Validation of an impedance-based *in vitro* potency assay for repeatability and intermediate precision

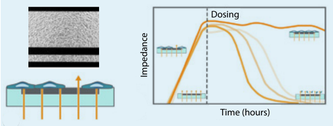
Danielle Califano[1], Jianjie Jiang[2], Dirk Windgassen[2], Caitlyn De La Flor[2], Stacie Chvatal[1], Denise Sullivan[1], and Daniel Millard[1]
[1]Axion BioSystems, [2]Miltenyi Biotec, Inc.

Introduction

Immune effector T cells are a promising cancer therapy due to their innate cytotoxicity. In addition, engineering chimeric antigen receptors (CAR) to target tumor-associated antigens or neoantigens can lend high specificity. Assessing the efficacy and potency of these label-free T cell therapies, *in vitro* and at high throughputs, is vital for the preclinical development of these promising therapies.

Axion BioSystems' Maestro Z platform offers impedance-based cell analysis for real-time, label-free monitoring of cell viability, morphology, cytolysis, and signaling. Here, we measured cytotoxicity data from several different potency assays using a variety of target cells and immune-effector cells.

Figure 1



The impedance is measured from electrodes embedded in the bottom of each well. As cells cover more of the electrode, impedance increases in proportion to the number of attached cells. If a perturbation kills the attached cells, impedance decreases as the cells lyse.

Methods and materials

1 Maestro Z product family

Figure 2A

Features	Maestro Z	Maestro TrayZ	Maestro ZHT
Throughput	96-well	Up to 8 x 96-well	384- and 96-well
Environmental controls	Built-in	External	Built-in
GxP compatible	☑	☑	☑
Barcode plate tracking	☑	☑	☑
Automation API	☑	No	☑
Dimensions (WxDxH)	280 x 413 x 225 mm	440 x 450 x 60 mm	280 x 452 x 225 mm

- Label-free, non-invasive tracking of cultured cells or spheroids/organoids
- Integrated environmental control provides a stable benchtop environment for short- and long-term toxicity studies
- Automatic and continuous cell monitoring from 96 or 384 wells simultaneously
- "One button setup" automatically docks the plate and adjusts temperature and CO_2 levels
- Powerful data analysis to focus on the science, while AxIS Z handles the details with simple setup and automatic experiment tracking
- See your cells with the viewing window included in each well of the CytoView-Z 96-well plate
- State-of-the-art electrode processing chip (BioCore v4) offers stronger signals, ultra-low frequency content, and enhanced flexibility

Impedance assay measures diverse cell properties

The Maestro Z records impedance at multiple frequencies simultaneously, enabling a thorough characterization of cell behavior, including:

- **Coverage/Density** – The change in impedance is directly related to the quantity of cells in a 2D- and 3D-culture covering the electrodes
- **Cytotoxicity** – Dynamic monitoring of cell viability provides measures of the degree and speed of cell death
- **Morphology** – The cell size, shape, and intercellular-tight junctions significantly impact the measured impedance
- **Signaling** – Small changes in cell shape or cytoskeleton organization are detected in response to intracellular signaling events

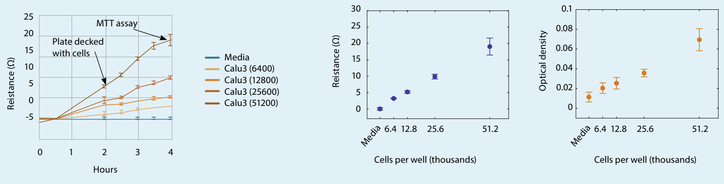
Figure 2B

2 Direct correlation of impedance assay with cell number

To validate impedance-based monitoring of cell viability, Calu-3 cells were added to a CytoView-Z plate with a varying number of cells per well and monitored for four hours on the Maestro Z platform. The change in resistance was correlated with the number of cells initially seeded, and the resistance continued to increase as the cells adhered and flattened on the surface. At four hours post-seeding, the plate was removed and an MTT assay was performed in the CytoView-Z plate.

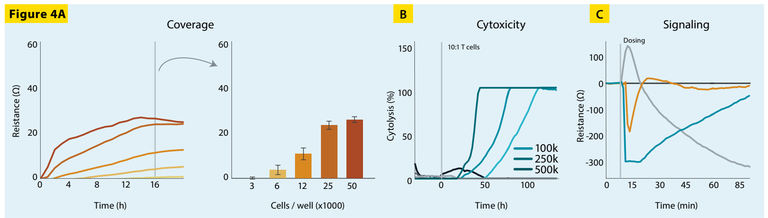
The resistance measured with the Maestro Z platform was linear, with respect to the cell number, and directly correlated to the MTT assay readings from the same wells.

Figure 3



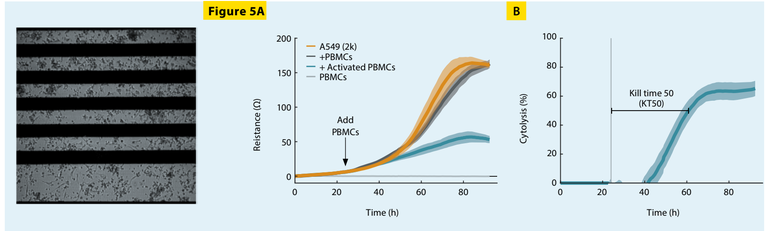
3 Impedance-based assay for cell-mediated cytotoxicity

Figure 4A



The impedance measurement is sensitive to the attachment of adherent or tethered target cells, but not the presence of non-adherent immune effector cells. In this way, the assay is naturally sensitive and specific to target cell attachment and cytotoxicity. The attachment and proliferation of the A549 target cells (orange) is measured via the resistance over time. At 24 hours, the peripheral blood mononuclear cells (PBMCs) were added across various conditions. First, the PBMCs were added to some wells alone (light gray), and did not affect the resistance measurement, confirming that the measurement in this assay is specific to the target cells. The PBMCs were also added to the wells containing the target cells, with (blue) and without (dark gray) anti-CD3 and IL-2 to activate the immune effector cells. The resistance measure was significantly lower when activated PBMCs were added to the target cells, indicating immune cell-mediated cytotoxicity. The dynamics of the cytotoxicity were quantified as the KT50, defined as the time duration required for 50% cytolysis of the target cells. In this example, the KT50 was 39 ± 3 hours for activated PBMCs added at a 10:1 effector to target ratio.

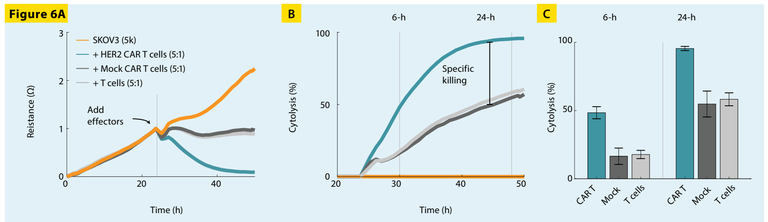
Figure 5A



4 CAR T cells demonstrate antigen-specific killing

CAR T cell therapy uses genetically engineered T cells that express a CAR that binds to a specific antigen on tumor cells. In HER2-overexpressing SKOV3 cell line, donor-matched mock CAR T cells, which lack the tumor antigen-recognizing domain, and non-transduced T cells were used to separate non-specific killing from specific CAR T cell killing. The HER2-targeted CAR T groups demonstrated approximately twice as much target-cell killing as mock CAR T cells and non-transduced T cells as shown by the resistance (fig. 6A) and cytolysis (fig. 6B) time courses for SKOV3 killing by CAR T cells and the comparison (fig. 6C) of % cytolysis at 6 and 24 hours, following the effector cell addition at E:T = 5:1.

Figure 6A

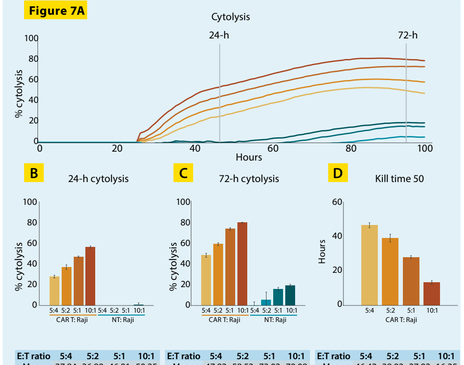


Results

1 High repeatability of CAR T cell-mediated cytotoxicity assay

Development and validation of potency assays is a crucial step in the immunotherapy pipeline. To qualify a potency bioassay, researchers must report the intra-assay precision or repeatability. To assess repeatability, the cytolysis of CD19-expressing Raji cells by CD19-specific CAR T cells was measured across replicates at four different E:T ratios (10:1, 5:1, and 2.5:1). Time series and endpoint (24- and 72-hours post-CAR T addition) % cytolysis of target cells treated with either CAR T or non-transduced T cells was measured. As expected, the number of CAR T cells highly correlated with the percent cytolysis of the target cells, with the highest E:T ratio reaching ~80% cytolysis. This was further confirmed by comparing KT50 values for each E:T ratio. The mean and standard deviation, as well as the % CV, were calculated for both endpoint cytolysis and KT50.

Figure 7A



E:T ratio	5:4	5:2	5:1	10:1
Mean	27.84	36.89	46.91	50.25
STDev	0.90	1.04	1.64	3.35
%CV	3.23	2.82	3.49	5.95

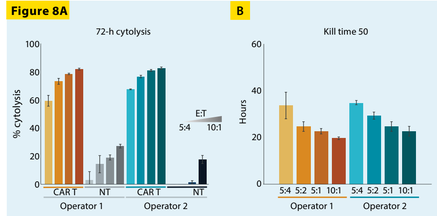
E:T ratio	5:4	5:2	5:1	10:1
Mean	47.92	58.52	73.02	79.08
STDev	3.33	2.22	2.08	0.9
%CV	6.95	3.79	2.85	1.13

E:T ratio	5:4	5:2	5:1	10:1
Mean	46.42	38.92	27.92	16.25
STDev	2.12	2.65	1.73	1.53
%CV	4.57	6.80	6.20	9.41

2 Low analyst-to-analyst variability of liquid tumor cell killing

Intermediate precision results were generated by two analysts who prepared replicate sample preparations with their own cell vials and reagents. Each analyst seeded Raji target cells to anti-CD40-coated plates at a density of 25K cells. After 24 hours, the analysts added CD19 CAR T cells or non-transduced T cells (NT) at four different E:T ratios (10:1, 5:1, 2.5:1). Endpoint cytolysis and KT50 values were compared between operators and overall mean, standard deviation and %CV were determined. The % cytolysis at 100 hours was 63.36%, 74.81%, 79.63%, and 82.16% for each increasing E:T ratio, respectively. The KT50 values inversely correlated with the number of effector cells, whereby as the number of effector cells increased, the time required for 50% cytolysis of the target cells decreased. The % CV was less than 15% for all conditions.

Figure 8A

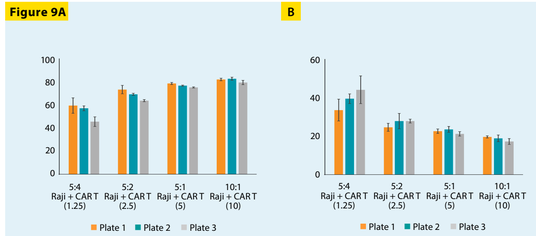


E:T ratio	5:4	5:2	5:1	10:1
Mean	63.36	74.81	79.63	82.16
STDev	6.13	3.12	1.65	1.19
%CV	9.67	4.17	2.08	1.45

E:T ratio	5:4	5:2	5:1	10:1
Mean	34.16	26.99	23.66	21.16
STDev	3.71	3.03	1.86	2.14
%CV	10.86	11.24	7.87	10.10

3 Interassay precision highlights plate-to-plate consistency

Figure 9A



Cytolisis reproducibility (intra-assay precision)

E:T ratio	5:4	5:2	5:1	10:1
Mean	53.88	68.56	76.62	81.19
STDev	7.44	4.79	1.71	1.71
%CV	13.81	6.98	2.23	2.11

KT50 reproducibility (intra-assay precision)

E:T ratio	5:4	5:2	5:1	10:1
Mean	39.17	26.83	22.50	18.61
STDev	5.31	1.88	1.17	1.23
%CV	13.56	7.02	5.21	6.60

The aforementioned setup was repeated on three separate days and plates to evaluate inter-assay precision. Endpoint cytolysis and KT50 for each plate are displayed in figures A and B, respectively. The overall mean, standard deviation, and % CV are shown in the tables below. Endpoint cytolysis values show high plate-to-plate consistency across all E:T ratios with % CV less than 15%. KT50 values were used to compare overall cytolysis between plates and exhibited a low % CV of around 15% or less.

Conclusion

Potency test validation is critical to ensure cell-based therapies' efficacy, safety, and reproducibility. Validation procedures validate the therapeutic potential, support regulatory approval processes, and facilitate improved patient outcomes. Here, we demonstrate a qualified method for *in vitro* immune cell-mediated killing assays with non-adherent target cell types. Low variability between replicates, operators, and plates of CAR T killing reflects the robustness of the Maestro Z assay, as well as the proficiency and expertise of CDMO analysts. In summary, the potency of CAR T cell killing of liquid tumor cell lines can be quantified using the Maestro Z, making it a valuable tool for CAR T cell discovery and manufacturing.

- The Maestro Z allows for simple, non-invasive, real-time monitoring of immune-cell mediated killing of target cells, providing a sensitive, quantitative assay for evaluating potency *in vitro*
- This impedance-based potency assay serves to evaluate and characterize immunotherapy products, with high consistency and minimal analyst-to-analyst variability
- These data taken together indicate that the Maestro Z platform meets the criteria for repeatability and precision for potency testing when using a tethered suspension cell line

Miltenyi BioIndustry, a division of Miltenyi Biotec, Inc. | 2825 N. First Street | San Jose, CA 95134, USA | Phone 530-888-8871 | macuss@miltenyi.com | **www.miltenyibioindustry.com**

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