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screening solutions for life

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Apoptosis is a tightly regulated cell death program that can be triggered as a defense mechanism against toxic events or executed by cells that are no longer necessary. There are numerous methods by which apoptosis can be initiated and established markers that can be assayed to determine if a cell has become apoptotic. Because apoptosis is intimately related to the overall health of a cell and fundamentally involved in all of cell biology, it is important in drug discovery. The need for characterizing apoptosis and apoptotic processes occurs throughout the drug discovery process, from primary screening to toxicity profiling.

In the area of apoptosis testing, there is a wealth of methodologies for both research and screening applications. IntelliCyt has developed the no-wash MultiCyt™ 4-Plex Apoptosis Screening Kit that allows simultaneous detection of Caspase 3/7 activation, Annexin V binding, cell viability, and mitochondrial membrane depolarization from a single sample. Additionally, cell count is an inherent capability of the platform and may be useful for identifying overtly toxic treatments. The MultiCyt 4-Plex Apoptosis Screening Kit is the only kit commercially available that allows simultaneous detection of these 4 endpoints, enabling the ability to construct phenotypic apoptotic profiles for compounds of interest (Luu, *et. al,* 2011).

This proprietary kit uses a streamlined workflow and ultimate ease-of-use with a single-step addition of a cocktail containing all the necessary dyes and reagents to each sample. All the individual assay components have been titrated for maximal signal to noise and they seamlessly multiplex to provide a phenotypic profile. After reagent addition and a 1-hour incubation at room temperature, plates are ready to read (no wash assay).

In this application note, we report on the development of this 4-plex apoptosis screening assay in a no-wash format. Validation of the assay performance using Jurkat as the cell model yields z' values of > 0.8 for all endpoints, demonstrating broad applicability of this assay for phenotypic screening campaigns as well as toxicity profiling.

The MultiCyt 4-Plex Apoptosis Screening Kit uses a unique set of 4 dyes that are matched to the detection capabilities of IntelliCyt's screening platforms:

The activation of Caspase 3/7 is detected by the use of a Caspase substrate that, upon cleavage by activated enzyme, results in a fluorescent signal

Surface expression of phosphatidylserine is detected by the binding of Annexin V

Cell viability is determined by the uptake of dye through compromised (porous) membranes

Mitochondrial membrane potential is determined by a dye that localizes in the mitochondrial lumen when mitochondria are healthy and able to maintain a membrane potential. Upon, mitochondrial depolarization, the dye leaks into the cytoplasm and loses its ability to fluoresce

Figure 1. Apoptotic markers measured by the MultiCyt Apoptosis Screening Kit

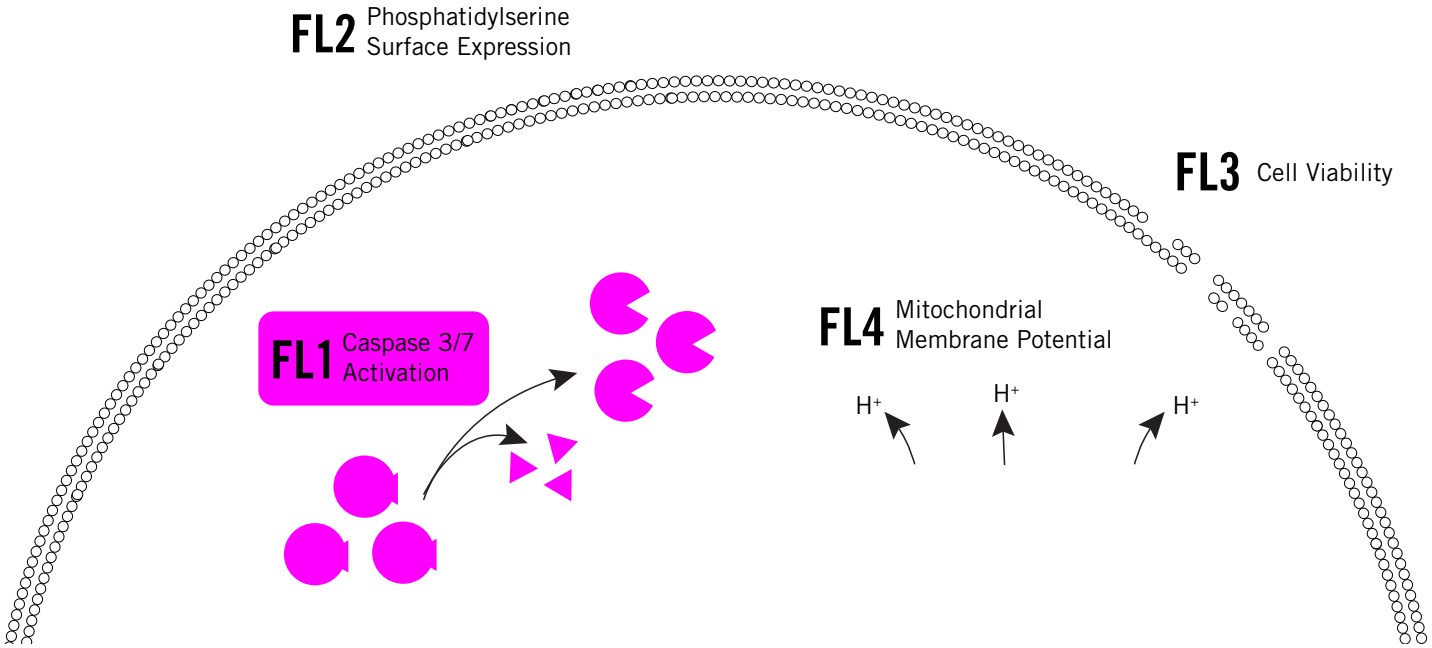

Figure 1. The MultiCyt Apoptosis Screening Kit simultaneously measures 4 apoptosis-specific markers: 1) Caspase 3/7 activation, 2) Phosphatidylserine surface expression, 3) Cell viability, and 4) Mitochondrial membrane potential. Cell numbers are also determined for each sample.

The MultiCyt 4-Plex Apoptosis Screening Kit requires a single 1-hour staining step before plates are ready to be analyzed on IntelliCyt screening systems. All four dyes are added as a cocktail, requiring only a single addition to your assay volume. Stained plates do not require a wash step, allowing a true mix-and-read workflow that is perfect for high throughput screening environments. After staining is complete, data can be acquired from each 384-well plate in about 30 minutes.

We designed a 6-plate (384-well plates) validation strategy to quantify the reproducibility of the Apoptosis Screening Kit. Each plate was seeded with Jurkat E6.1 cells at a final concentration of 1 x 10^6 cells / mL. Duplicate plates of maximum effect, vehicle only, and staurosporine dose response were made. For the maximum effect (Max) plates, cells were treated with 5 µM staurosporine in 0.5% DMSO, which is a concentration known to saturate the response to all 4 measured endpoints of apoptosis. Vehicle only (Min) plates were treated with 0.5% DMSO in media. Finally, two dose response plates were created, starting at a max dose of 5 µM and proceeding in a 1:2 serial titration. Dose response series were laid out in columns, resulting in plates that contained 24 replicates of 16-point titrations.

All plates were prepared by adding equal volumes (10 µL each) of cells and compounds to the specified final concentrations. After 24 hours of incubation at 37°C and 5% CO_2, plates were removed from the incubator and stained by direct addition of 20 µL of the prepared staining cocktail. After a one-hour incubation at room temperature, data was acquired using the iQue™ Screener.

Using staurosporine to induce apoptosis in Jurkat cells, we investigated well-to-well and plate-to-plate reproducibility of the MultiCyt Apoptosis Kit for 5 endpoints – 4 apoptosis-specific endpoints and cell count (Table 1). Two 384-well plates were treated with 5 µM staurosporine. As expected, we observed a nearly complete response (> 99% of cells) for Caspase 3/7 activation, Annexin binding, viability, and mitochondrial membrane depolarization. Importantly, the well-to-well CVs was less than 3% for all endpoints. Cell counts had CVs of 9.6% (Max 1) and 17.2% (Max 2). Comparison of cell counts from treated wells to control wells provides a useful measurement of general cytotoxicity.

Table 1. Well-to-well reproducibility of the MultiCyt Apoptosis Screening Kit

	Mean Number of cells	CV	Mean % of cells	CV	Mean % of cells	CV	Mean % of cells	CV	Mean % of cells	CV
Max 1 (5 µM staurosporine)	597	9.6	99.4	0.6	99.7	0.6	90.0	2.8	99.0	0.8
Max 2 (5 µM staurosporine)	626	17.2	99.3	0.5	99.6	0.4	91.7	2.4	99.3	0.5
Untreated 1	2025	10.8	2.7	19.0	2.6	19.0	3.6	18.8	2.7	19.0
Untreated 2	2125	9.7	2.7	20.0	2.6	19.4	3.4	21.8	2.7	17.9

Using the two max-treated and two vehicle treated plates, we calculated a Z' value to determine the performance of the assay in a high throughput screening environment. We calculated the mean (µ) and standard deviation (σ) across the total of 768 wells in the max treated plates as our positive control. We did the same for untreated plates as our negative control. The final Z'-factor calculation was made using the following formula:

$$Z' = 1 - \frac{(3(\sigma_p + \sigma_n)}{| \mu_p - \mu_n |}$$

Across all 4 apoptosis-specific endpoints, the MultiCyt 4-Plex Apoptosis Screening Kit exhibited excellent Z'-factors (Table 1, range 0.89 – 0.97).

The speed with which plates can be stained and read using the 4-Plex Apoptosis Screening Kit makes it practical to screen compounds in dose response series (Figure 2).

Table 2. Z'-values for 4 apoptosis-specfic endpoints, measured by the MultiCyt 4-Plex Apoptosis Screening Kit Screening Kit

Caspase+	0.97
Annexin	0.97
Non-Viable	0.89
Depolarized Mitochondria	0.96

We tested the reproducibility of dose-response titrations by creating two dose response plates, each containing 24 replicates of 16-point staurosporine titration series (top dose, 5 µM). We found generally excellent agreement between EC_{50} values within and between plates (Table 3).

Table 3. Summary of dose response curves

	EC_{50} (µM)	R^2	EC_{50} (µM)	R^2	EC_{50} (µM)	R^2	EC_{50} (µM)	R^2
Dose Response Plate 1	0.084	0.995	0.087	0.995	0.088	0.997	0.091	0.995
Dose Response Plate 1	0.082	0.996	0.086	0.996	0.102	0.994	0.091	0.997

Figure 2. Dose response curves for 4 apoptotic responses

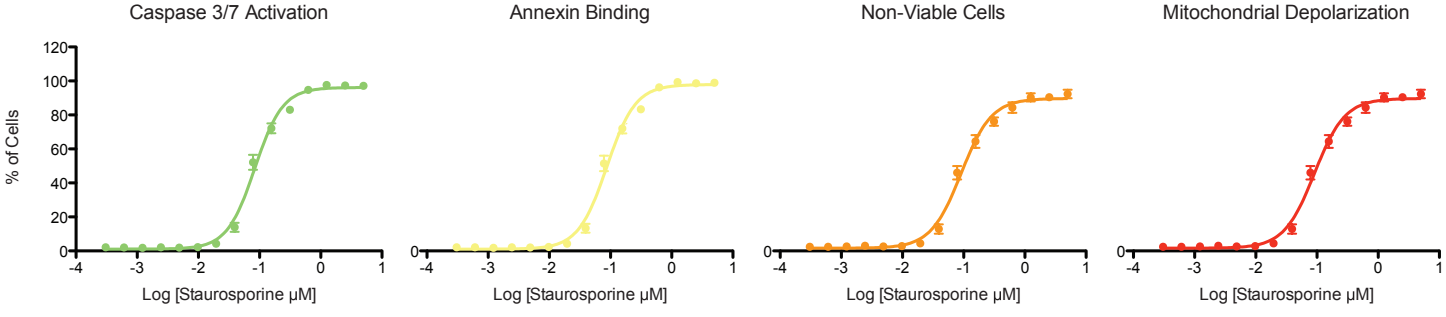

Figure 2. Jurkat cells were treated for 24 hours with a titration series of staurosporine, then stained with the MultiCyt 4-Plex Apoptosis Screening Kit. Two 384-well plates each contained 24 replicates of a 16-point 1:2 dose response series. Shown are the mean and standard deviation of 24 replicates for each dose. The data are fit with a four parameter logistic function.

The MultiCyt 4-Plex Apoptosis Screening Kit combines a high throughput screening-friendly protocol with the multiplexing capabilities of IntelliCyt's Screening Systems to create robust and high content data sets. Here we demonstrated the performance of our second generation apoptosis kit, using a unique combination of 4 apoptosis markers in a completely no wash protocol. The simultaneous detection of multiple markers in different apoptotic pathways enables the ability to create apoptotic "profiles" for the compounds in a screening campaign, enabling researchers to make more informed decisions.

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