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Faster and more reproducible Cell Viability Assays with the Eppendorf ep*Motion*® 96.

Eric Gancarek[1], Maud Brasseur[1], Muriel Art[1], Hanaë A. Henke[2]
[1]Eppendorf Application Technologies S.A., Namur, Belgium; [2]Eppendorf AG, Hamburg, Germany

Abstract

Cell Based Assays are an established standard tool in life science or pharmaceutical research. The most common formats for these assays are 96 and 384 well plates, requiring multichannel pipetting to ensure quick, efficient and reproducible handling – especially when multiple plates need to be processed.

In this application note, a cell viability assay was used to determine the speed, reliability and reproducibility of different pipetting systems. A manual 12-channel pipette, an electronic 12-channel pipette and a semi-automated 96-channel electronic pipette (Eppendorf ep*Motion* 96) were used for cell seeding, cell treatment by a cytotoxic agent and assay reagent addition. It was shown that the ep*Motion* 96 and electronic 12-channel pipette delivered most reproducible results, with the ep*Motion* 96 being significantly faster. Assays performed with a manual 12-channel pipette showed higher deviations and required the most time for completion.

In addition to the results the ep*Motion* 96 allows synchronous processing of 96 wells, which increases the throughput and at the same time negates the risks of time dependent intra-plate gradients and human errors.

Taken together, the ep*Motion* 96 is the ideal tool for plate based routine cell based assay tasks, including cell seeding, media change, compound and assay reagent addition – in particular because the ep*Motion* 96 comes with a small footprint and fits well into standard biosafety cabinets.

Introduction

Drug discovery is a long and very costly process involving the screening of a large number of potentially therapeutic compounds. Over the past decades, cell-based assays became a key tool for pharmaceutical companies. This approach allows a limited use of animal models and provides more representative information generated in a biological context like a normal physiological situation [1]. A large panel of cell responses can be tackled by this method such as cell proliferation, activation of specific signaling pathways, changes in morphology, cytotoxic impact of a molecule, apoptosis or cell viability. If cell-based assays need to be implemented routinely, automation has to be considered as well. Compatibility of the liquid handling workstation with the cell-based assay has to be demonstrated. All instrument settings have to be defined to ensure a correct delivery of solutions without damaging mono-layers of adherent cells by mechanical forces. Finally, assay reproducibility is an essential factor which has to be evaluated [2]. We already demonstrated the capability to automate various cell-based assays on the Eppendorf ep*Motion* 5075t automated liquid handling workstation [3, 4, 5]. Nevertheless, this equipment can be an investment smaller labs cannot afford. The ep*Motion* 96, semi-automatic pipette equipped with 96 channels, could be an alternative solution for scientists interested in increasing their throughput. This study clearly illustrates the benefits of using the ep*Motion* 96 for cell based assays.

Materials and Methods

Materials

Instruments

> ep*Motion* 96 (Eppendorf, order no.: 5069 000.012)
> electronic pipette, 12-channel, 50 µL-1200 µL
> manual pipette, 12-channel, 10 µL- 100 µL
> electronic pipette, 12-channel, 5 µL-100 µL
> MixMate® (Eppendorf, order no.: 5353 000.014)

Consumables

> epT.I.P.S.® Motion as Reload-System, with Filter, PCR clean and sterile, 50 µL (Eppendorf, order no.: 0030 014.529)
> epT.I.P.S. Motion as Reload-System, with Filter, PCR clean and sterile, 300 µL (Eppendorf, order no.: 0030 014.537)
> ep Dualfilter T.I.P.S® 1250 µL PCR clean/sterile (Eppendorf, order no.: 0030 077.750)
> ep Dualfilter T.I.P.S 100 µL PCR clean/sterile (Eppendorf, order no.: 0030 077.547)
> 96-well Reservoir Low Profile – Pyramid bottom (Porvair® sciences, order no.: PP 390 004)
> Cell Imaging Plate (Eppendorf, order no.: 0030 741.013)
> Cell Culture Flask T-75 TC treated, with filter cap (Eppendorf, order no.: 0030 711.122)

Cell assay

> Human hepatocellular carcinoma HepG2 cell line (DSMZ, order no.: ACC 180)
> Staurosporine from *Streptomyces staurospores* (Sigma-Aldrich®, order no.: S4400) dissolved in DMSO
> CellTiter-Blue® Cell Viability Assay kit (Promega®, order no.: G8081)
> CASY® Cell Counter and Analyser, model TT (Omni Life Science®, order no.: 2501126A).

Method

Cell culture

Human hepatocellular carcinoma HepG2 cell line is cultivated in RPMI 1640 medium supplemented with 10 % FBS and 1 % Penicillin-Streptomycin in a T75 Tissue culture treated flask. After a visual check with the microscope, cells are washed with PBS and detached by adding 2 mL of 0.25 % Trypsin-EDTA. After inactivation of Trypsin-EDTA, cells are centrifuged and diluted in 15 mL of fresh culture medium. Cell number is determined by using the CASY® Cell Counter.

Cell viability assay

A usual cell viability assay consists of three major steps: the sterile cell seeding, the cell treatment with a potentially cytotoxic agent and finally, the assay reagent addition. After cell counting, cells are seeded into 96-well Eppendorf Cell Imaging plates at a density of 10,000 cells per well and plates are placed in a CO_2 incubator at 37 °C for 24 hours. The day after, a concentration curve of staurosporine (STS) is prepared. Eleven increasing concentrations between 1 nM and 20 µM are used for inducing cell death. 10 µL of each concentration is dispensed from Deepwell plates to 96-well cell imaging plates. The final vehicle concentration (DMSO) does not exceed 1 % and is equivalent for all compound concentrations tested. The plates are mixed at 300 rpm for 1 minute and are incubated 48 hours at 37 °C (5 % CO_2) in presence of the cytotoxic agent. After incubation, the plates are removed from the CO_2 incubator and 20 µL of freshly prepared CellTiter-Blue Cell Viability reagent are added into each well of the 96-well plates. The CellTiter-Blue reagent is a buffered solution containing highly purified resazurin. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent (Figure 1). Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal.

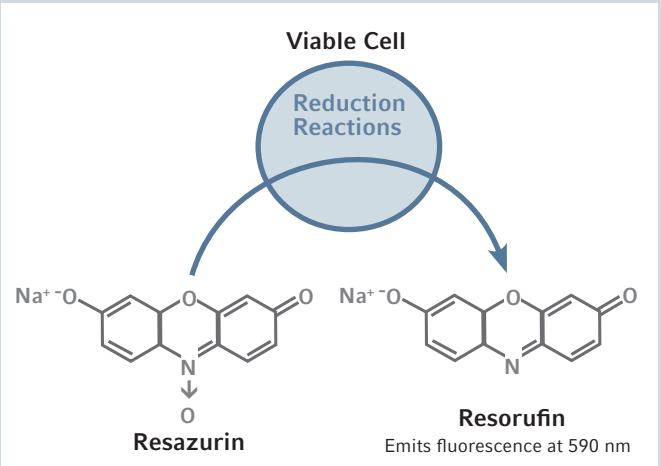

Figure 1: Conversion of resazurin (dark blue) to resorufin (pink and highly fluorescent) by metabolically active cells

The plates are mixed at 300 rpm for 1 minute and incubated at 37 °C in a humidified 5 % CO_2 atmosphere for 2 hours. Fluorescence generated by the resazurin metabolism is read in each well at two wavelengths (excitation at 535 nm and emission at 595 nm).

Results and Discussion

The workflow of a cell viability assay classically includes three successive steps: the cell seeding, the cell treatment by a cytotoxic agent and the assay reagent addition. All those steps involve liquid handling instruments. In the cell-based assay chosen for this study (CellTiter-Blue Cell Viability Assay kit from Promega), volumes dispensed are between 10 µL and 90 µL. Moreover, solutions handled vary from a cell suspension to a cytotoxic agent dissolved in DMSO. The instrument selected should consequently offer accuracy and precision for low volume dispensing independent of the composition of the respective solution.

Impact of liquid handling instrument on cell-based assay performances

Signal-to-background ratio and Z' factor

The possibility to integrate the ep*Motion* 96 in a traditional cell-based assay protocol was firstly assessed by using it for the first and last assay step. This semi-automated electronic pipette with 96 synchronous channels was used to seed HepG2 cells into 96-well plates (90 µL) and to add the assay reagent (20 µL) to all wells. In parallel, those assay steps were also performed with a 12-channel electronic pipette and a 12-channel manual pipette. The ep*Motion* 96 and electronic pipette were used in dispensing mode with the speed set at level 5 and for each instrument, 3 plates were processed in parallel. The HepG2 cells, viability was evaluated after treatment with a staurosporine dose-response curve. An electronic 12-channel pipette was used to add 8 replicates of this curve into the 9 microplates. Staurosporine is a cytotoxic agent and a reduction of the viable cell number was expected when increasing concentrations of this reagent were applied. As nonviable cells rapidly lose their metabolic ability to reduce resazurin to resorufin, the fluorescent signal decreased according to the staurosporine concentration. To limit the impact of a potential edge effect, data generated from curves displayed in lines A and H in all plates were considered as outliers and removed from data analysis, so 6 replicates were used for calculation. Profiles obtained for each instrument tested are shown in figures 2 to 4.

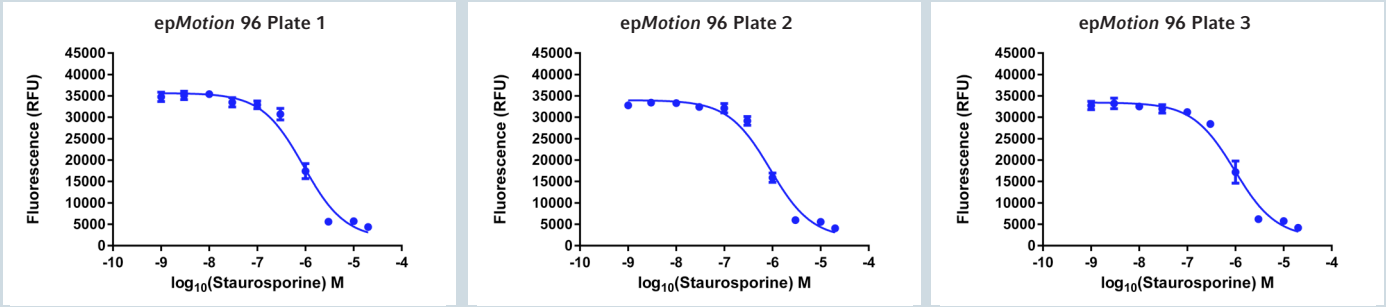

Figure 2: Dose-dependent staurosporine toxic effect on HepG2 cells. Cell seeding and assay reagent addition performed with the ep*Motion* 96 used in dispensing mode (n=6 replicates, line B-G).

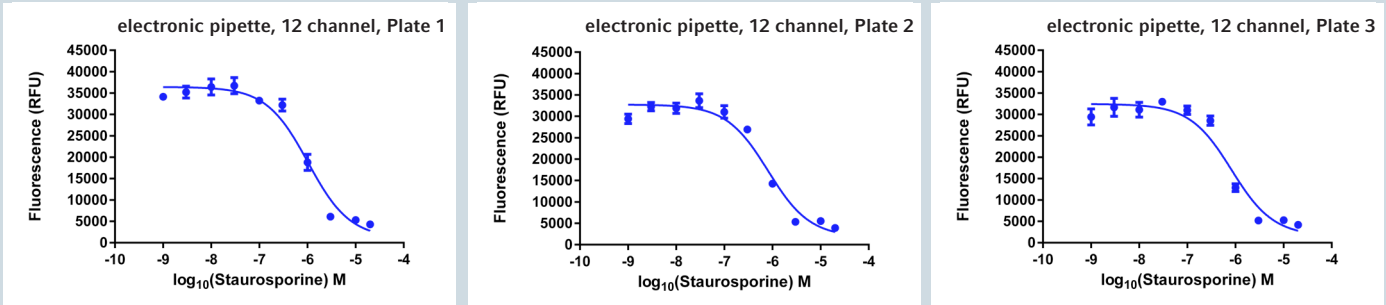

Figure 3: Dose-dependent staurosporine toxic effect on HepG2 cells. Cell seeding and assay reagent addition performed with the electronic 12-channel pipette used in dispensing mode (n=6 replicates, line B-G).

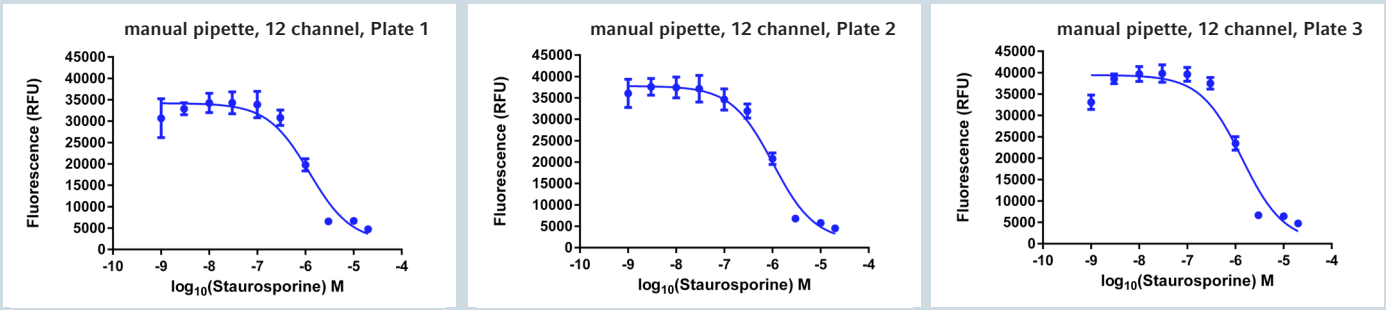

Figure 4: Dose-dependent staurosporine toxic effect on HepG2 cells. Cell seeding and assay reagent addition performed with the manual 12-channel pipette (n=6 replicates, line B-G).

Profiles generated were comparable for each instrument used for cell seeding and reagent adding. Nevertheless, with a manual pipetting system, the assay reproducibility was affected as indicated by higher standard deviations. Cell-based assay performances can be analyzed in a number of different ways. The signal-to-background ratio (S/B) is often used as distinction of assay performance, even if it does not take assay variability into consideration. For this reason, the Z'-factor, a statistical value, is the industrial standard used as an indicator of assay robustness. This coefficient takes the assay signal dynamic range as well as the data variation associated with the signal measurements into account. A value above 0.5 is the sign of an excellent assay quality [6]. Signal-to-background ratio and Z'-factor were calculated for each plate and are depicted in table 1.

As expected by similar profiles generated (see figures 2 to 4), S/B values were comparable for each instrument used for cell seeding and reagent adding. All values were between 5.85 and 7.87 indicating a good discrimination between untreated cells and cells exposed to the highest staurosporine concentration. When an electronic instrument was used, the assay robustness was perfectly guaranteed. Indeed, with the ep*Motion* 96 as well as with the electronic pipette, all Z'-factor values were higher than 0.7. With the manual pipette, one plate presented a Z'-factor value lower than 0.5 which was explained by the higher variability observed with this instrument.

Table 1: S/B and Z'-factor calculated for CellTiter-Blue Cell Viability Assay according to the instrument used for cell seeding and reagent adding (n=6 replicates)

n=6	S/B			Z'-factor		
	Plate 1	Plate 2	Plate 3	Plate 1	Plate 2	Plate 3
ep*Motion* 96	7.68	7.20	7.87	0.81	0.91	0.76
electronic pipette, 12 channels	7.70	5.85	6.01	0.85	0.74	0.79
manual pipette, 12 channels	6.72	7.74	6.13	0.42	0.78	0.72

Intra- and inter-plate reproducibility

The opportunity to use the ep*Motion* 96 for all steps of a cell-based assay was also evaluated. In this case, a single staurosporine concentration (1,000 nM) was applied to HepG2 cells to induce cell death. The ep*Motion* 96 was compared to a 12-channel electronic pipette and a 12-channel manual pipette. All three successive assay steps (cell seeding, cell treatment by a cytotoxic agent and assay reagent addition) were performed with those instruments. As the staurosporine solution contained DMSO, an organic solvent, and as the volume dispensed was particularly low (10 µL), a prewetting step was applied for adding the cytotoxic agent whatever instrument used. Moreover, for this step, the dispensing speed was set at the maximal level for the electronic pipettes. ep*Motion* 96 and the electronic pipette were used in dispensing mode. For each instrument, 3 plates were processed in parallel. The assay reproducibility was assessed by calculating intra-plate and inter-plate coefficients of variation (CV) presented in table 2.

Table 2: Intra- and inter-plate CVs calculated for CellTiter-Blue Cell Viability Assay for the respective instruments used (n=no. of replicates)

	Intra-plate CV (n=96)			Global Intra-plate CV (n=96 x 3)	Inter-plate CV (n=288)
	Plate 1	Plate 2	Plate 3		
ep*Motion* 96	9.4	7.9	8.8	8.7	9.8
electronic pipette, 12 channels	7.20	8.4	10.1	8.6	10.4
manual pipette, 12 channels	13.1	15.9	16.8	15.3	17.9

When an electronic instrument was used, the assay reproducibility was generally better than with the manual instrument. Indeed, the global intra-plate CV and the inter-plates CV generated by the ep*Motion* 96 and the electronic pipette were comparable and significantly lower than CV obtained with a manual instrument. By reducing human intervention, those instruments allow to eliminate human error as one of the major sources of variability.

Besides the assay reproducibility improvement, the use of an electronic instrument also allows to reduce the hands-on time as indicated in figure 5.

Among electronic instruments tested, the ep*Motion* 96 appears as the fastest option since a complete cell-based assay requires only 4 minutes, that means 2 times less hands-on time than what is needed with the electronic pipette (8 minutes). The benefit brought by a 96-channel pipette is still more obvious when the comparison is made with a manual 12-channel pipette as the cell-based assay fully performed with the manual pipette requires almost 20 minutes, which means almost 5 times more hands-on time than the ep*Motion* 96.

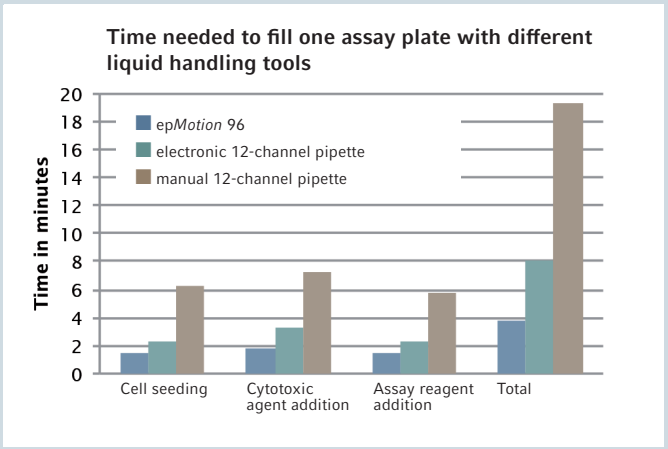

Figure 5: Hands-on time needed for one assay plate for each CellTiter-Blue Cell Viability assay step according to the instrument used.

Conclusion

In the present Application Note, we show the capability to use ep*Motion* 96, semi-automatic pipette equipped with 96 channels, for processing a cell-based assay. With all assay performance parameters studied (signal-to-background ratio, Z'-factor, intra-plate and inter-plate CV), it was demonstrated that the assay robustness and reproducibility were significantly better when an electronic pipette (ep*Motion* 96 or electronic pipette) was used. When a large number of tests have to be handled in parallel and multi-channel pipettes do not offer the needed throughput, the ep*Motion* 96 can represent a good choice for scientists interested in a low or medium throughput. This instrument allows saving hands-on time and combines the ease of use of pipettes with a high precision at a price more affordable than fully automated systems.

Literature

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[6] 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.

Ordering information

Description	Order no. international	Order no. North America
epMotion® 96	5069 000.012	5069000012
epT.I.P.S.® Motion as Reload-System, with Filter, PCR clean and sterile, 50 µL	0030 014.529	0030014529
epT.I.P.S.® Motion as Reload-System, with Filter, PCR clean and sterile, 300 µL	0030 014.537	0030014537
ep Dualfilter T.I.P.S.® 1250 µL PCR clean/sterile	0030 077.750	0030077750
ep Dualfilter T.I.P.S.® 100 µL PCR clean/sterile	0030 077.547	0030077547
Cell Imaging Plate	0030 741.013	0030741013
Cell Culture Flask T-75 TC treated, with filter cap	0030 711.122	0030711122

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