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Automating Cell-based Apoptosis Assays with the Eppendorf ep*Motion*® 5075t increases the reproducibility

Eric Gancarek, Sandrine Hamels, Muriel Art
Eppendorf Application Technologies S.A., Namur, Belgium

Abstract

Cell-based assays have become an established and widely used tool in modern life sciences, whether basic research or routine drug screening. Performing cell-based assays can be laborious and time consuming when multiple parameters such as different cell types, compounds, concentrations etc. have to be assessed at the same time. Medium scale benchtop automation can significantly facilitate this work, especially when multiple 96 or 384 well plates have to be processed, while simultaneously increasing quality and results consistency, making different sets of experiments more comparable.

In this study HeLa and Jurkat cell lines were cultivated in Eppendorf Cell Imaging plates and treated with different concentrations of staurosporine, a compound well characterized to induce apoptosis. The level of apoptosis was determined via the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega®) that generates a fluorescent signal measured by the Eppendorf PlateReader AF2200. A comparison of a manual and automated approach shows that automating the entire workflow of an apoptosis assay including cell seeding, compound dilution and addition, and assay setup with the Eppendorf ep*Motion* 5075t automated liquid handling system generates reliable and consistent results without the risk of contaminating the cultured cells and also increases the reproducibility.

Introduction

Originally, drug discovery researches were conducted on animal models. Over the past few years, the emergence of cell-based assays specifically developed for high or medium-throughput screening allowed to limit this practice to an absolute minimum, especially for ethical and economic reasons. Compared to biochemical assays, also commonly used for therapeutic agent screening, *in vitro* information provided by cell-based assays is considered as more pertinent as the drug response is evaluated in a cellular context which is more representative of the real-life [1]. Among all cell responses which can be evaluated, apoptosis is a subject being extensively studied since the early 1990s. This natural physiological process, also called programmed cell death, contributes to regulation of cell populations in the human body by balancing cell division. Apoptosis plays an essential role during nervous and immune system development, takes part in wound healing and is also needed to destroy cells representing a threat such as auto-aggressive immune cells or cells infected with virus. When this normal process is halted or impaired, it can also be involved in disease formation. Some cancers can be induced by cells not correctly removed by apoptosis, which are subsequently maintained and finally becoming immortal. Excessive apoptosis can also inflict tissue damages as observed in Alzheimer's, Huntington's and Parkinson's diseases [2]. Mechanisms of apoptosis are complex and characterized by specific morphological and biochemical features, including activation of caspases. Just like a large variety of assays developed for detecting apoptosis, the Apo-ONE Homogeneous Caspase-3/7 Assay provided by Promega and used in this study is based on caspase activity detection via cleaved fluorescent substrates [3]. If cell-based assays development is crucial for the drug screening process, automation has also to be considered. Compatibility between liquid handling workstation and cell-based assay has to be demonstrated. All instrument settings have to be defined to ensure correct solution dispensing without damaging mono-layers of adherent cells by mechanical forces. Finally, assay reproducibility is an essential factor which has to be evaluated [4]. This study shows the possibility to implement a widely used cell-based apoptosis assay on the Eppendorf ep*Motion* 5075t.

Material and Methods

Automation and Detection Instruments

- ep*Motion* 5075t with CleanCap configuration option including UV lights and HEPA filters (Eppendorf, order no. 5076 000.302 and 5075 751.607)
- Gripper (Eppendorf, order no. 5282 000.018)
- TS50 pipetting tool (Eppendorf, order no. 5280 000.010)
- TM50-8 pipetting tool (Eppendorf, order no. 5280 000.215)
- TM1000-8 pipetting tool (Eppendorf, order no. 5280 000.258)
- ReservoirRack for ep*Motion* (Eppendorf, order no. 5075 754.002)
- PlateReader AF2200 (Eppendorf, order no. 6141 000.002)

Consumables

- epT.I.P.S.® *Motion* 50 µL Filter (Eppendorf, order no. 0030 015.215)
- epT.I.P.S. *Motion* 1000 µL Filter (Eppendorf, order no.0030 015.258)
- ep*Motion* Reservoir 30 mL and 100 mL (Eppendorf, order no. 0030 126.505 – no. 0030 126.513)
- Reservoir Rack Module TC, for use in ep*Motion* Reservoir Racks, temperable, 4 x Safe-Lock tubes 0.5/1.5/2.0 mL (Eppendorf, order no. 5075 799.081)
- Eppendorf Safe-Lock Tubes, 1.5 mL (Eppendorf, order no. 0030 120.086)
- Eppendorf Deepwell Plate 96/1000 µL (Eppendorf, order no. 0030 502.205)

Cell Assay

- Human cervical carcinoma HeLa cell line (DSMZ, order no. ACC 57), cultivated in RPMI 1640 medium supplemented with 10 % FBS (Fetal Bovine Serum) and 1 % Penicillin-Streptomycin
- Human T Cell leukemia Jurkat cell line (DSMZ, order no. ACC 282), cultivated in RPMI 1640 medium supplemented with 10 % FBS, 1 % Penicillin-Streptomycin and 2 mM of L-glutamine
- Cell Imaging Plate (Eppendorf, cat # 0030 741.013)
- Staurosporine from *Streptomyces staurospores* (Sigma-Aldrich®, order no. S4400) dissolved in DMSO
- Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, order no. G7791)

Methods for automated Cell-based assay

The complete workflow is programmed to process three 96-well plates in parallel and is divided into three ep*Motion* methods. For the first two methods, the ep*Motion* 5075t surfaces and tools are cleaned using a disinfection solution (Umonium^{38}®; Laboratoire Huckert's International). UV-lights and HEPA filters are started one hour before using the system. The UV-lights stop automatically after 15 minutes. At the end of each method, a user intervention is requested to handle the plates to downstream steps (incubation, reading).

Method 1:

Cell seeding is performed during this first protocol step. Adherent HeLa cells are seeded into 96-well Cell Imaging plates at 30,000 cells per well. With suspension Jurkat cells, a density of 10,000 cells per well is dispensed. Before starting the ep*Motion* method 1, a cell solution at the appropriate concentration (10,000 cells per 90 µL for Jurkat cells and 30,000 cells per 90 µl for HeLa cells) is prepared in a sterile tube and transferred to a sterile autoclaved ep*Motion* reservoir of 100 mL on the ep*Motion* 5075t. Cell culture media, used as blank, is transferred to a sterile ep*Motion* reservoir of 30 mL size. These steps need to be performed under sterile cell culture conditions. The method 1 generates the dispensing of 90 µL of cells per well in column 1 to 11 in three 96-well microplates and 90 µL of culture medium without cells in column 12 in these three plates. At the end of the program, the lid is manually replaced on the plate and the plates are placed into the CO_2 incubator at 37 °C for 24 hours.

Worktable Layout Method 1

Position	Item
A2	epT.I.P.S. *Motion* 1000 µL Filter
B2	Reservoir Rack
B3	Cell Imaging Plate (96-well plate)
B4	Cell Imaging Plate (96-well plate)
B5	Cell Imaging Plate (96-well plate)

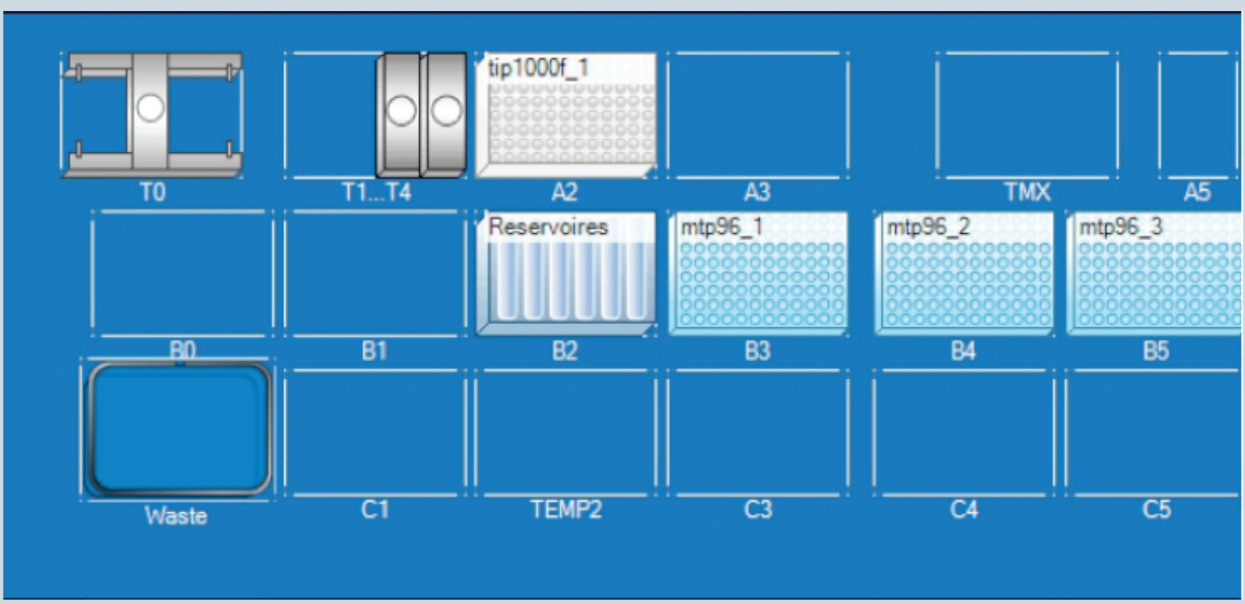

Figure 1: ep*Motion* worktable layout for method 1

Method 2:

The goal of the second method is to generate the concentration curve of the apoptosis inducer. Compound concentration range is between 1 nM and 20 µM for HeLa cells and between 3 nM and 100 µM for Jurkat cells.
16 increasing staurosporine (STS) concentrations are used for inducing apoptosis. In order to generate a concentration curve with the same amount of vehicle solution in each well, the dilution curve is produced by using two different dilution steps. The first dilution is performed into DMSO 99.9 % vehicle solution in a Deepwell plate for generating 16 STS concentrations 10 times more concentrated than desired. Culture medium without STS and DMSO being used as negative control is also added into the Deepwell Plate. At the end of this first part, the 96-well plates seeded with the cells are placed on the worktable. The second dilution step is performed into the 96-well plates and allows to obtain the 16 final STS concentrations. 10 µL of each STS concentration is dispensed from the Deepwell plate to the 96-well plates. 10 µL of culture medium without STS and DMSO is also added as control. The final vehicle (DMSO) concentration did not exceed 1 % and was equivalent for all staurosporine concentrations tested. The plates are transferred on the TMX of the ep*Motion* 5075t and mixed at 500 rpm for 30 sec. At the end of the method, the lid is replaced on the plates manually. The optimal apoptosis induction time is cell-type dependent, plates seeded with HeLa cells were incubated 24 hours at 37 °C (5 % CO_2) while 5 hours were optimal for stimulating Jurkat cells.

Worktable Layout Method 2

Position	Item
A2	epT.I.P.S. *Motion* 1000 µL Filter
A3	epT.I.P.S. *Motion* 50 µL Filter
B1	Reservoir Rack
B2	Deepwell Plate 96/1000 µL
B3	Cell Imaging Plate (96-well plate)
B4	Cell Imaging Plate (96-well plate)
B5	Cell Imaging Plate (96-well plate)

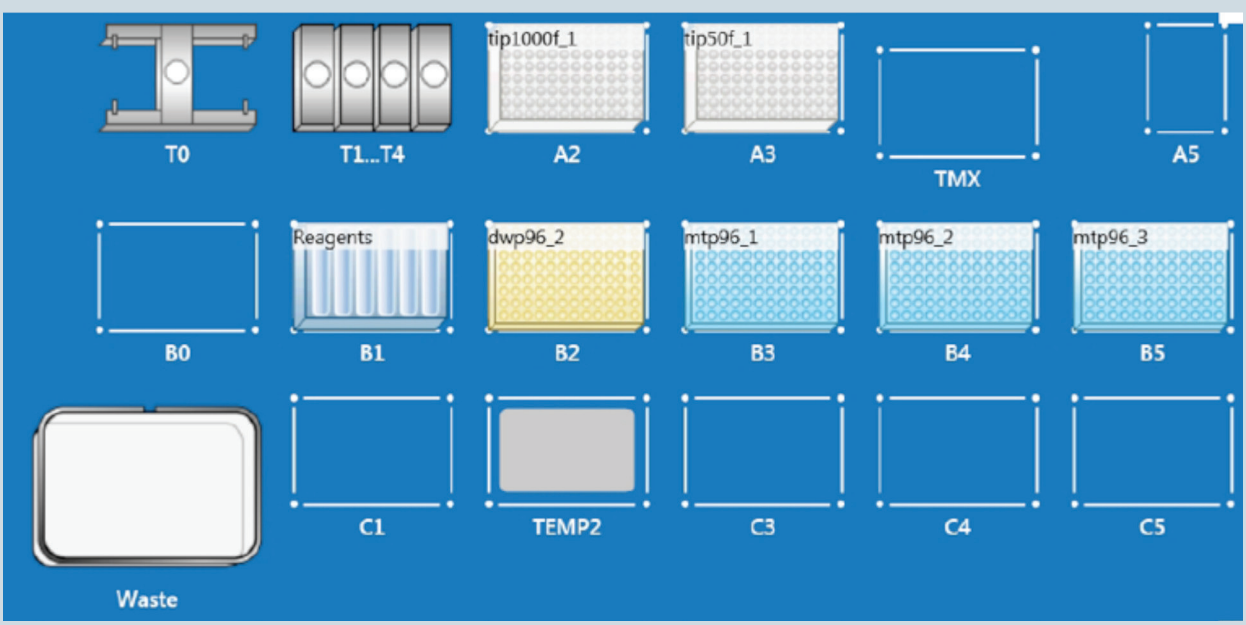

Figure 2: ep*Motion* worktable layout for method 2

Method 3:

After incubation, the plates are removed from the CO_2 incubator. This method includes the dispensing of 100 µL freshly prepared Apo-ONE Caspase-3/7 reagent to each well of three 96-well plates. The solution is first dispensed into the blank and negative controls. The plates are transferred to the ThermoMixer® and mixed at 500 rpm for 30 sec. At the end of this method, the lid is replaced on the plates manually and the plates are incubated for 1 hour at room temperature protected from light. Fluorescence generated by the caspase substrate cleavage was read in each well at two wavelengths (excitation at 485 nm and emission at 535 nm) with the Eppendorf PlateReader AF2200.

Worktable Layout Method 3

Position	**Item**
A2	epT.I.P.S. *Motion* 1000 µL Filter
B2	Reservoir Rack
B3	Cell Imaging Plate (96-well plate)
B4	Cell Imaging Plate (96-well plate)
B5	Cell Imaging Plate (96-well plate)

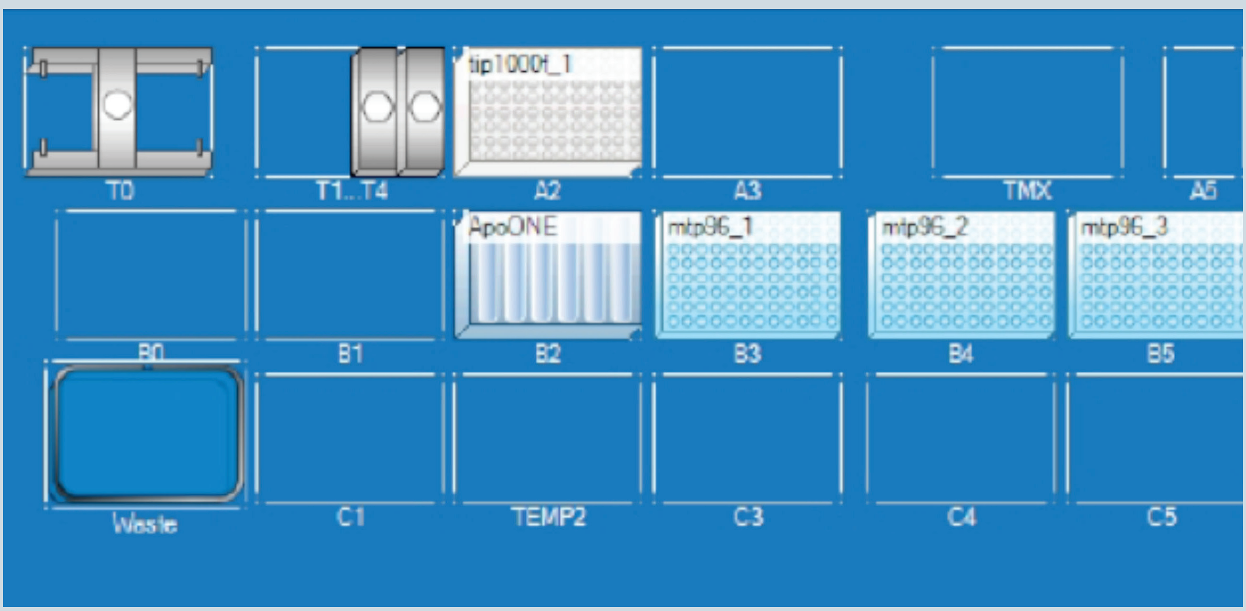

Figure 3: ep*Motion* worktable layout for method 3

Those methods allow the generation of three 96-well plates, but can be easily adapted for a different number of plates. In each plate, 16 apoptosis inducer concentrations are evaluated in five replicates. Blank and negative controls are also included. The final 96-well plate layout is presented below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC 1	CC 1	CC 1	CC 1	CC 1	CC 9	CC 9	CC 9	CC 9	CC 9	Negative control	Blank
B	CC 2	CC 2	CC 2	CC 2	CC 2	CC 10	CC 10	CC 10	CC 10	CC 10		
C	CC 3	CC 3	CC 3	CC 3	CC 3	CC 11	CC 11	CC 11	CC 11	CC 11		
D	CC 4	CC 4	CC 4	CC 4	CC 4	CC 12	CC 12	CC 12	CC 12	CC 12		
E	CC 5	CC 5	CC 5	CC 5	CC 5	CC 13	CC 13	CC 13	CC 13	CC 13		
F	CC 6	CC 6	CC 6	CC 6	CC 6	CC 14	CC 14	CC 14	CC 14	CC 14		
G	CC 7	CC 7	CC 7	CC 7	CC 7	CC 15	CC 15	CC 15	CC 15	CC 15		
H	CC 8	CC 8	CC 8	CC 8	CC 8	CC 16	CC 16	CC 16	CC 16	CC 16		

CC: Compound Concentration

Figure 4: Final plate layout.

Results and Discussion

Cell-based Assay Automation

Cell-based assays commonly contain three successive manipulation stages: sterile cell seeding, specific cell treatment including compound dilution and assay reagent addition. Essentially based on liquid handling, implementation of cell-based assays on automation platforms has been demonstrated as feasible. Depending on integrated equipment (shaker, incubator, plate reader), the assay procedure can be partly [5][6], or completely automated. Through the CleanCap configuration available for the Eppendorf ep*Motion* 5075t, the workstation enclosure can be used to maintain a clean environment and the entire cell-based assay protocol can be carried out with the automation platform. The complete workflow has been divided into three methods (Table 1). For steps requiring sterile environment, ep*Motion* 5075t surface and tools were beforehand cleaned with Umonium[38] disinfectant (steps 1 and 3). Each method corresponds to a protocol part which is independently processed by the ep*Motion* 5075t. Once a method is running, all manipulations are performed by liquid handling workstation. Only plate incubation at 37 °C and data reading were performed offline.

Table 1: General Workflow for implementation of a cell-based assay on the Eppendorf ep*Motion* 5075t

Method Method	Steps description	
Cell seeding (day 1)	1	Preparation of the ep*Motion* 5075t
	2	Cell transfer into 96-well plates
Compound dilution preparation (day 2)	3	Preparation of the ep*Motion* 5075t
	4	Compound dilution
	5	Compound addition into wells
Apo-ONE Homogeneous Caspase-3/7 Assay (day 3)	6	Preparation of the ep*Motion* 5075t
	7	Addition of Assay Reagent into wells

Comparison of manual versus automated cell-based assay performances.

The transferability of the Apo-ONE Homogeneous Caspase-3/7 Assay to the ep*Motion* 5075t was assessed with HeLa and Jurkat cell lines, both treated with a staurosporine dose-response curve. In parallel, the assay was performed manually using the same cellular models. Five replicates of each staurosporine concentration were tested and three plates were processed in parallel. The data obtained confirmed the possibility to automate the Apo-ONE Homogeneous Caspase-3/7 Assay on the ep*Motion* 5075t. As expected, apoptosis is induced by staurosporine in both cell lines. Automated assays provided caspase 3/7 activity profiles comparable to those achieved when assays were manually performed (Figures 5 and 6).

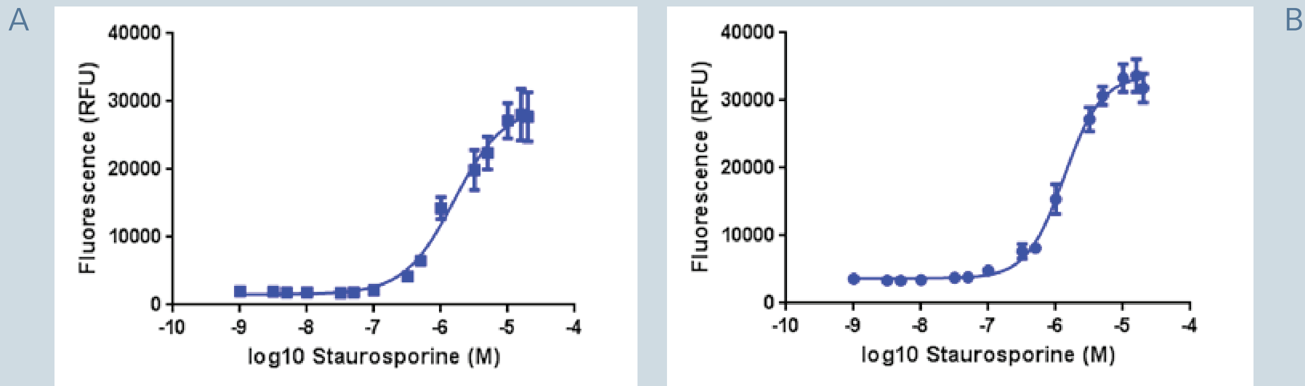

Figure 5: Dose-dependent apoptosis induction of HeLa cells. Cell-based assay performed manually (A) or on ep*Motion* 5075t (B)

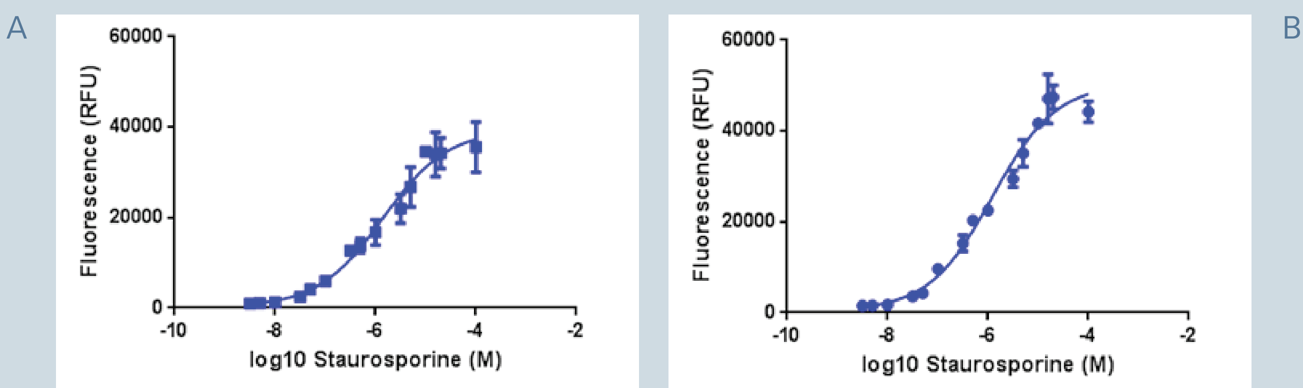

Figure 6: Dose-dependent apoptosis induction of Jurkat cells. Cell-based assay performed manually (A) or on ep*Motion* 5075t (B)

The Z′-factor, a statistical value comparing the assay's dynamic range to its data variation, was used to estimate and compare the assay quality (Table 2). Z′-factor is commonly used as an indicator of assay robustness [7]. A value above 0.5 is the sign of an excellent assay quality. All assays had a Z′-factor value equal or higher than 0.75 indicating that automated assays are as robust as assays performed manually.

Table 2: Z′-Factor for manual and automated Apo-ONE Homogeneous Caspase-3/7 assays

Z′-Factor Analysis		ep*Motion* 5075t	Manual
HeLa cells	Plate 1	0.82	0.75
	Plate 2	0.8	0.89
	Plate 3	0.9	0.81
Jurkat cells	Plate 1	0.86	0.85
	Plate 2	0.88	0.81
	Plate 3	0.8	0.77

Assay automation does not only guarantee robustness, performing cell-based assays with the ep*Motion* 5075t also permits to reduce assay variability. Comparison of intra- and inter-plate precision calculated for manual and automated assay clearly proves that using the ep*Motion* results in a significantly higher precision (Table 3). By reducing human intervention, automation of cell-based assays allows to eliminate human error as one of the major sources of variability. Errors can also be efficiently reduced especially when a broad concentration range of apoptotic compound has to be prepared.

Through the flexible and intuitive epBlue™ software, this error prone protocol step can completely be handled by the workstation ensuring reproducible dilutions and correct dispensing. Depending on the cellular models used for this study, the cell-based assay variability can be decreased by 30 % to 73 % when automated.

Table 3: Global intra- and inter-plate coefficient of variation (CV) calculated for manual and automated Apo-ONE Homogeneous Caspase-3/7 assays

		ep*Motion* 5075t		**Manual**	
		Global intra-plate CV	**Global inter-plate CV**	**Global intra-plate CV**	**Global inter-plate CV**
HeLa cells	Plate 1	6.11 %		11.90 %	
	Plate 2	7.15 %		7.69 %	
	Plate 3	5.80 %		15.84 %	
	Plates 1-2-3		5.53 %		19.87 %
Jurkat cells	Plate 1	9.43 %		12.71 %	
	Plate 2	6.15 %		12.71 %	
	Plate 3	7.83 %		13.50 %	
	Plates 1-2-3		8.47 %		11.81 %

Automation Benefit

Cell-based assays are often used to screen chemical compound libraries. To rapidly identify active molecules, scientists want to perform large series of assays reliably and quickly. Assay variability is one of the parameters evaluated to determine reliability. A simple approach for estimating assay variability is to perform a large number of replicates (at least 20) in a single run on a single day. Automated Apo-ONE Homogeneous Caspase-3/7 assay variability was determined by seeding 30,000 HeLa cells per well and treating those cells with two staurosporine concentrations. The first concentration belonged to the exponential phase (1 µM) and the second was at the beginning of the plateau phase (10 µM) as determined in previous experiments. For each concentration, 22 replicates were tested and three plates were included (Figure 7).

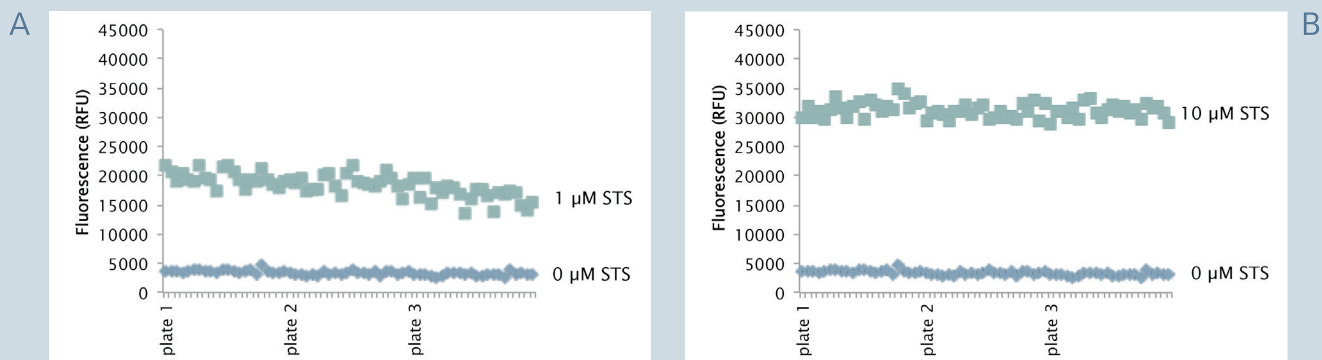

Figure 7: Caspase 3/7 activity induced by 1 µM (A) or 10 µM (B) of staurosporine (STS), determined in three plates with 22 replicates each.

Table 4: Apo-ONE Homogeneous Caspase-3/7 assay variability evaluation on the ep*Motion* 5075t

	Intra-plate 1 CV	Intra-plate 2 CV	Intra-plate 3 CV	Mean intra-plate CV	Inter-plates CV
1 µM STS	6.7 %	7.5 %	10.0 %	8.0 %	8.5 %
10 µM STS	4.3 %	3.5 %	3.7 %	3.8 %	1.5 %

The variability when the Apo-ONE Homogeneous Caspase-3/7 assay is implemented on the ep*Motion* 5075t is excellent. For the lowest staurosporine concentration tested (1 µM), the mean variability observed within a plate is 8.0 %, while the inter-plate precision is 8.5 %. When a higher staurosporine concentration (10 µM) is used, variability is reduced to maximum 3.8 % and 1.5 % respectively for the intra- and inter-plate precision (Table 4). With all Z'-factors being above 0.5, the assay robustness is also guaranteed when a large number of replicates is handled within an assay run (data not shown). Besides the high precision offered to researchers by automation, assay implementation on a platform as the ep*Motion* 5075t also allows to reduce time spent for manipulation. As a result, 75 % of the time needed to perform the assay does not require the presence of an operator.

Conclusion

In the present Application Note, we demonstrate the possibility to automate the Apo-ONE Homogeneous Caspase-3/7 assay on the Eppendorf ep*Motion* 5075t. Because this workstation can be equipped with the CleanCap configuration, a clean environment can be maintained and the complete cell-based assay protocol can be carried out on the automation platform. Assay transferability has been evaluated with two cell lines and all assays performed had a Z′-factor value equal or higher than 0.75, indicating an excellent robustness.

Comparison to the manual method showed that automation with the ep*Motion*® 5075t significantly increases the assay reproducibility. Depending on the cellular models used for this study, the cell-based assay variability can be decreased by 30 % to 73 % when automated. Excellent intra-plate and inter-plate precision is preserved when the number of samples handled in parallel increases. This clearly shows that automation of cell-based assays on the ep*Motion* 5075t is an excellent solution for scientists interested in a low to a medium-throughput screening.

Literature

[1] Zang R, Li D, Tang I-C, Wang J, Yang S-T. Cell-based Assays in High-Throughput Screening for Drug Discovery. *International journal of Biotechnology for Industries* 2012; 1:31–51.
[2] Elmore S,. Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology* 2007; 35:495–519.
[3] Apo-ONE® Homogeneous Caspase-3/7 Assay *Technical Bulletin* #TB295, Promega Corporation, 2009
[4] An W.F, Tolliday N. Cell-based Assays for High-Throughput Screening. *Mol Biotechnol* 2010; 45:180–186.
[5] Worzella T, Larson B. Automating Promega Cell-Based Assays in Multiwell Formats. Promega Notes, number 85, 2005.
[6] Bracht D, Baranowski S. Apoptosis assay with the Promega Apo-ONE® Homogeneous Caspase-3/7 Assay on the ep*Motion*® 5075 TMX. Eppendorf Application Note 209, 2009; www.eppendorf.com
[7] Zhang J, Chung T, Oldenburg K. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screening* 2009; 4:67–73.
[8] Hardware Manual Eppendorf ep*Motion*® 5075t, 00/062013 www.eppendorf.com

Ordering information

Description	Order no. international	Order no. North America
Equipment & Accessories		
ep*Motion*® 5075t with CleanCap configuration option including UV lights and HEPA filters including UV lights and HEPA filters	 5076 000.302 5075 751.607	 5076000302 5075751607
Gripper	5282 000.018	5282000018
TS50 pipetting tool	5280 000.010	5280000010
TM50-8 pipetting tool	5280 000.215	5280000215
TM1000-8 pipetting tool	5280 000.258	5280000258
ReservoirRack for ep*Motion*®	5075 754.002	5075754002
PlateReader AF2200	6141 000.002	6141000002
Consumables		
epT.I.P.S.® *Motion* 50 µL Filter	0030 015.215	0030015215
epT.I.P.S.® *Motion* 1000 µL Filter	0030 015.258	0030015258
ep*Motion*® Reservoir 30 mL 100 mL	 0030 126.505 0030 126.513	 0030126505 0030126513
Equipment & Accessories		
Reservoir Rack Module TC, for use in ep*Motion*® Reservoir Racks, temperable, 4 x Safe-Lock tubes 0.5/1.5/2.0 mL	 5075 799.081	 5075799081
Consumables		
Eppendorf Safe-Lock Tubes, 1.5 mL	0030 120.086	0030120086
Eppendorf Deepwell Plate 96/1000 µL	0030 502.205	0030502205
Cell Imaging Plate	0030 741.013	0030741013

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