xCELLigence System Real-Time Cell Analyzer

Focus Application
Compound-Induced Cytotoxicity

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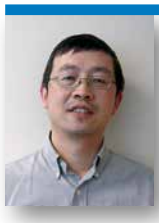
Featured Study:
Using the Time Resolving Function of the xCELLigence System to Optimize Endpoint Viability and Cytotoxicity Assays



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Introduction

Monitoring of cell viability and toxicity is critical to many areas of biological and biomedical research. This is true for understanding the molecular and biochemical pathways regulating cell viability, for developing therapeutic agents which modulate cell viability and for identifying potential cytotoxic side effects of potential therapeutic agents. Viability and cytotoxicity assays are routinely used to determine how various treatments potentially perturb cell homeostasis, leading to decreased viability and increased cytotoxicity. Many agents used for cancer therapeutics modulate the intricate balance between cell proliferation and cell death. The ultimate aim of cancer therapy is to reduce or eliminate cancerous cells in the body, thus shifting the balance by either increasing cell death or decreasing cell proliferation.

Cell death occurs through a spectrum of distinct morphological and biochemical pathways culminating in apoptosis, necrosis, and autophagy (1). It is important to understand the different modes of cell death mediated by cytotoxic agents to decipher the mechanisms of drug action and identify significant side effects.

The direct effect of cancer therapy on cell death is often transient and difficult to capture using standard endpoint assays. For example, apoptosis occurs only during a short window of time, often within just hours, and is frequently followed by secondary necrotic events. It is therefore crucial to conduct relevant assays, measuring either cell viability or cell death, at the optimal experimental time points Considering that the kinetics of cell killing is likely to be different for many compounds, it is important to monitor cell viability and toxicity continuously, to pinpoint the optimal time points for conducting endpoint assays and gain the necessary mechanistic information.

The xCELLigence System of real-time cell analyzers allows for label-free and dynamic monitoring of cellular phenotypic changes continuously by measuring electrical impedance. The system measures impedance using interdigitated microelectrodes integrated into the bottom of each well of the tissue culture E-Plates 96 (see Figure 1A). Impedance measurements are displayed as Cell Index (CI) values, providing quantitative information about the biological status of the cells, including cell number, cell viability, and cell morphology. Impedance-based monitoring of cell viability correlates with cell number and MTT-based readout (2). The kinetic aspect of impedance-based cell viability measurements provides the necessary temporal information when compounds of interest are used to induce cytotoxic effects. In particular, the xCELLigence System is ideal for pinpointing the optimal time points when the cytotoxic compounds achieve their maximal effect, as indicated by the lowest CI values, in cytotoxicity and cell death assays.

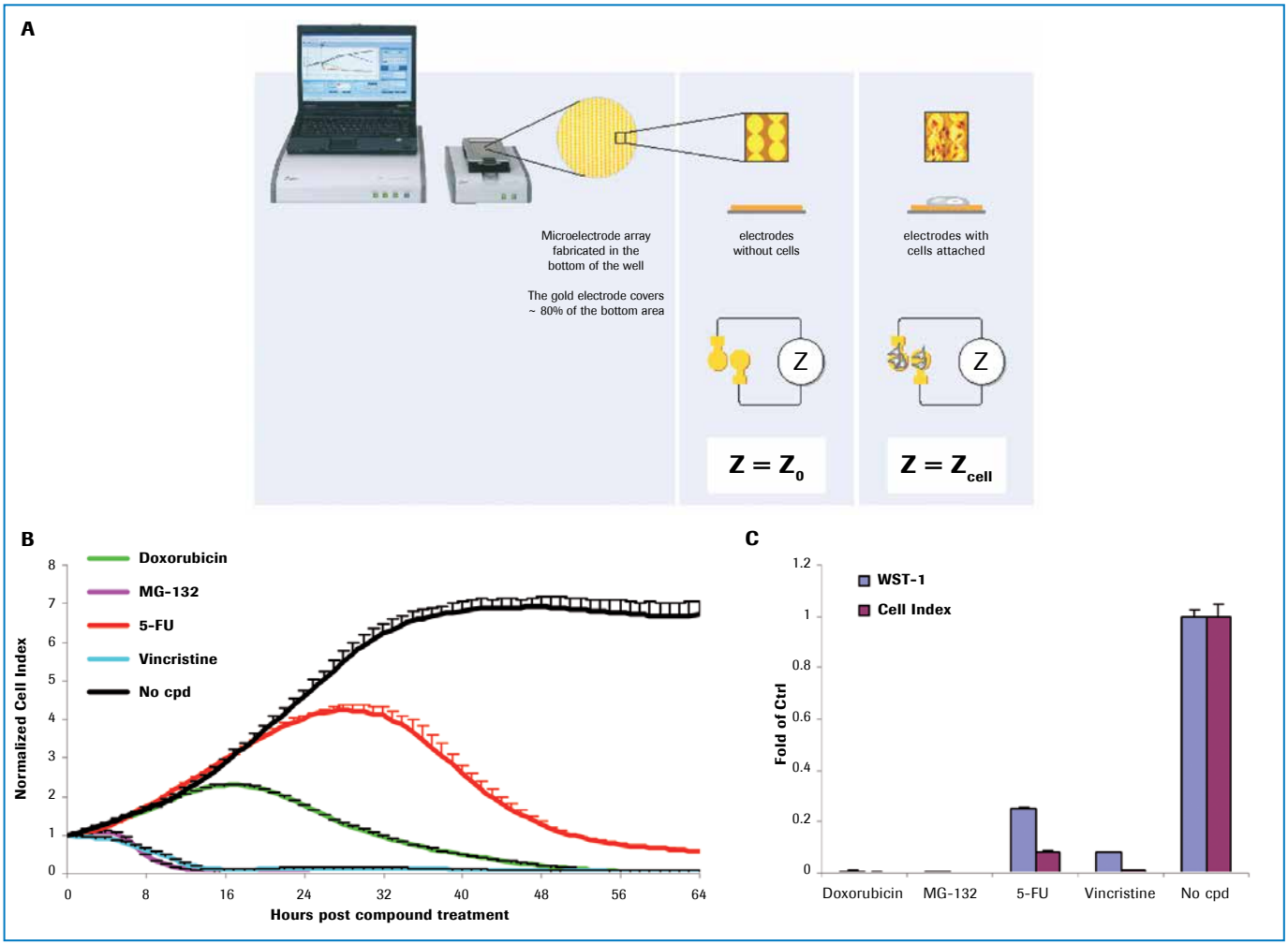

Figure 1: Real-time monitoring of cell viability using the xCELLigence System.
A. The RTCA SP Instrument is shown, consisting of the RTCA Control Unit, RTCA SP Station, E-Plate 96, and RTCA Analyzer. Interdigitated gold microelectrodes at the bottom of each well of the E-Plate 96 are shown, with and without cells. Impedance, expressed as Cell Index (CI) values, is determined for media alone (Z_0), or when cells attach and proliferate in contact with the electrodes (Z_{cell}).
B. HeLa cells seeded in the E-Plate 96 for 24 hours were treated with different compounds: **MG-132** (3.3 µM), **doxorubicin** (0.33 µM), **vincristine** (6.2 nM), and **5-FU** (11.1 µM). Cell cultures not treated with compound were used as control. Cell Index values were monitored every 15 minutes for 64 hours. The average of duplicate samples is shown; error bars represent the standard deviation of the mean.
C. At the end of the experiment (shown in panel B), 64 hours post compound addition, cells were stained with the Cell Proliferation Reagent WST-1. Both CI values and WST-1 findings are shown as fold changes relative to controls. The average of duplicate experiments is shown; error bars represent the standard deviation of the mean.

Materials and Methods

Cell culture and Cell Index calculation

HeLa cells were obtained from ATCC. Cells were maintained in DMEM media with 10% FBS and 1% penicillin and streptomycin. Cells were cultured in a +37°C incubator with 5% CO_2. Cell attachment, spreading, and proliferation were continuously monitored using the xCELLigence System (ACEA). The electronic readout of cell-sensor-detected impedance is displayed as arbitrary units called Cell Index (CI) values. The CI value at each time point is defined as **(Rt-Rb)/15** where **Rt** is defined as the cell-electrode impedance of the well with the cells at different time points, and **Rb** is defined as the background impedance of the well with the media alone. The normalized Cell Index is calculated by dividing the Cell Index value at a particular time point by the Cell Index value at the time of interest.

Compound treatment, cell proliferation, and apoptosis assays

For compound treatment, 2500–5000 cells were seeded in wells of the E-Plate 96. Approximately 24 hours later, compounds were serially diluted and added to the wells containing the cells. Cell-electrode impedance was monitored using the xCELLigence System every 15–30 minutes to produce time-dependent cell response dynamic curves. Doxorubicin, vincristine, MG-132, and 5-FU were obtained from Sigma. For staining with the Cell Proliferation Reagent WST-1 (Roche), the E-Plate 96 was removed at the desired time point and media removed and replenished with 100 µl media containing 10% WST-1. The E-Plate 96 was incubated at +37°C for 1 hour before media containing WST-1 were transferred to a 96-well culture plate and absorbance at 450 nm was measured using a microplate

reader (DTX 880, Beckman Coulter). For the apoptosis assay, the Cell Death Detection ELISA^{PLUS} kit was obtained from Roche and used following the manufacturer's instructions.

Results and Discussion

The xCELLigence System allows cell viability to be measured continuously and in real-time. The kinetic profiles for several cytotoxic compounds, including the DNA-damaging agents doxorubicin and 5-FU, proteasome inhibitor MG-132, and anti-mitotic agent vincristine, are shown in Figure 1B. Even though all four compounds induce cytotoxicity as shown by the WST-1 assay (see Figure 1C), kinetic profiles provided by the xCELLigence System indicate that the rate and dynamics of cytotoxicity vary significantly between the compounds. MG-132-mediated cytotoxicity starts at 4 hours post compound addition and reaches maximal effect at 10 hours. In contrast, vincristine-induced cytotoxicity starts immediately after compound addition, reaching a maximal effect at 15 hours. Doxorubicin and 5-FU take a longer time before cytotoxic effects are observed at 12 and 24 hours post compound addition respectively, and maximal effects were not found until 48 hours after treatment.

We tested whether Cell Index changes can be used to select optimal time points for performing functional endpoint assays. For this purpose, we carried out apoptosis assays using two apoptosis-inducing agents, MG-132 and 5-FU (3-5), with different cell-killing kinetics (see Figure 1B). HeLa cells were seeded in the wells of the E-Plate 96. Twenty-four hours later, threefold serial dilutions of each of the above compounds were added to the wells. Cell Index values were monitored continuously for 64 hours. As observed previously, MG-132 was found to induce a maximal decrease in Cell Index values during the first 10 hours after compound treatment (see Figure 2A), while 5-FU required at least 48 hours (see Figure 2B). Interestingly, cell-killing kinetics were shown to be concentration-dependent such that higher concentrations induced cytotoxicity much faster compared to lower concentrations, as indicated by the rate of decline in Cell Index values (see Figure 2B).

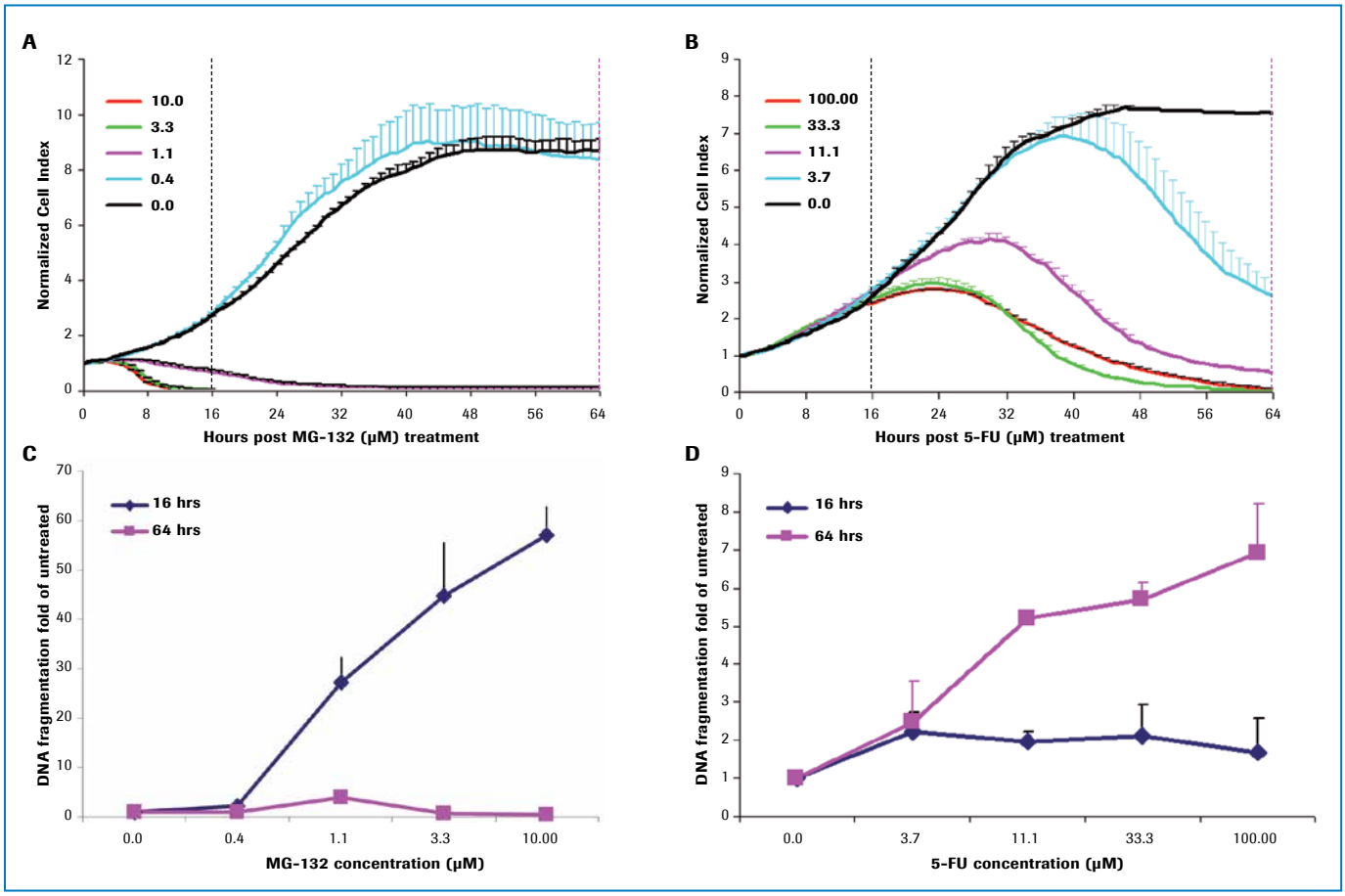

Figure 2: Real-time monitoring of apoptosis using the xCELLigence System. HeLa cells were treated with various concentrations of the proteasome inhibitor **MG-132** (panels A & C) and DNA-damaging agent **5-FU** (panels B & D). Cell Index values were monitored continuously for 64 hours (panels A & B). Parallel experiments were performed in E-Plates 96 and samples harvested at 16 and 64 hours post compound addition for apoptosis assays (panels C & D). Apoptosis was determined using the Cell Death Detection ELISA^{PLUS} kit and expressed in terms of the fold change relative to untreated cell cultures.

Functional apoptosis assays were performed at two different time points, 16 hours post compound addition, when the maximal CI changes were observed for the higher concentrations of MG-132, and 64 hours post compound addition when maximal effects were observed for the higher concentrations of 5-FU. For MG-132, a dose-dependent apoptotic induction was observed at 16 hours, but not at 64 hours. This finding confirms the transient nature of apoptosis, underscoring the importance of performing the assay at an optimal time point to best capture short-lived (transient) cellular responses (see Figure 2C). For 5-FU, a dose-dependent induction of apoptosis was observed at 64 hours when maximal CI changes were observed for the higher concentrations, but not at 16 hours, when the CI values for treated cells just started to diverge from the control samples (see Figure 2D).

The kinetic curves obtained using the xCELLigence System were then used to determine the optimal time point for conducting the apoptosis assays described below. With respect to the xCELLigence data obtained above, it is clear that the apoptosis assays should be carried out as soon as the CI profile reaches its lowest value, indicating the lowest level of cell viability. Assaying for apoptosis too early or too late may not provide the best window for assessing the effect of each of the different compounds tested.

After establishing the correlation between lowest Cell Index values and apoptotic induction, we examined the role of CI changes in predicting apoptosis induction more systematically. For this study, MG-132 was used due to its faster kinetics. Assays were performed within 12 hours post treatment. Cell Index values were monitored continuously for 12 hours post compound addition. Parallel samples were set up and assayed using the Cell Proliferation Reagent WST-1 and the Cell Death Detection ELISA^{PLUS} kit at 1, 4, 8 and 12 hours post treatment (see Figure 3). Highest-level apoptotic induction was observed when CI values reached their near-lowest level (see Figures 3B & 3C).

Importantly, CI changes appeared to be more sensitive in predicting apoptosis induction than the WST-1 assay (see Figures 3B & 3C). For example, at 8 hours post compound addition, while CI values showed >80% change compared to untreated cell cultures, the WST-1 assay showed only a <20% change. Correspondingly, a very robust apoptotic induction was observed at this time point, sixfold higher than untreated samples. The short-term sensitivity observed from the CI changes obtained using the xCELLigence System is best explained by the compound's faster kinetic effect on cell adhesion, morphology, and membrane integrity, compared to measuring the mitochondrial function assayed using the WST-1 assay (probably because the latter events take longer to complete). This high-level sensitivity of CI measurements in detecting transient cellular responses is ideal for pinpointing the best time point(s) for further examining the compound-mediated changes produced by agents leading to apoptosis (such as gene and protein expression analyses focused on identifying underlying molecular mechanisms).

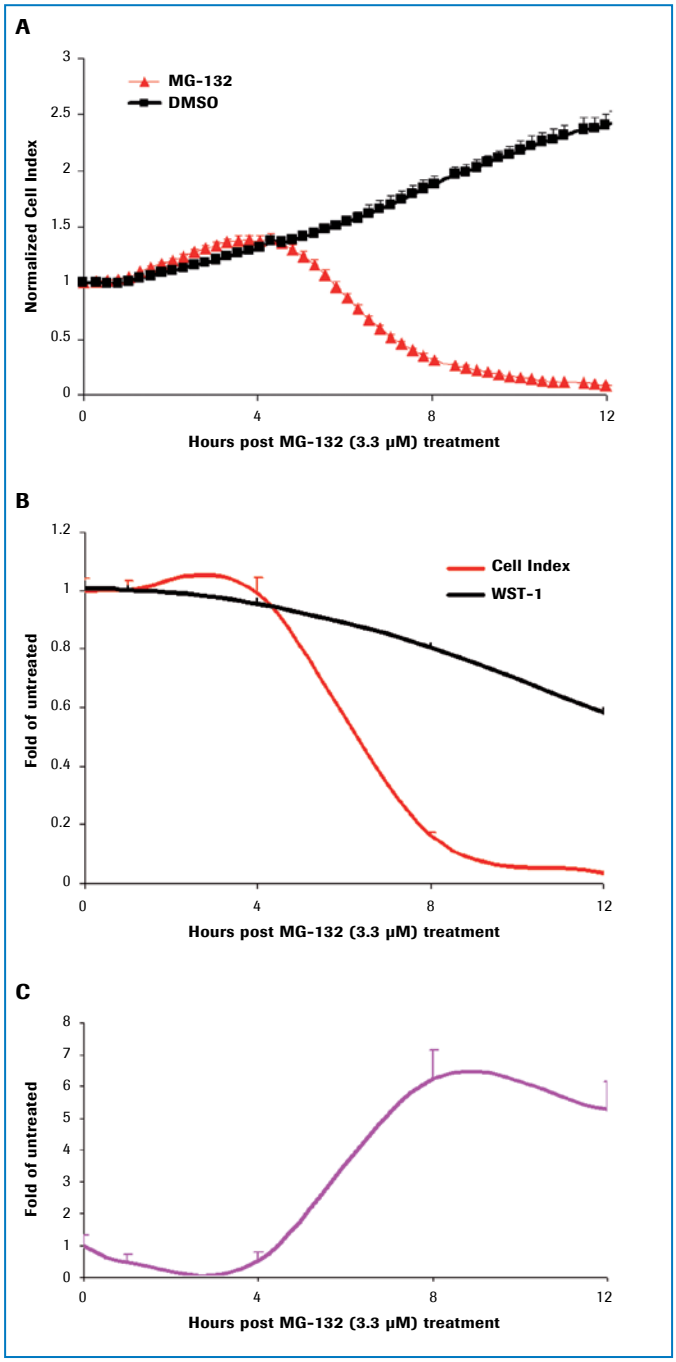

Figure 3: Time resolution of the xCELLigence System makes it possible to optimize the timing of apoptosis assays. HeLa cells were treated with 3.3 µM **MG-132**. CI values were continuously monitored for 12 hours (A). Parallel samples were carried out and tested using the Cell Proliferation Reagent WST-1 (B) and Cell Death Detection ELISA^{PLUS} kit (C) at 1, 4, 8, 12 hours post compound addition. Cell Index values were normalized with respect to untreated cell cultures. The average of triplicate experiments is shown; error bars indicate the standard deviation of the mean.

Conclusion

The results presented in this Focus Application Note demonstrate that the xCELLigence System is ideal for continuously monitoring cell viability and cytotoxicity in real time without using exogenous labels. Cellular kinetic impedance-based information permits pinpointing the optimal time window for performing downstream endpoint assays, to examine cell death, apoptosis, and other cell responses in more detail. xCELLigence data also provide very sensitive and accurate information about the earliest time points when cells first exhibit compound-mediated effects after treatment.

In summary, the xCELLigence System is ideal for studying cell viability and cell death when testing cytotoxic compounds. Real-time impedance-based cell analyzers, such as the RTCA SP Instrument with the E-Plate 96, can:

1. Identify the onset of cytotoxicity using continuous impedance-based monitoring of cell viability, pinpointing when maximal effects occur.
2. Identify the best time point, when CI values are at their lowest value, for conducting functional endpoint assays that examine cell fate transitions, such as apoptosis and cell cycling, in greater detail.
3. Identify culture wells with inappropriate cell numbers at the beginning of the assay, thus minimizing the role of cell-seeding and culture plate edge artifacts during data analysis.

References

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Ordering Information

Product	Cat. No.	Pack Size
xCELLigence RTCA DP Instrument	**00380601050**	**1 Bundled Package**
RTCA DP Analyzer	05469759001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA SP Instrument	**00380601030**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA SP Station	05229057001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA MP Instrument	**00380601040**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA MP Station	05331625001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
E-Plate 16	05469830001	6 Plates
	05469813001	6 x 6 Plates
E-Plate VIEW 16	06324738001	6 Plates
	06324746001	6 x 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)
CIM-Plate 16	05665817001	6 Plates
	05665825001	6 x 6 Plates
E-Plate 96	05232368001	6 Plates
	05232376001	6 x 6 Plates
E-Plate VIEW 96	06472451001	6 Plates
	06472460001	6 x 6 Plates
E-Plate Insert 96	06465412001	1 x 6 Devices (36 16-Well Inserts)
E-Plate Insert 96 Accessories	06465455001	6 Units (6 Receiver Plates + 6 Lids)

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Key Words:
MG-132, 5-FU, TCRP, xCELLigence System, impedance measurement, cell viability, cell index, apoptosis

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