Application Note

Cell Analysis

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Bispecific T Cell Engager (BiTE) Mediated Cancer Cell Lysis Assay

Using the Agilent xCELLigence real-time cell analysis (RTCA) eSight

Authors

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Abstract

This application note evaluates the Agilent xCELLigence RTCA eSight as a tool to monitor BiTE antibody-mediated cancer cell lysis. RTCA eSight technology combines cellular impedance with live cell imaging into a dual readout system to quantify cellular behavior in real time. The study presents an *in vitro* real-time potency assay using an xCELLigence RTCA eSight to assess BiTE-mediated immune cell cytolysis of epithelial cell adhesion molecule-1 (EpCAM) expressing cancer cells. EpCAM is a well-characterized tumor-associated antigen (TAA) expressed exclusively on epithelia and epithelial-derived neoplasms under investigation as a prognostic marker and potential immunotherapeutic strategy for cancers of epithelial origin (for example, breast, colorectal, ovarian, ampullary pancreas, gall bladder, and liver cancers).[1,2] The data demonstrate that the RTCA eSight real-time potency assay is a robust method to screen and characterize BiTE activity *in vitro*.

Introduction

As of 2022, over 60 FDA-approved immunotherapies collectively covered treatment for almost every major cancer type. Moreover, thousands of immuno-oncology agents and remedies are currently undergoing evaluation in clinical trials worldwide, including BiTEs.[3] BiTEs—engineered antibody constructs—enable polyclonal T cell response to cell-surface TAAs, bypassing narrow T cell receptor specificities and major histocompatibility pathways (MHC) pathways. To redirect immune response, BiTE constructs target both an antigen expressed on cancer cells (for example, CD19 in B cell linage malignancy) and the CD3 receptor on immune cells, simultaneously.[4] The FDA approved the first BiTEs antibody, blinatumomab (BLINCYTO), developed by Amgen, in December 2014 to treat relapsed or refractory B cell precursor acute lymphoblastic leukemia (ALL).[5] While clinical efficacy is the final assessment for all treatments, they must travel a long and complex developmental path to get there. Assays acting as intermediate indicators of efficacy capable detecting and quantifying T cell mediated, antigen-specific immune responses are needed to cross the chasm between preclinical and clinical development.

Assay principle

The RTCA eSight is currently the only instrument that integrates microscopic live cell imaging with real-time impedance detection within one well. The instrument features five cradles. Three cradles collect both impedance and imaging using specialized plates, while the other two are suitable for common cell culture plates and only collect imaging data. Gold biosensors at the bottom of each well in Agilent E-Plate VIEW 96 PET microwell plates measure cellular impedance and record the signal at a user-defined time frequency (every minute, hour, etc.), reported as a unitless parameter called the Cell Index. Changes in target cells (cell shrinkage, detachment, death, lysis, etc.) associated with immune cell killing are easily detected as a drop in Cell Index (impedance) and correlate to cell number, proliferation rate, cell size and shape, and the strength of cell-matrix adhesion. Note that impedance provides a sensitive readout of immobilized target cells; nonadherent immune cells do not contribute to impedance.

At the center of each E-Plate VIEW well, a window allows the RTCA eSight to track cell health, behavior, and viability through live cell imaging in brightfield and three fluorescent colors (red, green, and blue). RTCA eSight software can automatically convert immune cell killing via impedance readout and decrease in cancer cell number via live cell imaging to the percentage cytolysis.

Experimental

Target cell

Human breast cancer cell line T47D purchased from ATCC (part number HTB-133) was initially isolated from a pleural effusion obtained from a patient with an infiltrating ductal breast carcinoma. Cells were cultured in RPMI1640 medium, 10% FBS and 1% penicillin/streptomycin in a 37 °C CO_2 incubator.

For live cell imaging, T47D-Red, a stable cell population, was generated from the parental T47D cells by transduction with lentiviral particles harboring the gene encoding for red fluorescent protein (Agilent eLenti Red reagent, part number 8711011) and subsequent short puromycin selection. The expression level of EpCAM in T47D-Red cells was analyzed and confirmed using EpCAM-PE antibody conjugates (BioLegend, part number 369805) in conjunction with the Agilent NovoCyte flow cytometer (part number 2010047).

Effector cell

Fresh PBMCs were isolated from the whole blood of healthy human donors with Ficoll-Paque using the protocol provided by Miltenyi Biotec. PBMCs were activated with 7.5 µL/mL of ImmunoCult Human CD3/CD28 T Cell Activator (StemCell Technologies, part number 10971) for two days in the T25 cell culture flask. Before mixing with BiTEs, the cells were spun down and washed with RPMI1640 culture medium, followed by counting with a hemocytometer.

BiTE-mediated cytolysis assay

T47D-Red cells constitutively expressing mKate fluorescent protein in the nucleus were seeded at 8,000 cells per well in Agilent E-Plate VIEW 96 PET microwell plates (part number 300601030) on day 1. Cellular impedance and live cell imaging data acquisition were initiated at 15 minutes and 2-hour intervals, respectively, using the RTCA eSight. Exposure time for the red fluorescent channel was 300 ms. On day 2, activated PBMCs were premixed with CD3xEpCAM BiTE antibody (EpCAM BiTE) (G&P Biosciences, part number FCL2028B) and then added to T47D-Red cells. E-Plate data acquisition was resumed at 15-minute intervals for impedance readout and two-hour intervals for imaging. The experiment was terminated 72 hours after treatment.

Software for data analysis

All eSight data in this manuscript were processed with the latest version of eSight software V1.2.0 and the Immunotherapy module. The dose-response curves were generated with GraphPad Prism 9.5.1.

Results and discussion

Quantification of EpCAM BiTE-mediated cytolysis using the Agilent xCELLigence RTCA eSight

The dose-dependence of EpCAM BiTE-mediated cytolysis

To develop a potency assay to assess EpCAM BiTEs using both impedance and live cell imaging simultaneously on the RTCA eSight, we generated T47D-Red, a target cancer cell line that constitutively expresses red fluorescent protein in the nucleus as described in the Experimental section. Cells seeded in E-Plate VIEW 96 microwell plates were allowed to adhere and proliferate overnight. Approximately 24 hours after cell seeding, PBMCs (40,000 cells/well) were premixed with increasing concentrations of EpCAM BiTE, added to the wells containing T47D-Red cells, and monitored for an additional three days. As shown in Figure 1A, BiTE addition resulted in a dose- and time-dependent decrease in Cell Index, suggesting a loss in T47D-Red cell viability as a result of BiTE-mediated PBMC cytolysis. Normalized Cell Index data can be converted to percentage cytolysis using RTCA eSight software (Figure 1B). Taking the area under the curve (AUC) of percentage cytolysis, a clear dose-dependent response appears and derives a 175.7 ng/mL EC_{50}, demonstrating EpCAM BiTE-mediated target cell killing (Figure 1C).

As indicated by both the Normalized Cell Index curve and derived percentage cytolysis, at a dose of 0 ng/mL BiTE, 30 to 40% cell lysis is detected. Considering PBMCs are a highly heterogeneous cell population comprised of varying percentages of lymphocytes, dendritic cells, and monocytes, BiTEs-independent target cell killing is expected.

To confirm that the decrease in Normalized Cell Index is directly correlated to BiTE-induced cell death and provide additional context to the potency assay, live cell images of T47D-Red cells in the presence of different doses of EpCAM BiTE and PBMCs were recorded in conjunction with impedance.

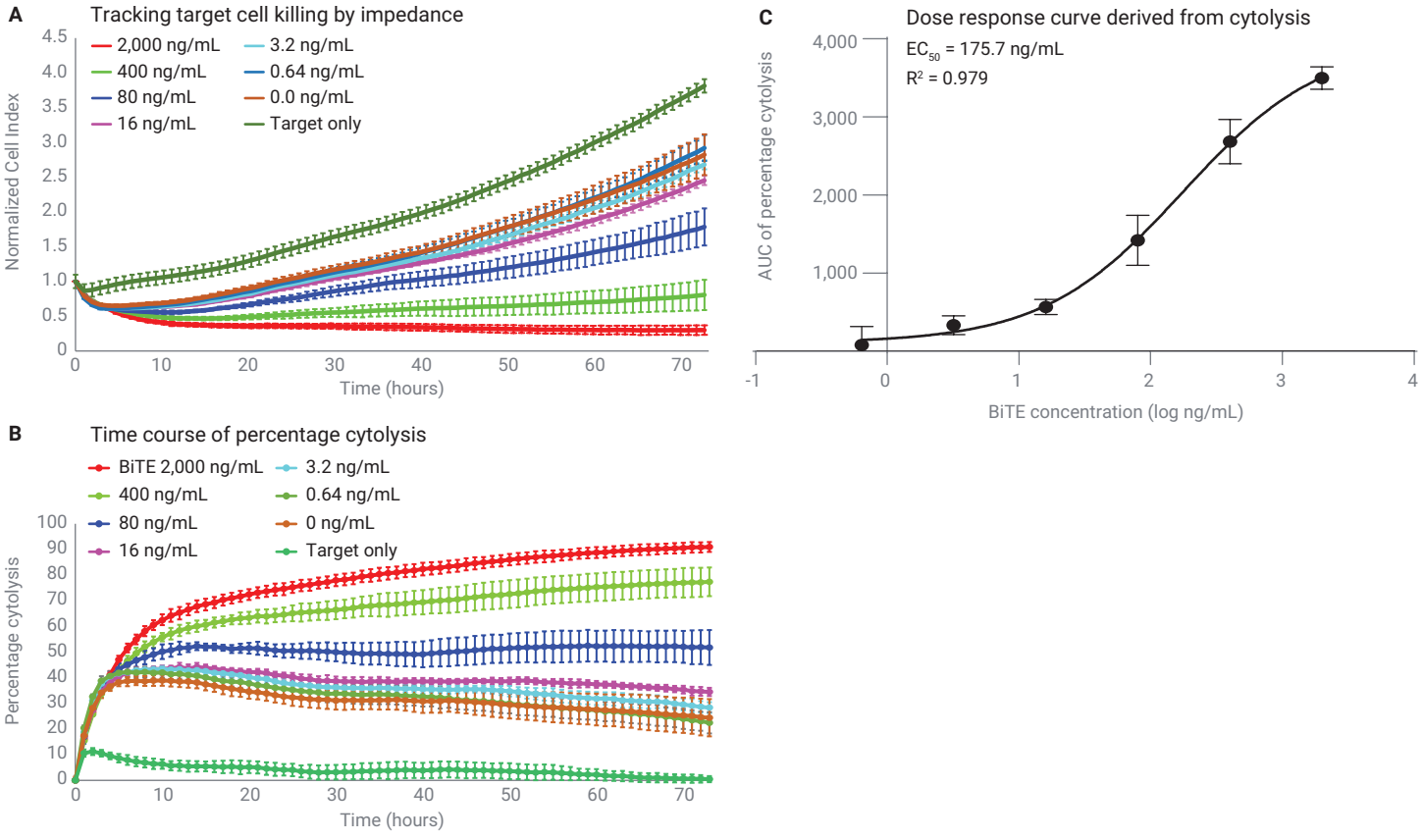

Figure 1. EpCAM BiTE-mediated PBMC cytolysis. (A) Time course of cytolysis measured by impedance. (B) The percentage cytolysis derived from the Normalized Cell Index. (C) Dose-response curve including extrapolated EC_{50} and R^2. Assays were run in triplicate; error bars represent standard deviation.

As shown in Figure 2A, the presence of 400 ng/mL EpCAM BiTE significantly decreased the number of red fluorescent target cells in a time-dependent manner compared to the control, confirming that BiTE inclusion facilitates T47D-Red cells cytolysis. Fluorescent target cells are automatically detected and quantified by eSight software (Figure 2A, lower panel). Inclusion of EpCAM BiTE results in a significant decrease in the number of T47D-Red cells as quantified by fluorescent nuclei (Figure 2B).

The impact of effector cell to target cell ratio on EpCAM BiTE-mediated cytolysis

In addition to BiTE-mediated cell killing dose-dependence, the impact of varying the effector-to-target cell ratio (E:T) on BiTE killing potency was assessed. Target cells seeded and cultured as outlined prior were treated with a mixture of PBMCs at different E:T ratios and final EpCAM BiTE concentration of 80 ng/mL. Increasing PBMCs in the presence of EpCAM BiTE resulted in an impedance decrease dependent on the number of effector cells (Figure 3A). Results indicate cytolysis at different E:T ratios is linear with the number of effector cells added (Figures 3B and 3C).

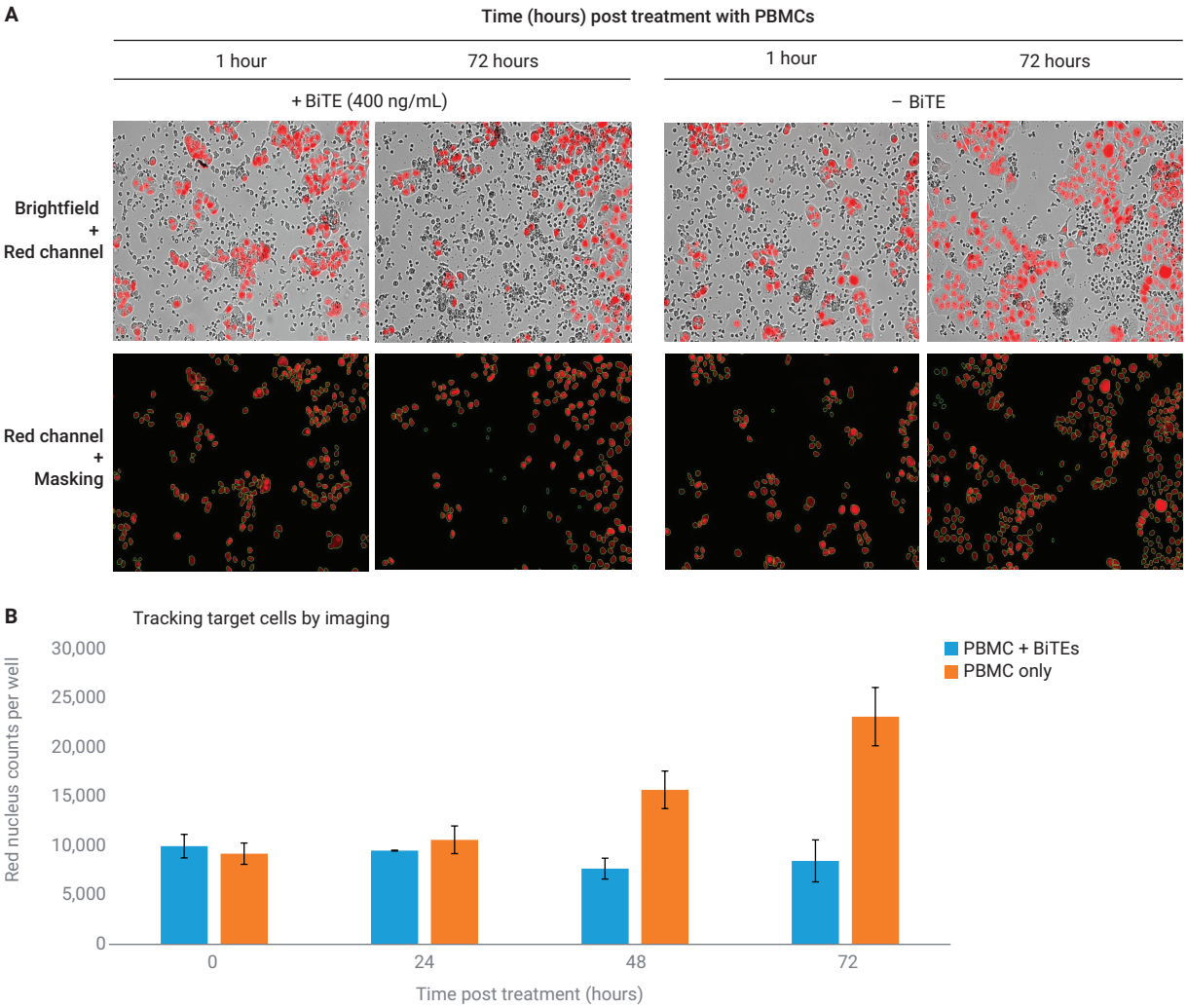

Figure 2. Quantitative imaging evaluation of target cell lysis using the Agilent xCELLigence RTCA eSight. (A) Images of brightfield and red fluorescent channel (upper panel) and red fluorescent channel with masking (lower panel) recorded at two time points following PBMC addition +/– BiTE (400 ng/mL). (B) Dynamic recording of target cell nucleus numbers counted by the eSight software. Cytolysis assays were run in triplicate; error bars represent standard deviation.

Live cell images simultaneously captured with impedance were also evaluated and quantified (Figures 3D to 3F). Red fluorescent nuclei quantification indicates a decrease in T47D-Red cells in a PBMC and BiTE-dependent manner. Converting red fluorescent nuclei to percentage cytolysis shows that increasing the E:T ratio increases time-dependent cytolysis. Like impedance data (Figure 3C), direct correlation exists between percentage cytolysis and the number of PBMCs added in the presence of EpCAM BiTE (Figure 3F). Overall, tracking the killing response using impedance (Figures 3A to 3C) and live cell imaging (Figures 3D to 3F) provides a complementary insight into different aspects of the same biological process; the rapid and sensitive real-time data acquisition of impedance monitoring allowing users to continuously track cellular events with minimal perturbation. It stands out when assessing cell-substrate attachment strength and detecting early-stage cellular changes that might be missed by other methods. Alternatively, live cell imaging offers spatial and morphological information crucial to understanding cellular dynamics and interactions. It enables

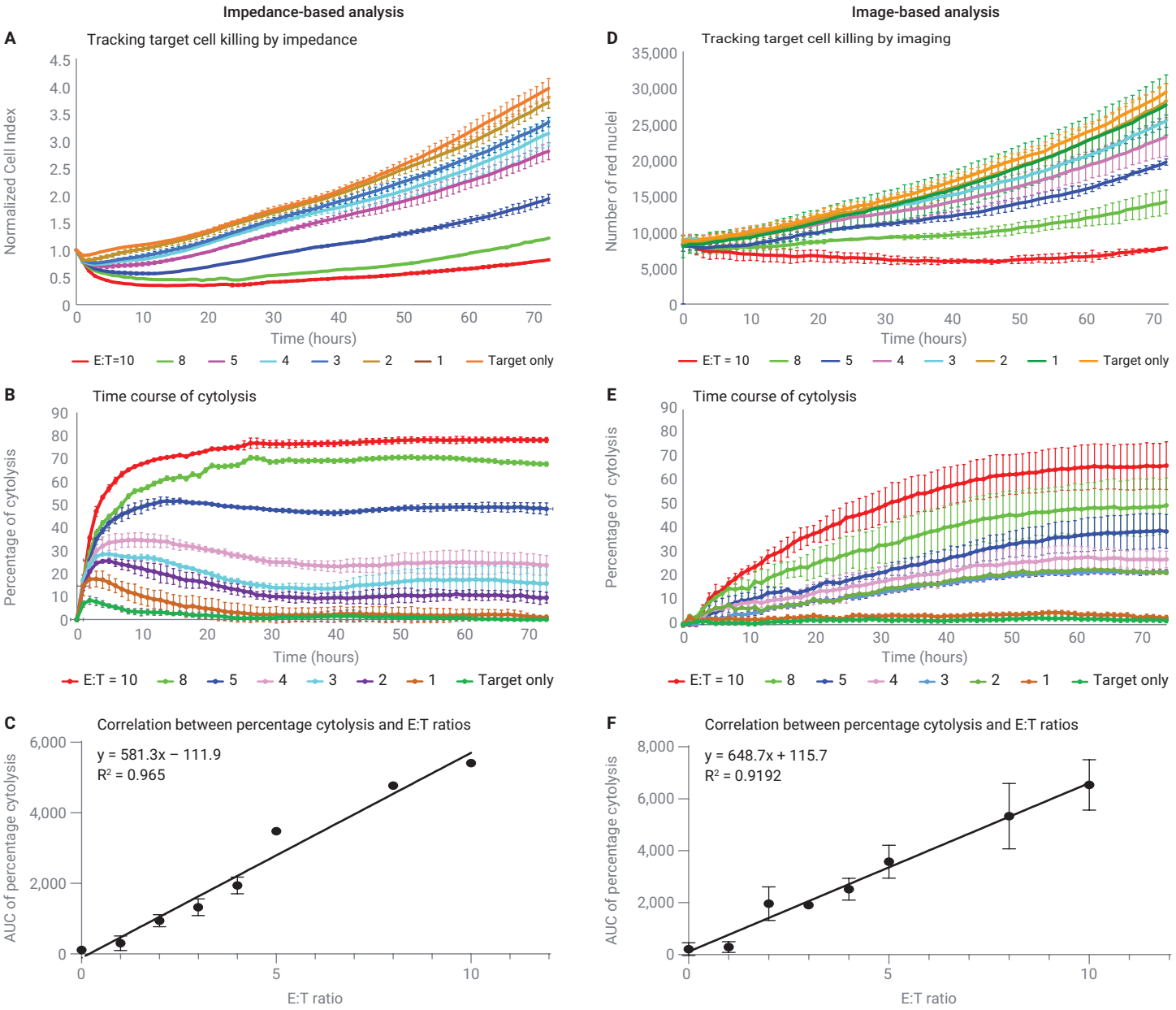

Figure 3. Cytolysis of T47D-Red at different E:T ratios measured by impedance (A–C) and imaging (D–F) using the Agilent xCELLigence RTCA eSight. Upper panels (A, D) display the Normalized Cell Index, middle panels (B, E) display percentage cytolysis, and lower panels (C, F) display the relationship between AUC of percentage cytolysis and E:T ratios derived from impedance and live cell images respectively. Cytolysis assays were run in triplicate; error bars represent standard deviation.

the visualization of specific cellular markers, allowing the identification and tracking of different cell types such as target cells and effector cells in the same field of view.

Conclusion

In summary, the data demonstrate that the Agilent xCELLigene RTCA eSight potency assay effectively analyzes BiTEs in a quantitative, dose-, and time-dependent manner. More generally, experiments with EpCAM BiTE demonstrate the ability of the xCELLigence RTCA eSight to simultaneously analyze killing efficiency across multiple conditions, ensuring assay optimization is rigorous and efficient. In addition, combining the advantages of real-time impedance monitoring—including simplicity, analytical sensitivity, and objectivity—with the readout specificity of live cell imaging, the xCELLigence RTCA eSight delivers a wealth of information from a single killing assay without increasing workload. This cutting-edge platform not only boosts confidence in the data obtained, but also provides researchers with comprehensive, in-depth insights into cellular behavior.

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