating tumor cell death. Continuous impedance data acquisition produces real-time killing curves.

Image Attribution

Generated in xCELLigence software by Amanda Russell, Melissa Khaw, Quinlan Kile and Shee Kwan Phung (University of Minnesota).

Guidelines

Principles of achieving even monolayers

- Mix cells/drug solutions well before adding them into a well to ensure consistent replicates.
- When adding effectors/drugs into the well, do not mix contents of the well, pipette gently to avoid disturbing monolayers.
- It is good practice to check your cell layers under a benchtop microscope to see if an adherent monolayer has formed (after first plating targets) and at the end of the assay to see if the impedance readings align with the confluence of the wells (did they slough off, so are giving low impedance despite surviving well?).

Effector cells

- The standard assay uses an enriched effector population *e.g. NK cells enriched from blood or expanded on feeder cells.*
- It is possible to run these assays with mixed populations *e.g. peripheral blood mononuclear cells, but greater effector:target ratios must be used e.g. 20:1*

Materials

E-Plate 96 PET e.g. cat. H063028 or H063027

Preparation - plate design

1
- Include 'media alone' and 'target alone' wells in the plate map. Mark them appropriately on the Xcelligence software as they are needed for internal calculations.
- Consider including 'effector alone' and 'target + drug, no effectors' as additional controls. If using ascites, you need to include effector alone.
- Aim to plate in triplicate. This can be reduced with practice if necessary or increased to evaluate reproducibility.
- Consider including a range of effector:target (E:T) ratios *(e.g. 5:1, 2:1, 1:1, 1:2)* for greatest sensitivity to differences. If using PBMC, E:T ratio will need to be increased (e.g. 10:1, 20:1).
- Edge effects - the edge of the plate evaporates differently over 2-3 days. Although analysis can be used to compensate for this, avoid replicates for a condition falling at the edge of the plate together. Avoid placing essential controls, e.g. tumor alone to which all data is normalized, at the edge of the plate.

Preparation - target cell titration

2 Target cell density should be titrated, so that the full assay can be run with logarithmic cell growth, with the greatest impedance and maximum sensitivity/reliability possible.

Note

- Titrations of target cells should be performed for each new target line prior to killing assays in order to select the most appropriate number of target cells/well.
- The ideal density of target cells provides a sizeable impedance signal and an upward slope of impedance for the duration of the assay. This allows us to be confident that the reduction in impedance is 100% attributed to the effectiveness of the drug in killing the target cells, and not because the cells are dying due to media exhaustion/confluence/sloughing.
- Suggested target cell titration: 100k, 75k, 50k, 25k, 12.5k, 10k, 7.5k, 5k, 2.5k, 1.25k, 0/well

- *e.g. If target cells reach confluence at 48h, this gives you 24h for target cell adherence and 24h for the cytotoxicity assay.*

3 Useful cell densities for a 96 well plate format are as follows:
- HT29 - 35,000 cells/well (48h until confluence)
- OVCAR8-12,500 cells/well
- Cal33-12,500 cells/well
- LN229- 25,000 cells/well
- DU145-25,000 cells/well
- C42-25,000 cells/well

Background readings

4 Add 50µl of media to each well.

5 Place your plate in the xCELLigence.

1. Open the incubator, check which bays are occupied (red or green light on the numbered panel at the front). This should match the online booking form.
2. Lift the handle to an unoccupied position, place your plate in with position A1 back right (aligning with the numbering on the top of the holders).
3. Lower the handle to lock the plate. That bay light will now be illuminated red on the front panel (='idle').

6 Set up the assay in the xCELLigence software and take a scan of all your wells with the media alone.

1. On the individual window for your bay (e.g. [#1 …] on the window header for bay 1 in the machine), press the 'lock' button.
2. On the main tab for the software (not the individual window for your bay) press the 'play' button.
3. The software will give you two options for the new experiment. For a cytotoxicity assay, select 'immunotherapy'.
4. Give your experiment an appropriate name to help you locate it later (e.g. date, your name, some experimental details)
5. Under the appropriate tabs within the window fill out the experimental details. If adding multiple NK cell lines, you can use the 'quantity' of NK cells to differentiate between donors. E.g. rather than saying you have 20,000 NK cells say you have 1 NK or 2 NK for your different donors (the software will then analyze them separately).
6. On the scheduling screen, right click to add a step e.g. 1 image for media alone and then many images at 15 min intervals for e.g. 48 hours.

7 Check all wells have sensible readings.

- Very high impedance values can result from air bubbles in the plate. Check for these on a benchtop microscope. Tap the plate on the side to dislodge the bubble and then take the reading again.

Add adherent target cells

8 Remove your plate and add your target cells in 50µl of media, bringing your total media to 100µl.

- To remove plate, pause and unlock the individual plate within its own window in the software before trying to physically open the plate holder.
- Add additional media to your 'effector alone' and 'media alone' wells up to 100µl for consistency.

9 Let the plate sit for 10 min at room temperature or 37°C to ensure an even monolayer of cells
- Check the cells under a benchtop microscope to ensure cells are not gathered on one side or rolling around.

10 Return your plate to the xCELLigence and begin scanning again to monitor confluence of target cells.
- Confluence will ideally take 48 to 72 h depending on seeding density and cell type.

Add effector cells

11 Pause the scanning of your plate in the software (see Step 8) and retrieve your plate.

12 Gently add effectors and/or treatments to a total of 200 µL per well.
- Do NOT MIX wells. This can disrupt the adhered monolayer on the plate bottom.
- Also top up 'media alone' and 'effector alone' wells

13 Return your plate to the machine and continue the scan.

Exporting data and analysis

14 Export the data from the xCELLigence Software into Excel Spreadsheet.
- Check individual wells cell index and make sure you have no outliers.
- If you see outliers in the triplicate wells, right click on the well and click 'mask well'. This will exclude the well from the analysis.
- Drag the vertical bar in the graph to set the normalization timepoint. The cell index should be normalized for all wells to the stable point when effectors and drugs are added (24 h).
- % Cytolysis depends upon this normalization point and estimates the % of target cells that have been killed at any given timepoint.
- For data export, in the Data Analysis tab of your experiment, right click on the graph.
- Click "copy data in list format" from the drop down.

(Unchecking "average" and "standard deviation" boxes will provide individual replicate values)
- Transfer data to Excel for processing. Invert the data (100% - Cytolysis = % Tumor cell survival)
- Plot in GraphPad Prism for statistical comparisons.

Protocol references

xCELLigence Manual: https://www.agilent.com/cs/library/usermanuals/public/user-guide-cytotoxicity-assays-xcelligence-5994-1896en-agilent.pdf