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iQue® Human 4-Plex Apoptosis Kit Using Advanced High Throughput Flow Cytometry

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Introduction

Apoptosis or programmed cell death is an essential process with roles in maintenance of cell populations, the immune system and development[1]. Deviations from homeostatic apoptosis can cause a number of diseases. Genetic mutations that increase apoptosis are implicated in neurodegeneration, whilst decreased apoptosis is linked to cancer[2]. Therefore, drugs that modulate levels of apoptosis have long been a target for drug discovery in a multitude of therapeutic areas. For example, many chemotherapy drugs kill cancer cells by upregulating apoptosis.

Numerous factors can induce apoptosis, such as: infection; severe DNA damage; hypoxia and disrupted cell cycle signalling[2,3]. These cellular stresses begin a molecular cascade through one of two main apoptotic pathways: extrinsic or intrinsic. The early phases of the intrinsic pathway involve depolarization across the mitochondrial membrane whilst the extrinsic pathway relies on signaling by 'death ligands'[1]. Both pathways join to follow a final execution pathway, beginning with activation of Caspase 3: an enzyme key for molecular cleavage during downstream events. The execution pathway involves mass biochemical and morphological changes, including: protein degradation, DNA fragmentation, membrane blebbing and extrinsic phosphatidylserine expression to promote phagocytosis[1].

Flow cytometry assays offer significant benefits for studying the hallmarks of apoptosis—with the ability to quantify multiple cellular parameters at once. These assays also interrogate apoptosis at the single cell level, rather than as an estimate from the bulk population, unlike some of the more traditional colorimetric assays.

Here we illustrate how the iQue® and iQue® Human 4-Plex Apoptosis Kit can be used to measure apoptosis in a simple, mix and read assay format. The kit provides an in-depth analysis of cell health by simultaneous measurement of up to 4 markers of apoptosis:

1. Mitochondrial depolarization determined by leakage from the mitochondrial lumen to the cytoplasm reduces signal from a fluorescent dye.
2. Caspase 3/7 activity on a substrate that releases a fluorescent dye upon cleavage.
3. Annexin V binding to phosphatidylserine on the cell surface.
4. Cell viability via a dye that binds specifically to dead cells.

Jurkat cells were treated with compounds in 96 or 384-well plate formats for 24 hours then labeled with the apoptosis reagents for 1 hour. The no-wash protocol combined with the high throughput capability of the iQue® meant that, once cells were labeled, a library of 8 cytotoxic compounds could be profiled in only 20 minutes. The gating strategy and data analysis parameters included with the kit provide instantaneous pharmacological readouts, including EC_{50} values. The low volume sample requirements (20 µL) meant multiple samples could be taken from a single assay plate to obtain temporal information.

Methods

Cell Culture and Assay Protocol

Non-adherent Jurkat cells (immortalized Human T-lymphocyte cell Line) were grown in RPMI 1640 (Gibco) supplemented with 10% FBS and 1% Pen-Strep. Compounds (Camptothecin, Dexamethasone and those listed in Table 1) were serially diluted 2-fold in RPMI and added to 96 or 384-well plates (3-6 replicates per series). Cell culture media (containing no compound) was used as a negative control and Camptothecin (10 µM) or Staurosporine (10 or 20 µM) were included as positive controls. Jurkats were added at $1e^5$-$1e^6$ cells/mL. Total assay volumes ranged from 20–100 µL. Plates were incubated at 37° C, 5% CO_2. For temporal assays, 20 µL samples were taken and analyzed at 2 and 6 hours.

Data Acquisition and Analysis

Endpoint analysis was performed at 24 hours using the iQue® Human 4-Plex Apoptosis Kit (Cat#90053) and the iQue® 3 (Figure 1). The 4 kit components measure cell viability, Caspase 3/7 activity, Annexin V binding, and mitochondrial depolarization. All four reagents can be run simultaneously, or individual reagents are available separately to "mix and match" according to experimental objectives. The kit provides an acquisition protocol and data analysis, including event gates and gating strategy (Figure 2), percentage expression metrics, heat maps, and concentration-response curves, which are imported into the iQue Forecyt® software.

1. Combine treatment and cells of choice in 96-well or 384-well plate.
2. Transfer 20 mL of cells to new plate.
3. Add 4-plex apoptosis or individual reagents, incubate for 1 hour and run.

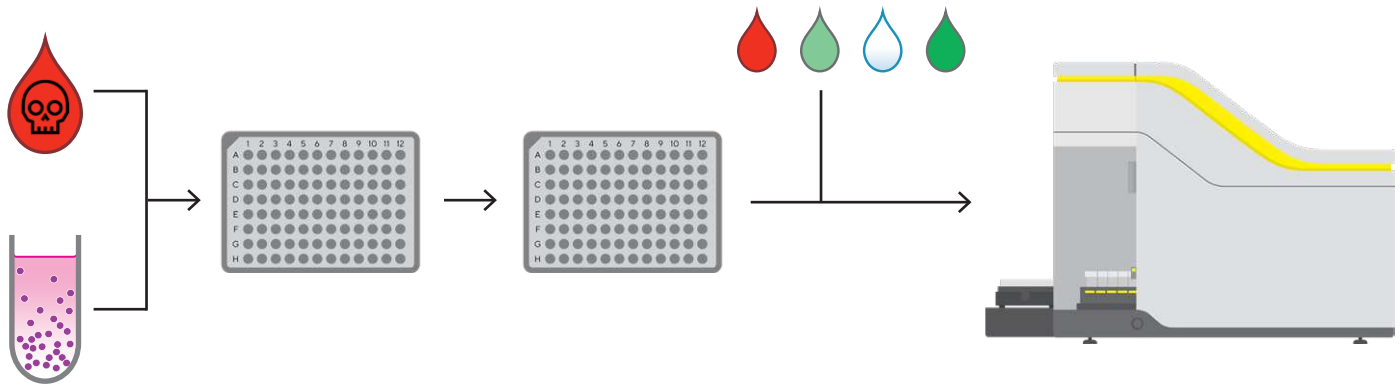


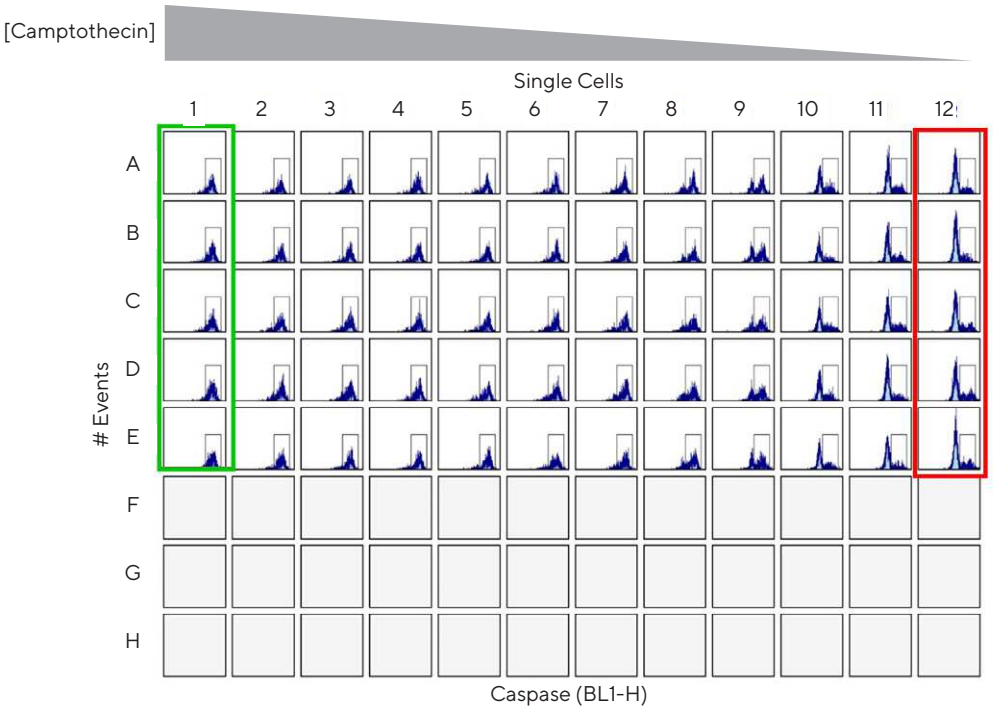
Figure 1: Schematic of the simple, mix and read iQue® Human 4-Plex Apoptosis Kit workflow

Results and Discussion

Drug-Induced Apoptosis can be Measured with the IQue® and Apoptosis Reagents

Many chemotherapy drugs work by causing DNA damage which, through the action of p53, activates apoptosis via the intrinsic pathway[1]. Initial experiments measured changes in the proportion of apoptotic cells in response to various concentrations of Camptothecin—a compound that induces DNA damage. Jurkats (1e^5 cells/mL) were added to a 96-well plate containing Camptothecin for 24 hours then labeled with the iQue® Human 4-Plex Apoptosis Kit and analyzed on the iQue® advanced flow cytometry platform. Figure 2A shows a plate view of the distribution of Caspase 3/7 activity in each individual well. There is a clear shift towards a greater proportion of cells in the Caspase positive gate as drug concentration increases. The gates for the positive (10 µM Camptothecin) and negative (media only) controls are highlighted with green and red boxes, respectively. Caspase activity in these control wells was used to precisely adjust the positioning of the Caspase positive gate (Figure 2B).

A.



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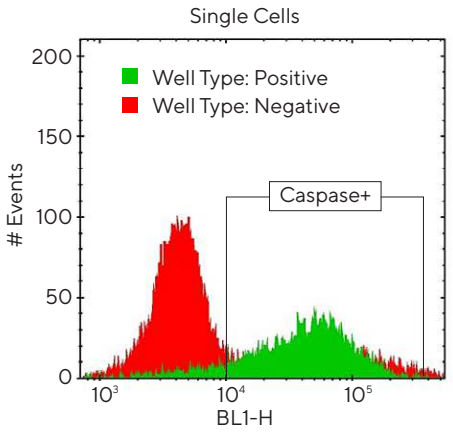

Figure 2: Histograms are gated to distinguish positive and negative apoptotic responses. (A) 96-well plate view shows Jurkat cell response to Camptothecin (concentration decrease left to right; Row 2 to Row 12 = 1 in 2 dilution from 1 µM). Green and red boxes show positive (10 µM) and negative (0 µM) controls. (B) Histogram comparing the proportion of Caspase positive cells in the positive and negative control wells.

Apoptotic Markers Can Be Quantified in High Throughput Using a 384-Well Plate Format

Drug discovery is reliant on high throughput methods to provide fast profiling of large compound libraries. To illustrate the use of the iQue®advanced flow cytometry platform as a high throughput tool for quantification of apoptosis, we screened the response of Jurkat cells to 8 cytotoxic compounds. These compounds are common in scientific literature and induce apoptosis through well-defined mechanisms of action (Table 1). Jurkats were added to serial dilutions of each compound in a 384-well plate. Concentrations of each compound required to produce curves with defined maximal and minimal levels of apoptosis (over 24 hours) had been determined by previous experiments. Results in Figure 3 show the damaging effect (%) each compound had on cells' mitochondria. Each compound displayed a unique pharmacological profile for induction of apoptosis. The iQue Forecyt® panorama feature was used to rank the compounds from most to least potent based on depolarization of mitochondria. The ordered list of compounds and their corresponding EC_{50} values were exported directly from iQue Forecyt® and inserted into Table 1.

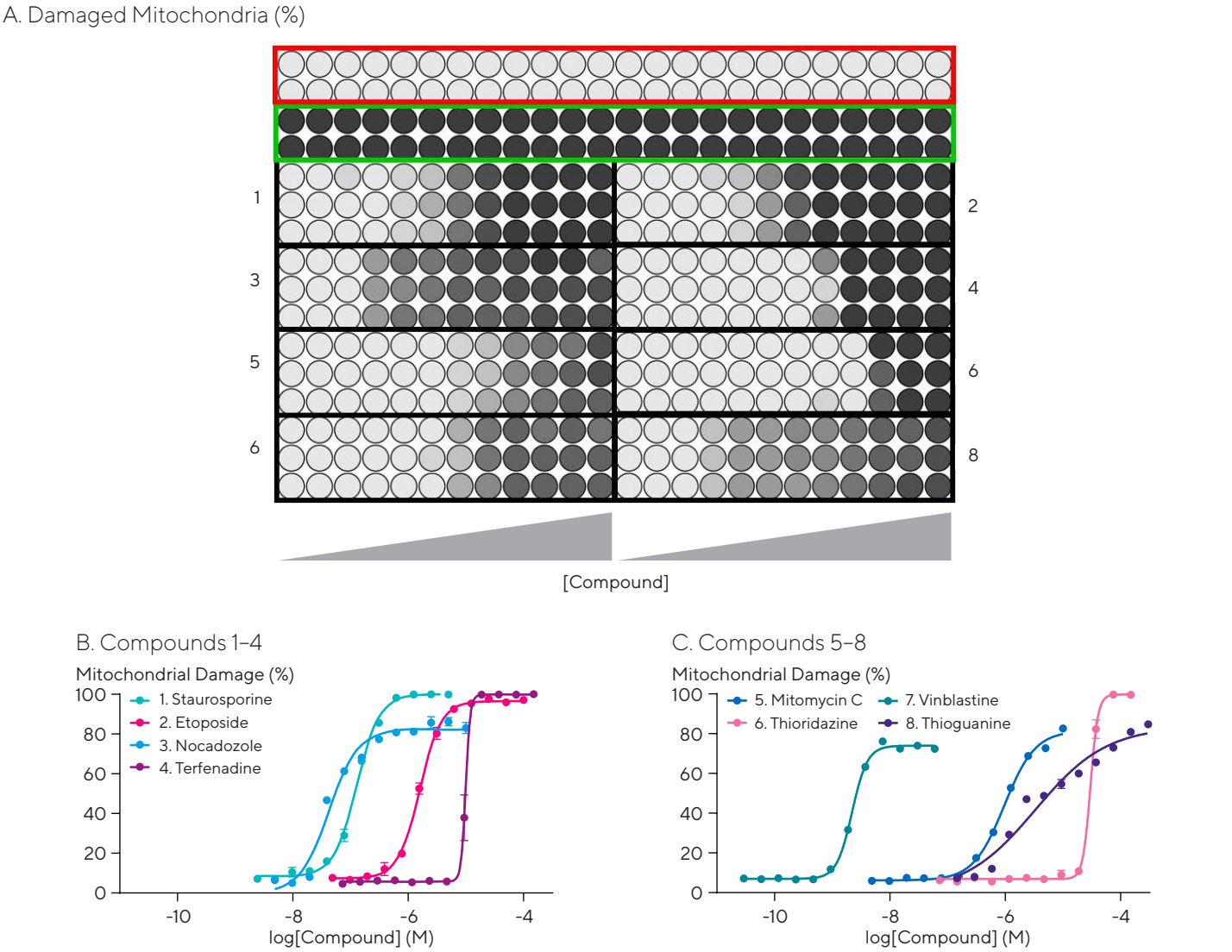

Figure 3: Screening cytotoxic compounds reveals varied pharmacological profiles. Jurkat cells (1e^6/mL) were incubated in a 384-well plate for 24 hours with a range of concentrations of 8 cytotoxic compounds (n=3). (A) Heat map from iQue Forecyt® compares the proportion of cells with mitochondrial damage in each well. Red and green boxes show the negative and positive controls, respectively. Black boxes separate wells containing each compound ((1=Staurosporine, 2=Etoposide, 3=Nocodazole, 4=Terfenadine, 5=Mitomycin C, 6=Thioridazine, 7=Vinblastine and 8=Thioguanine). (B and C) Concentration-response curves for mitochondrial damage response (%). These were used to calculate EC_{50} values for each compound.

Drug	EC_{50} (nM)*	Mechanism of Action from Literature
Vinblastine	2.2	Chemotherapeutic; induces cell cycle arrest through disruption of microtubules[4]
Nocodazole	46.6	Induces microtubule depolymerization[5]
Staurosporine	126.7	Pan-inhibitor of protein kinase activity[6]
Mitomycin C	971.0	Chemotherapeutic; alkylating agent that cross-links DNA[7]
Etoposide	1582.1	Anti-cancer drug that induces DNA damage by binding to topoisomerase II[8]
Thioguanine	4114.9	Chemotherapeutic; incorporates into DNA as a nucleotide to exert toxicity[9]
Terfenadine	9533.9	H1 histamine receptor agonist; can induce DNA damage and activate caspases[10]
Thioridazine	30093.1	Inhibits the PI3K/Akt/mTOR/p70S6K signalling pathways[11]

* EC_{50} values exported from iQue Forecyt®

Table 1: Cytotoxic Drugs Ranked from Most to Least Potent Based on Mitochondrial Depolarization Effect

Cytotoxic and Cytostatic Compounds Induce Different Cell Health Effects

Both cytotoxic (death-inducing) and cytostatic (growth inhibiting) drugs play important roles in the fight against tumor progression[12]. We examined the ability of the iQue® Human 4-Plex Apoptosis Kit to reveal the different cell health profiles induced by these drug types. Jurkat cells were treated with a range of concentrations of cytotoxic compound—Mitomycin C (Table 1)—or Dexamethasone: a glucocorticoid with known cytostatic activity[13]. Results after 24 hours are shown in Figure 4. As expected, there was a concentration dependent increase in all 4 markers of apoptosis in response to Mitomycin C. Contrastingly, no such effect was observed with Dexamethasone; expression of each marker remained constant across the concentration range. This illustrates how compounds affecting cell health could be characterized based on their mechanism of action using this technique.

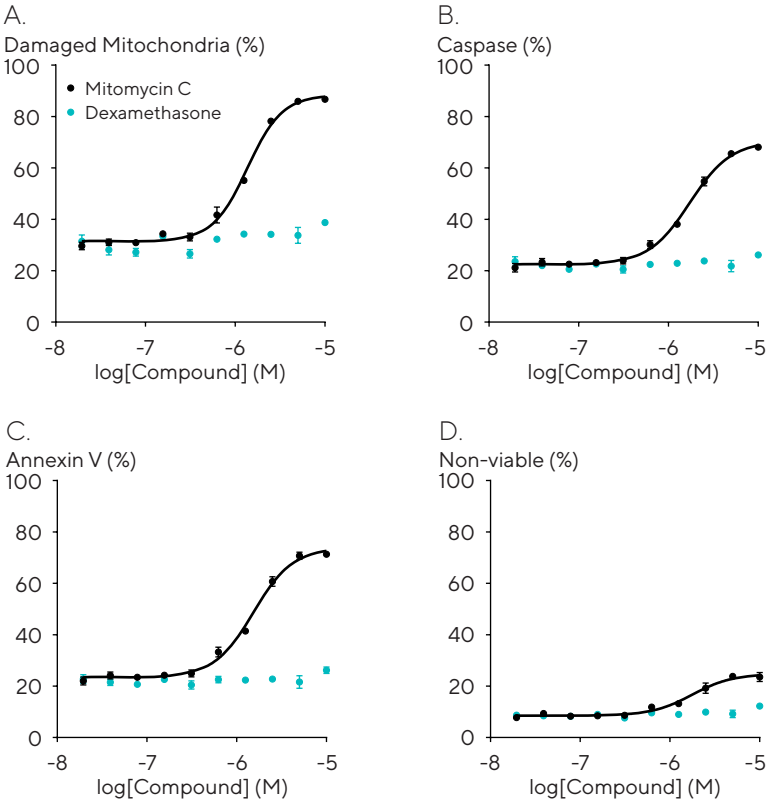

Figure 4: Cytotoxic and cytostatic compounds can be characterized based on difference in effect on cell health. Jurkats (1e6/mL) were treated with various concentrations of cytotoxic compound (Mitomycin C) or cytostatic compound (Dexamethasone). After 24 hours cells were analyzed using all 4 components of the iQue® Human 4-Plex Apoptosis Kit. Graphs show the percentage of cells with (A) Damaged mitochondria (B) Caspase 3/7 activity (C) Annexin V binding and (D) Loss of viability.

These data also show how expression of each marker of apoptosis differs at a given concentration. For example, at the highest concentration of Mitomycin C, 87 ± 0.6% of cells have damaged mitochondria, while only 24 ± 1.8% are labeled with the dead cell dye. This indicates a temporal difference in induction of each marker once a cell has been committed to apoptosis. These data fit with the cascade of events in the intrinsic apoptosis pathway as described in the literature, with early changes in mitochondrial polarization leading to downstream Caspase activation and phosphatidylserine externalization (facilitating Annexin V binding)[1]. Integrity of the plasma membrane (which prevents dead cell dye staining) is maintained until the later stages of apoptosis[2].

Apoptosis is Dependent on Both Time and Concentration of Drug Treatment

To verify that this technique can reveal temporal and concentration dependent changes in apoptosis, we treated Jurkats with 3 concentrations of Staurosporine and analyzed samples at 2, 6 and 24 hours. There was a clear increase in apoptosis over time, as displayed in Figure 5A, with histograms in iQue Forecyt® showing a gradual increase in Caspase 3/7 activity across the time course: from 29.3% at 2 hours to 38.3% at 6 hours, almost doubling to 73.9% by 24 hours. This progressive increase in apoptosis was observed across multiple different markers and across the range of Staurosporine concentrations (Figures 5B and 5C). All wells containing Staurosporine saw greater levels of apoptosis compared to control wells (containing media only).

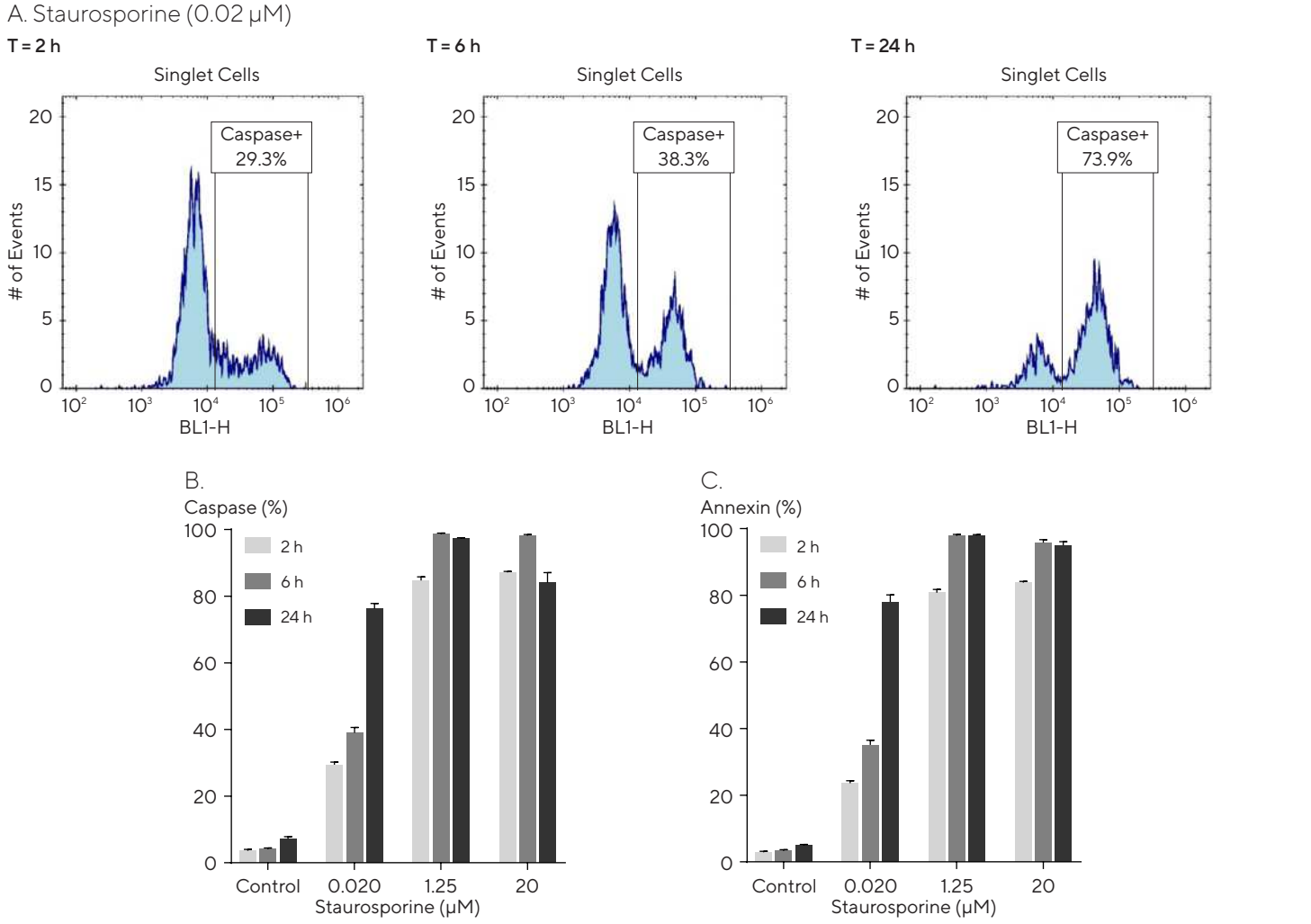

Figure 5: Staurosporine induces a time and concentration dependent increase in apoptosis. Jurkat cells (1e6/mL) were treated with Staurosporine (0.02, 1.25 or 20 µM). Control wells contained media only (no Staurosporine). Samples taken at 2, 6 and 24 hours were analyzed using iQue® Human 4-Plex Apoptosis Kit. (A) Histograms show the proportion of Caspase positive cells at each time point in wells treated with 0.02 µM Staurosporine. (B and C) Percentage of Caspase and Annexin V expressing cells over time at each concentration of compound.

To increase efficiency, many high-throughput screening methods test compounds in singlet[14]. This means that the assays used must be highly sensitive and reproducible. We used statistical analysis of Jurkat response to Staurosporine in order to evaluate the suitability of this assay for high-throughput screening. Z' values for all 4 apoptosis parameters were calculated using the positive (10 µM Staurosporine) and negative (growth media only) controls. The calculated Z' values (Table 2) were all excellent— ranging from 0.74 to 0.95—indicating a high quality screening assay.

Apoptosis Marker	Control	Mean (%)	SD	Z'
Mitochondrial damage	-	5.61	1.55	0.92
	+	98.78	0.94	
Caspase	-	5.40	2.00	0.92
	+	99.40	0.63	
Annexin V	-	3.25	0.87	0.95
	+	99.18	0.58	
Non-viable	-	1.91	0.43	0.74
	+	89.62	7.04	

Table 2. Statistical analysis of Z' values to evaluate assay quality as screening tool

Conclusions

The iQue® Human 4-Plex Apoptosis Kit used in conjunction with the iQue® advanced flow cytometry platform provides a robust assay for measuring cellular progression through apoptosis via multiple pathways. This delivers valuable insight into the mechanism of action and pharmacological profile of cytotoxic drugs. Utilizing the speed of the iQue® 3 and the pre-defined analysis parameters defined in the inbuilt iQue Forecyt® software enables rapid profiling of compound libraries.

The experiments described in this note have highlighted:

1. The iQue Forecyt® software and iQue® Human 4-Plex Apoptosis Kit template auto-generate gating strategy and analysis metrics to provide immediate data outputs. This can be used to study the effects of cytotoxic compound concentration on the apoptotic response.
2. Assays can be run in 96 or 384-well plate formats. The high throughput of the iQue® advanced flow cytometry platform means a full 384-well plate can be run in only 20 minutes. This facilitates gathering of pharmacological information, such as EC_{50} values, about multiple drugs in a short space of time.
3. Readouts can reveal mechanistic differences between drugs. We exemplified this by comparing the apoptotic response between a cytotoxic and cytostatic compound.
4. Small sample requirements (20 µL) to feed into the kit enables temporal data on apoptosis to be collected from a single assay plate.

Combined, these benefits make this technique a powerful tool with potential applications in drug discovery and cell biology.

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