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Reliable and robust automation of Cell Viability Assays with the ep*Motion*® 5075t

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Abstract

Cell viability assays as the Promega® CellTiter-Blue® assay are widely used to determine potential toxic effects of various substances on cultured cells. As one step in characterizing the toxic potential of a certain compound, dose response curves are generated with different cell types, which can be a tedious procedure as it includes compound dilution series, preparing complex patterns of replicates, different compounds etc. Taken together with the fact that often these assays will be performed in 96 or 384 well plate formats to allow a certain throughput, this is a good reason for automating these assays to reduce manual workload and overall variability.

In this study a dose response curve for Staurosporine was generated by challenging HepG2 cells grown in three 96 well plates with multiple concentrations of this cell death inducing compound and subsequent conduction of the above mentioned cell viability assay. The direct comparison of a manual procedure with an automated approach using the ep*Motion* 5075t liquid handling system showed that besides the reduced hands on time, the ep*Motion* could increase the robustness of the experiments.

In a second set of fully automated experiments, HepG2 and HeLa cells were both treated with different concentrations of Staurosporine and Camptothecin, to show the compatibility of the ep*Motion* system with different test setups. Here all scenarios resulted in an excellent assay robustness as indicated by high Z'-Factor values.

The entire workflow – cell seeding, compound dilution plus addition to the cells and the addition of the assay reagents was carried out automatically with the ep*Motion* 5075t liquid handling workstation, allowing to reduce the hands on time to a minimum. In total, these experiments emphasize the usability of the ep*Motion* liquid handling workstation for cell based assays.

Introduction

Two major approaches are commonly used by pharmaceutical companies for drug discovery: the phenotypic drug discovery (PDD) when substances screening is performed on whole organisms and the target-based drug discovery (TDD) when new medication research is based on a known biological target. For a long time, PDD was the only strategy employed and led to plenty of medicines still on the market. As originally carried out on living animals and isolated organs, a shift from PDD to TDD has been progressively observed from the mid 1980's. Unfortunately, this new strategy being too reductionist, it has not fulfilled expectations. For a few years, the trend is to adopt a balanced approach incorporating both strategies [1]. In parallel, the emergence of cell-based assays specifically developed for medium or high-throughput screening allowed to limit the use of animal models and contributed to make the phenotypic drug discovery attractive again.

Among cell responses which can be evaluated, the cell viability is central during drug discovery process. For infectious diseases as well as for cancer investigations, the potential therapeutic effect on the targeted cell has to be evaluated by determining its effective concentration (product concentration which results in the death of 50 % of the targeted cells). For promising compounds, viability assays are combined with cytotoxicity assays (determining the product's toxic impact on host cells) in order to define the selectivity index, a ratio indicating how safe (or toxic) a drug is [2]. Cell-based assays developed to monitor cell viability can be divided into two categories based either on membrane integrity evaluation or on cell metabolic activity. Among all metabolic assays available, CellTiter-Blue Cell Viability Assay provided by Promega being used in this study is based on the ability of viable cells to reduce resazurin, a cell wall permeable redox indicator, into resorufin which is highly fluorescent [3]. If cell-based assay development is crucial for the drug screening process, automation also has to be considered. This study shows how a commonly used cell-based viability assay can be implemented on the Eppendorf ep*Motion* 5075t.

Material and Methods

Materials

Automation and Detection Instruments

> ep*Motion* 5075t with CleanCap configuration option including UV lights and HEPA filters (Eppendorf, order no.: 5075 000.302 and 5075 751.607)
> Reservoir Rack Module TC, for use in ep*Motion* Reservoir Racks, temperable, 4 x Eppendorf Safe-Lock tubes 0.5/1.5/2.0 mL (Eppendorf, order no.: 5075 799.081)
> 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)
> TM300-8 pipetting tool (Eppendorf, order no.: 5280 000.231)
> 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)
> Galaxy® 48 R incubator (Eppendorf, order no.: CO48R-230-000)

Consumables

> epT.I.P.S.® Motion 50 µL Filter, sterile (Eppendorf, order no.: 0030 015.215)
> epT.I.P.S. Motion 300 µL Filter, sterile (Eppendorf, order no.: 0030 015.231)
> epT.I.P.S. Motion 1000 µL Filter, sterile (Eppendorf, order no.: 0030 015.258)
> ep*Motion* Reservoir 30 mL and 100 mL (Eppendorf, order no.: 0030 126.505 - 0030 126.513)
> 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 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 hepatocellular carcinoma HepG2 cell line (DSMZ, order no.: ACC 180) cultivated in RPMI 1640 medium supplemented with 10 % FBS and 1 % Penicillin-Streptomycin
> Cell Imaging Plate (Eppendorf, order no.: 0030 741.013)
> Staurosporine from Streptomyces staurospores (Sigma-Aldrich®, order no.: S4400) dissolved in DMSO
> Camptothecin (Sigma-Aldrich, order no.: C9911) dissolved in DMSO
> CellTiter-Blue Cell Viability Assay kit (Promega, order no.: G8082, G8081, G8080)

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 ep*Motion*. The UV-lights stopped automatically after 15 minutes. At the end of each method, a user intervention is requested to perform downstream steps (incubation, reading).

Method 1:
Cell seeding is performed during this first protocol step. Irrespective of the cell line used, cells are seeded into 96-well Eppendorf Cell Imaging plates at a density of 10,000 cells per well. Before starting the ep*Motion* method 1, a cell solution at a concentration of 10,000 cells per 90 µL 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. These steps need to be performed under sterile cell culture conditions. The method 1 dispenses 90 µL of the above described cell suspension per well in columns 1 to 11 of three 96-well microplates and 90 µL of culture medium without cells in column 12 of these three plates. At the end of the program, the lid is manually replaced on the plates and the plates are placed into the Galaxy 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)

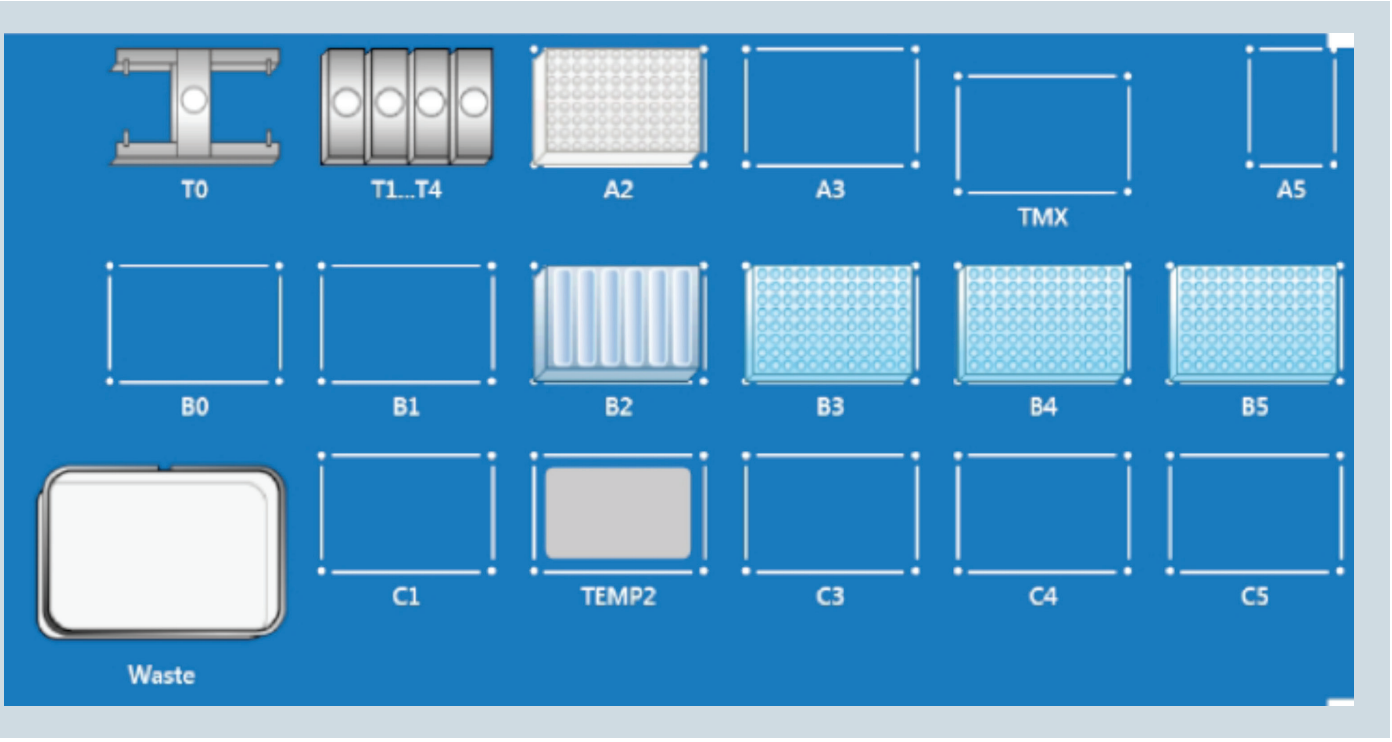

Figure 1: ep*Motion* worktable layout for method 1

Method 2:
The goal of the second method is to generate the concentration curve of one cytotoxic agent (Staurosporine or Camptothecin). Staurosporine concentration range is between 0.1 nM and 2 µM while Camptothecin concentrations are between 0.05 µM and 150 µM. For both compounds, 11 increasing concentrations are used for inducing cell death. In order to generate a concentration curve with the same amount of vehicle solution into each well, the dilution curve is produced by using two different dilution steps. The first dilution is performed into a 99.9 % DMSO vehicle solution in a Deepwell plate for generating 11 compound concentrations 10 times more concentrated than desired. Culture medium without toxic agent 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 11 final compound concentrations. 10 µL of each concentration is dispensed from the Deepwell plate to the 96-well plates. 10 µL of culture medium without toxic agent and DMSO is also added as control. The final vehicle (DMSO) concentration did not exceed 1 % and was equivalent for all compound concentrations tested. The plates are transferred to the TMX position and mixed at 500 rpm for 30 sec.
At the end of the method, the lid is replaced on the plates manually. With both cellular models used for this study (HeLa or HepG2 cells), seeded plates were incubated 48 hours at 37 °C (5 % CO_2) in the Galaxy incubator.

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)

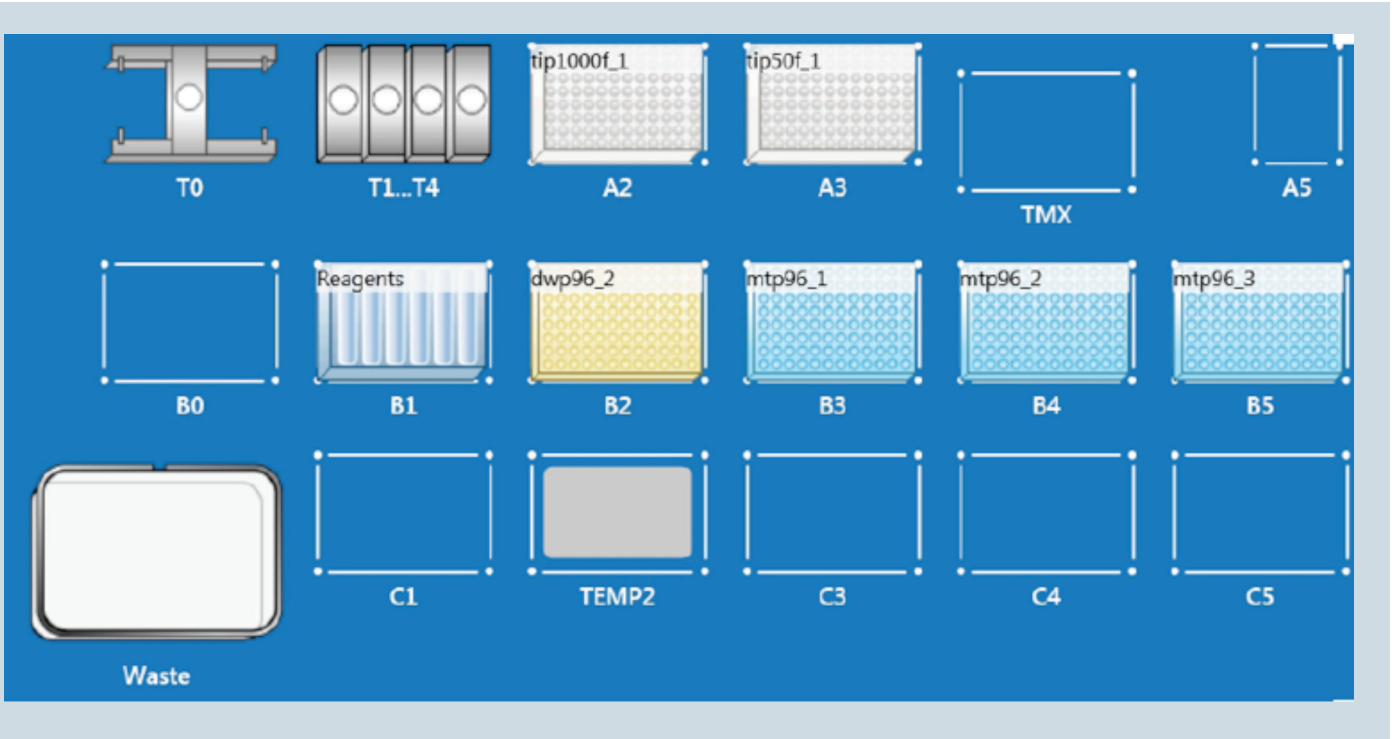

Figure 2: ep*Motion* worktable layout for method 2

Method 3:
After incubation, the plates are removed from the CO_2 incubator. This method includes the dispensing of 20 µL freshly prepared CellTiter-Blue Cell Viability reagent to each well of three 96-well plates. The solution is firstly dispensed into the blank and negative controls. The plates are transferred to the TMX position 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 at 37 °C in a humidified 5 % CO_2 atmosphere for 2 hours. Fluorescence generated by the resazurin metabolism was read in each well at two wavelengths (excitation at 535 nm and emission at 595 nm).

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)

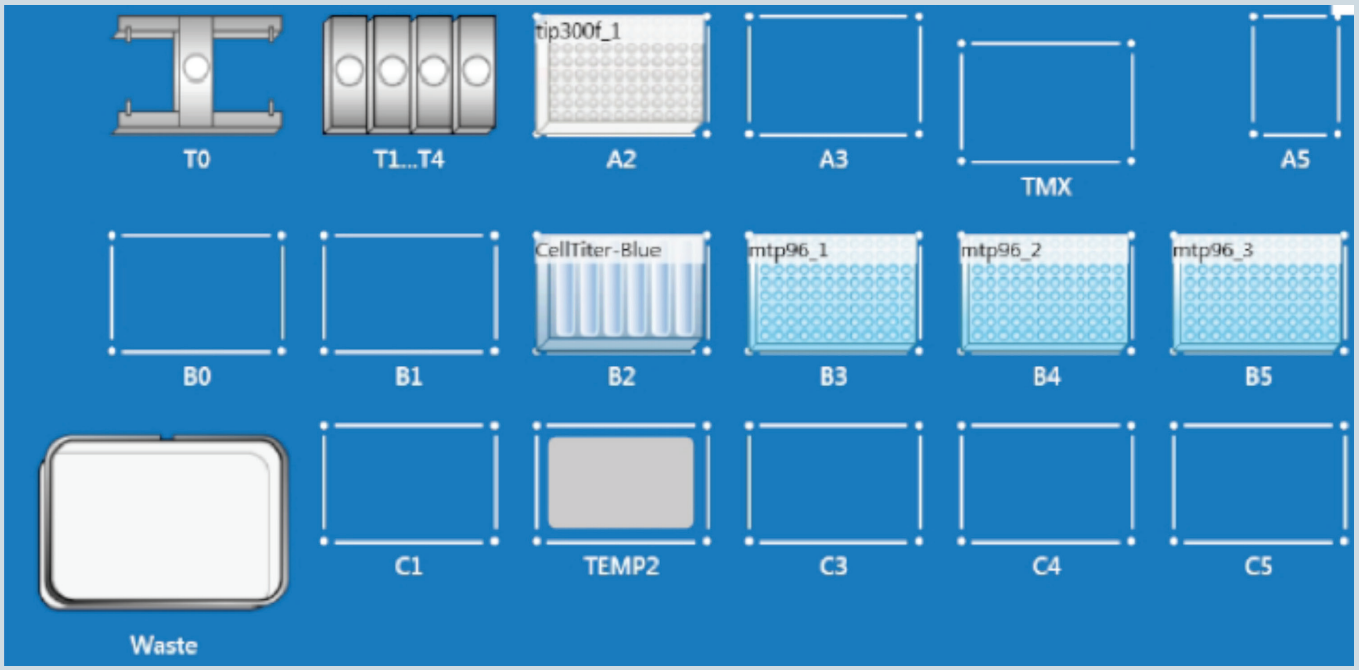

Figure 3: ep*Motion* worktable layout for method 3

Those methods allow the generation of three 96-well plates. In each plate, 11 compound concentrations are evaluated in four replicates. Blank and negative controls are also included.

Results and Discussion

The workflow of the automated cell viability assay is divided into three methods, each corresponding to a usual cell-based assay step: the sterile cell seeding, the specific cell treatment including compound dilution and the assay reagent addition. The CleanCap configuration available for the Eppendorf ep*Motion* 5075t is used to maintain a clean environment within the workstation enclosure. 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. The entire cell-based assay protocol, with the exception of plate incubation at 37 °C and assay readout, is therefore carried out on the automation platform.

Comparison of manual versus automated cell-based assay performances.

The transferability of the CellTiter-Blue Cell Viability Assay to the ep*Motion* 5075t was studied by treating HepG2 cells with a staurosporine dose-response curve. In parallel, the assay was performed manually using the same cellular model. Four replicates of each staurosporine concentration were tested and three plates were processed in parallel. Results obtained demonstrated the capability to automate the CellTiter-Blue Cell Viability Assay on the ep*Motion* 5075t. With staurosporine being a cytotoxic agent, a reduction of the number of viable cells is expected when increasing concentrations of this reagent are applied. As nonviable cells rapidly lose their metabolic ability to reduce resazurin into resorufin, the fluorescent signal decreases according to the staurosporine concentration and clearly proves the loss of viable cells. Automated assays provided profiles comparable to those achieved when assays were manually performed (Figure 4).

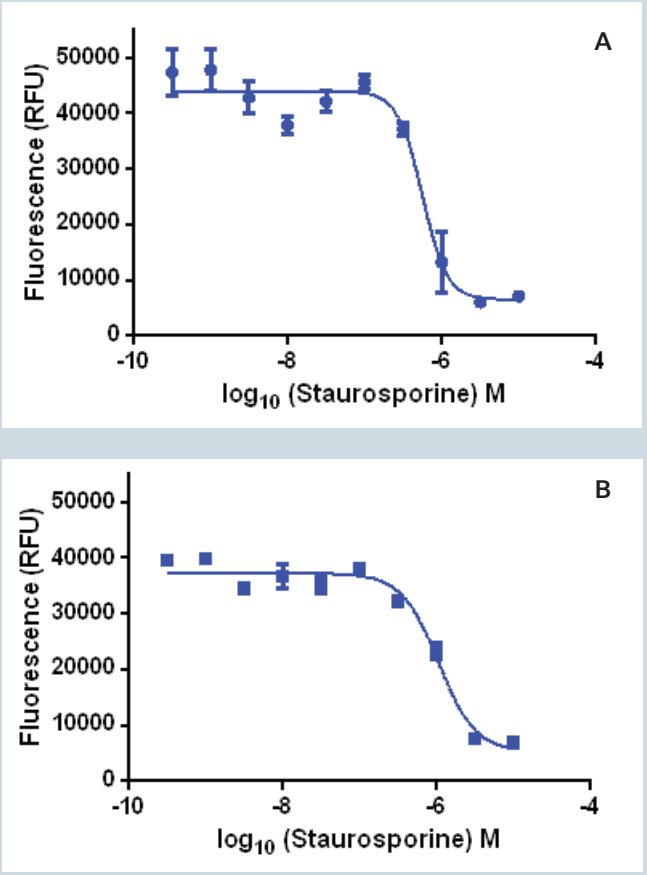

Figure 4: Dose-dependent staurosporine toxic effect on HepG2 cells. Measurement of resazurin metabolism as an indicator of cell viability. Cell-based assay performed manually (A) or on ep*Motion* 5075t (B)

The Z'-factor, a statistical value, being the industry standard as an indicator of assay robustness, was used to estimate and compare the assay quality (Table 1). A value above 0.5 is the sign of an excellent assay quality [4]. All assays had a Z'-factor value higher than 0.80 indicating that automated assays are as robust as assays performed manually.

Table 1: Z'-Factor for manual and automated CellTiter-Blue Cell Viability Assay

Z'-Factor Analysis		ep*Motion* 5075t	Manual
HepG2 cells treated with staurosporine	Plate 1	0.88	0.90
	Plate 2	0.89	0.87
	Plate 3	0.83	0.85

By reducing manual intervention, automation of cell-based assays allows to eliminate human error as one of the major sources of variability. As the comparison of intra- and inter-plate precisions calculated for manual and automated shows, this reproducibility increase is particularly observed when several plates are processed in parallel (Table 2). With an inter-plate CV of 2.9 %, the automated CellTiter-Blue Cell Viability Assay offers a higher consistency, essential for generating reliable results during a drug screening.

Table 2: Global intra- and inter-plate coefficient of variation (CV) calculated for manual and automated CellTiter-Blue Cell Viability Assay

	ep*Motion* 5075t		Manual	
	Global intra-plate CV	Global inter-plate CV	Global intra-plate CV	Global inter-plate CV
Plate 1	5.0 %		6.4 %	
Plate 2	6.4 %		7.7 %	
Plate 3	8.1 %		7.0 %	
Plate 1-2-3		2.9 %		9.3 %

In vitro models automation

Depending on the studied pathway, the target of a tested drug or the goal of a specific project, the *in vitro* cellular model selected by researchers can differ. Cell line and toxic agent are carefully chosen to be biologically relevant in the research context. As experimental conditions can be varied, the automated cell-based assay has to stay reproducible and consistent regardless of the cellular model used. In order to assess robustness of the automated CellTiter-Blue Cell Viability Assay in different situations, experiments were performed with two cell lines (HepG2 and HeLa cells) treated either with staurosporine or camptothecin. HepG2 is a perpetual cell line derived from the liver tissue of a young American male affected by a hepatocellular carcinoma. Those cells represent a suitable *in vitro* model system for studying hepatic functions and some human liver diseases. Because of involvement of the liver in drug metabolism, the HepG2 cell line is also one of the most widely used for evaluating the toxicity of chemicals and drugs. HeLa is an immortal cell line derived from a human cervical carcinoma. Plenty of research subjects have been tackled through HeLa cells such as cancer, AIDS or toxicology. Staurosporine and camptothecin are cell death inducers. Staurosporine is a protein kinase inhibitor while camptothecin inhibits the DNA enzyme topoisomerase I. For each toxic agent, a dose-response curve of 11 increasing concentrations has been prepared and applied on both cell lines. Four replicates of each compound concentration have been tested and three plates were processed in parallel. For all *in vitro* model systems, the Z' factor obtained for the automated CellTiter-Blue Cell Viability Assay is systematically higher than 0.80 (Table 3). Those values clearly prove that the robustness of the assay is guaranteed with multiple cellular models when transferred to the ep*Motion* 5075t.

Table 3: Z'-Factor for automated CellTiter-Blue Cell Viability Assay performed on four *in vitro* cellular models

Z'-Factor Analysis	HepG2	HeLa
Staurosporine treatment	0.83	0.92
Camptothecin treatment	0.90	0.85

Conclusion

During the long drug discovery process, various cell responses can and are also very likely to be studied. The capability to automate the Apo-ONE® Homogeneous Caspase-3/7 assay on the Eppendorf ep*Motion* 5075t has already been demonstrated [5]. In the present Application Note, we show that the CellTiter-Blue Cell Viability Assay can also be successfully automated on the same liquid handling workstation. Assay implementation has been evaluated on four *in vitro* cellular models and all assays performed had a Z'-factor value higher than 0.80, indicating the excellent robustness of the assay. Comparison with the manual method confirmed that automation with the ep*Motion* 5075t significantly increases the assay reproducibility, especially when several plates are handled in parallel. The overall hands-on-time was significantly reduced with automation. As the robustness and reproducibility are guaranteed independent of the cell response studied and the *in vitro* model chosen, automating cell-based assays on the ep*Motion* 5075t represents a perfect solution for scientists interested in a low to a medium-throughput screening.

Literature

[1] Terstappen G.C., Schlüpen C., Raggiaschi R., Gaviraghi G. Target deconvolution strategies in drug discovery. Nature Reviews Drug Discovery 2007; 6:891–903.
[2] Antiviral product development – Conducting and submitting virology studies to the Agency. Guidance for Industry U.S. Department of Health and Human Services – Food and Drug Administration 2006.
[3] CellTiter-Blue Cell Viability Assay Technical Bulletin #TB317, Promega Corporation.
[4] 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.
[5] Automated Cell-based Apoptosis Assays with the Eppendorf ep*Motion* 5075t increases the reproducibility, Eppendorf Application Note 329, 2014; www.eppendorf.com

Ordering information

Description	Order no. international	Order no. North America
ep*Motion*® 5075t with CleanCap configuration option		
including UV lights and HEPA filters	5076 000.302	5076000302
including UV lights and HEPA filters	5075 751.607	5075751607
Gripper	5282 000.018	5282000018
TS50 pipetting tool	5280 000.010	5280000010
TM50-8 pipetting tool	5280 000.215	5280000215
TM300-8 pipetting tool	5280 000.231	5280000231
TM1000-8 pipetting tool	5280 000.258	5280000258
ReservoirRack for ep*Motion*®	5075 754.002	5075754002
PlateReader AF2200	6141 000.002	6141000002
Galaxy® 48 R incubator	CO48R-230-000	CO48R-230-000
epT.I.P.S.® Motion 50 µL Filter, sterile	0030 015.215	0030015215
epT.I.P.S.® Motion 300 µL Filter, sterile	0030 015.231	0030015231
epT.I.P.S.® Motion 1000 µL Filter, sterile	0030 015.258	0030015258
ep*Motion*® Reservoir		
30 mL	0030 126.505	0030126505
100 mL	0030 126.513	0030126513
Reservoir Rack Module TC, for use in ep*Motion*® Reservoir Racks, temperable, 4 x Eppendorf Safe-Lock tubes 0.5/1.5/2.0 mL	5075 799.081	5075799081
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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