Applications

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Performing Cytotoxicity Assays with different substances on the Eppendorf ep*Motion*® 5070/5075

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Abstract

***In vitro* viability or cytotoxicity assays are used to determine the acute toxic effect of a given substance on cells or tissue. This application is critical in industry R&D, as a primary screen during development of novel agents or for testing of chemotherapeutic agents. Depending on the focus of research, a variety of cell viability assays is available. The application presented in this report utilizes the resazurin test as an example to demonstrate that cytotoxicity assays can be performed reliably on the ep*Motion* 5070/5075 and that the use of the ep*Motion* 5070/5075 provides advantages in both precision and time management.**

Introduction

The resazurin test determines the redox state of cells and extrapolates viability, i.e. the proportion of live cells, within the entire cell population. To this end, water soluble resazurin (Alamar Blue™) is incubated with the cells. Reduction of the non-fluorescing blue resazurin to the fluorescent red resofurin occurs only in live cells (Fig. 1). Hence, the assumption can be made that the amount of resofurin produced is directly proportional to the number of live cells present. Quantification of the newly created resofurin occurs via photometric measurement of fluorescence. If, following incubation of the cells with a test substance, reduced ability to reduce resazurin is observed, a cytotoxic effect, and therefore reduced viability, is indicated.

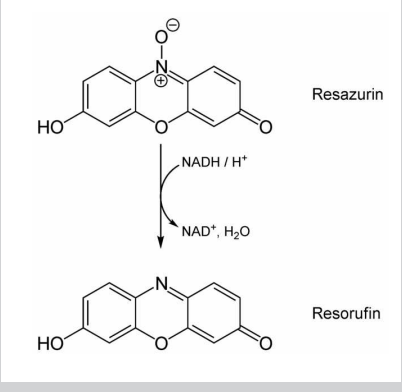

Fig. 1: Reduction of resazurin to resofurin

Materials and Methods

Eppendorf ep*Motion* 5070 CB or 5075 LH
equipped with:
- Dispensing tools TS 1000 and TM 1000-8
- Reservoir rack
- Reservoir rack module
- Thermorack for SafeLock tubes

Eppendorf Consumables
- 30-ml-Reservoir
- epTIPS Motion Filtertips 1,000 µl
- SafeLock tubes 1.5 ml

Research® pro

Multipette® stream

Consumables and reagents from other vendors:
- NIH/3T3 cells
- Cell culture medium: 0.9 x DMEM; 0.1 x FBS; 0.01 x Penicillin/Streptomycin
- 96-well cell culture plates
- Round bottom tubes 5 ml
- 8-channel pipetting unit, Geyer
- Hydrogen peroxide
- Sulforafan
- N-Acetyl-L-Cysteine (NAC)
- Menadion
- Ethanol, HPLC grade (99,8 %)
- Dimethylsulfoxide (DMSO)
- Alamar Blue™

eppendorf

Cell seeding using the ep*Motion*

The ep*Motion* was equipped as follows: A reservoir rack with 2 x 30 ml reservoirs, filled with cell culture medium or cell suspension, respectively, was placed in the source position, while a height adapter and a 96-well cell culture plate were placed in the destination position.

Each well in the 96-well cell culture plate received 80 µl of cell culture medium (1x pre-wetting, reagent transfer), followed by 80 µl of cell suspension per well (187,500 cells/ml, 1x pre-wetting, reagent transfer). The cells plated in this manner were incubated for 24 h at 95 % humidity and 5 % CO_2 to ensure growth.

Cell treatment using the ep*Motion*

Cell treatment requires simple positioning of the 96-well culture plate, which contains the seeded cells, into the ep*Motion*. The reservoir rack contains a reservoir rack module, which is equipped with 5 ml tubes. These tubes are filled with different cell culture media.

From these 5 ml round bottom tubes, 540 µl were pipetted into 20 empty SafeLock tubes (TS 1000, reagent transfer, multidispense). Here, the different media are employed; hence, it is possible to test substances with different solvents in one cell culture plate. Thus, all cells in one test group receive identical concentrations of solvent.

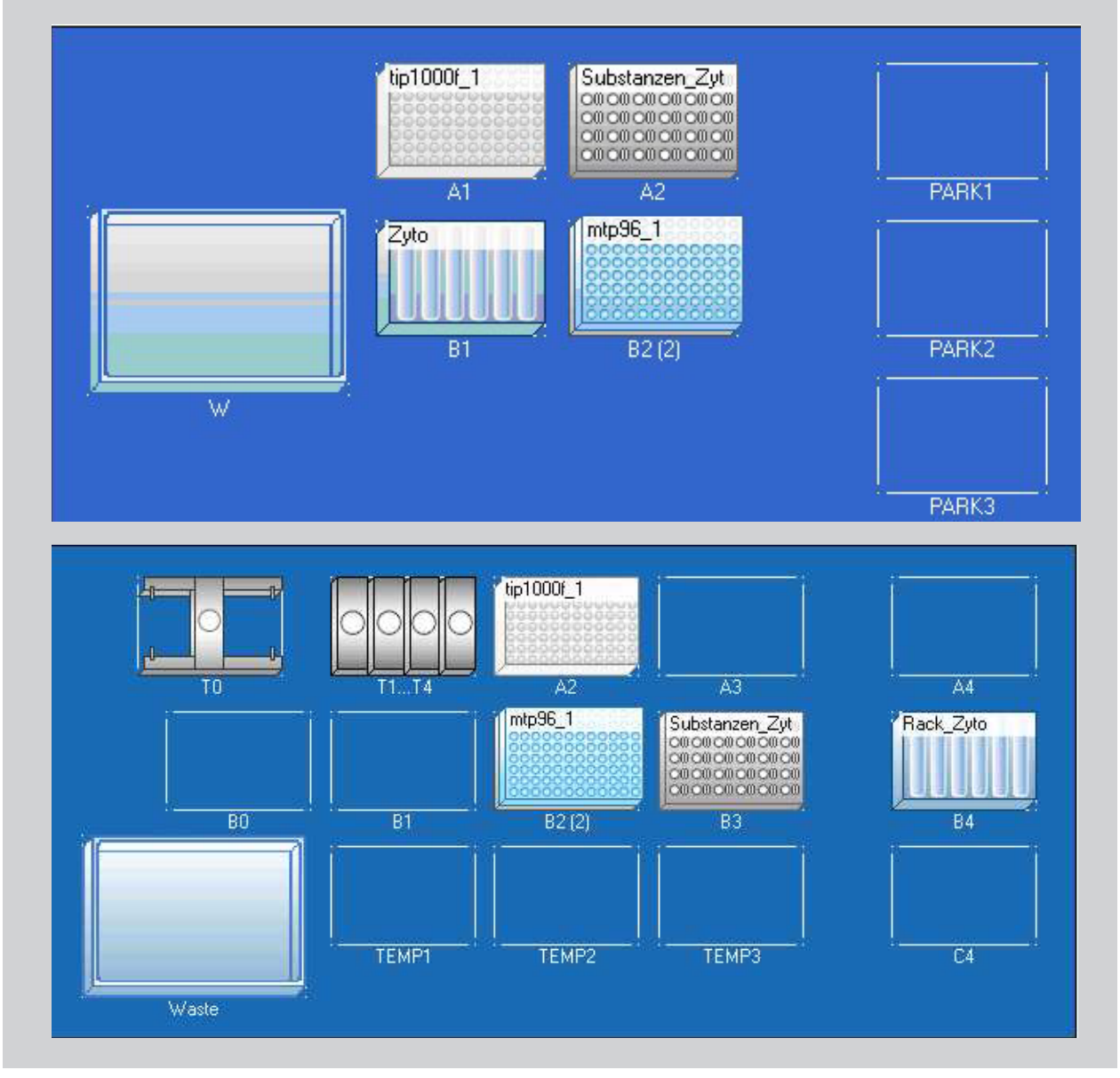

Figure 2: Worktable layout ep*Motion* 5070 CB (top) or 5075 LH (bottom) for cell seeding using the ep*Motion*

1 ml each was used for the serial dilution, where mixing occurred prior to aspiration as well as following dispensing of the cell suspension. Mixing parameters were chosen as follows: 2 cycles of 5 mm/s. Therefore the last row of the plate contained 2 ml of cell suspension. Whereas the number of cells per ml contained in each well of this last row was one half of the previous row, the total number of cells per well is the critical value, and thus one half of the cell suspension had to be removed from the wells in the last row. This was achieved with the command “PoolOneDest“ [1]. The empty 100 ml reservoir was used for waste disposal.

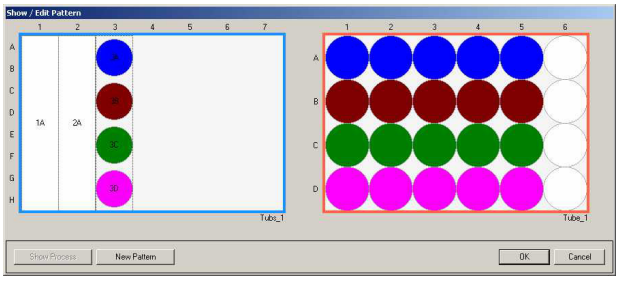

Figure 3: Diagram illustrating the filling of empty SafeLock tubes with different cell culture media for the purpose of stock dilution.

The last column of the thermo rack contains 4 SafeLock tubes, filled with stock solutions of the test agents to be used, in this case 465 µM menadion in medium with 1 % Ethanol, 1 mM hydrogen peroxide in medium, 50 µM sulforafan in medium with 0.1 % DMSO, and 100 mM NAC in medium. 60 µl from each container are transferred to the 4 reaction tubes lined up directly in front of the container and mixed twice. This process is then repeated three further times, so that serial dilutions are created. The first column of SafeLock tubes is excepted.

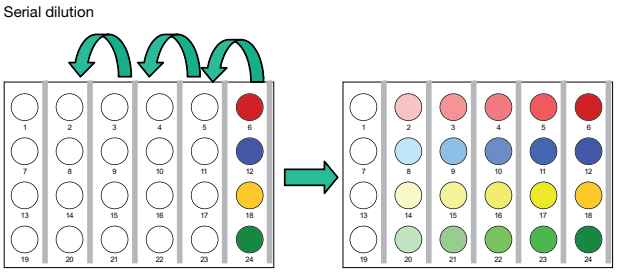

Figure 4: Schematic drawing of the serial dilution described

Subsequently, 80 µl of culture medium are removed from each well of the cell culture plate and discarded (TM 1000-8, pool in one destination, multiaspirate), followed by distribution of 80 µl of the prepared treatment media from the 1.5 ml SafeLock tubes into the appropriate wells of the cell culture plate (Figure 5) (TS 1000, reagent transfer, multidispense, 1x pre-wetting). Thus, a further dilution of 1:2 of the prepared substance concentration is achieved. The completed cell culture plate is incubated for 30 min in the incubator.

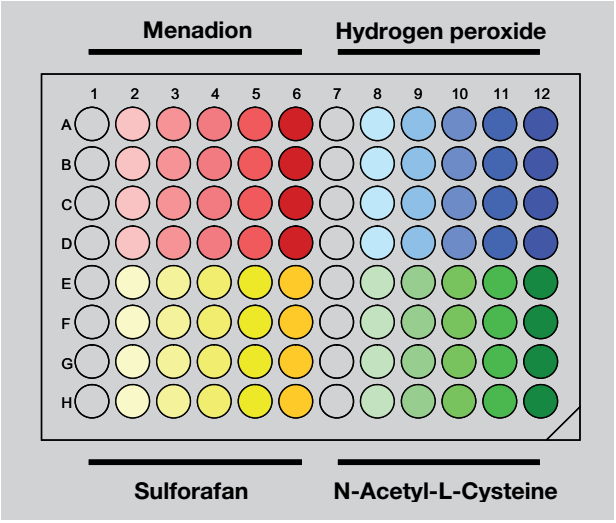

Figure 5: Pipetting diagram for the cell culture platefication.

Addition of dye using the epMotion

As a preparatory step, 17 ml of a 1:10 dilution of Alamar Blue-solution in cell culture medium is prepared manually in a 30 ml reservoir. The wells of the cell culture plate are emptied using a multichannel aspiration device with an aspiration pump (Geyer) and then filled directly with the dye solution. To this end, 80 µl are pipetted into each well (TM 1000-8, Reagent Transfer, multidispense, 1x pre-wetting). Following a change of pipette tips, this step is repeated.

Measurement of fluorescence

Fluorescence was measured at time 0 and again after 3 hours of incubation. A fluorescent plate reader Fluoroskan (excitation 530 nm, emission 590 nm) with Ascent software (Labsystem) was used. The calculated differences between both measurements were used for further analyses.

Comparative protocol (manual)

All work required for the comparative manual protocol was performed at a sterile work bench. The preparatory steps (preparation of cell suspension, stock solutions and dye solution) were performed as described for the ep*Motion* protocol. For cell seeding an automatic multichannel pipette (Eppendorf Research pro, 8-channel, 300 µl), equipped with the corresponding epTIPS, was used. For better comparison, ep*Motion* accessories, such as 30 ml reservoirs, were used. Distribution of dye solution was performed with the electronic repeater Multipette stream by Eppendorf.

Results

Measurement of fluorescence

For all substances used, dose-dependent reduction of cell viability was indicated by the measured fluorescence values.

Comparison of the semi-automatic method with a classic protocol

In order to compare results obtained with a semi-automatic process with those obtained using classic protocols, three independent experiments were performed under identical conditions.

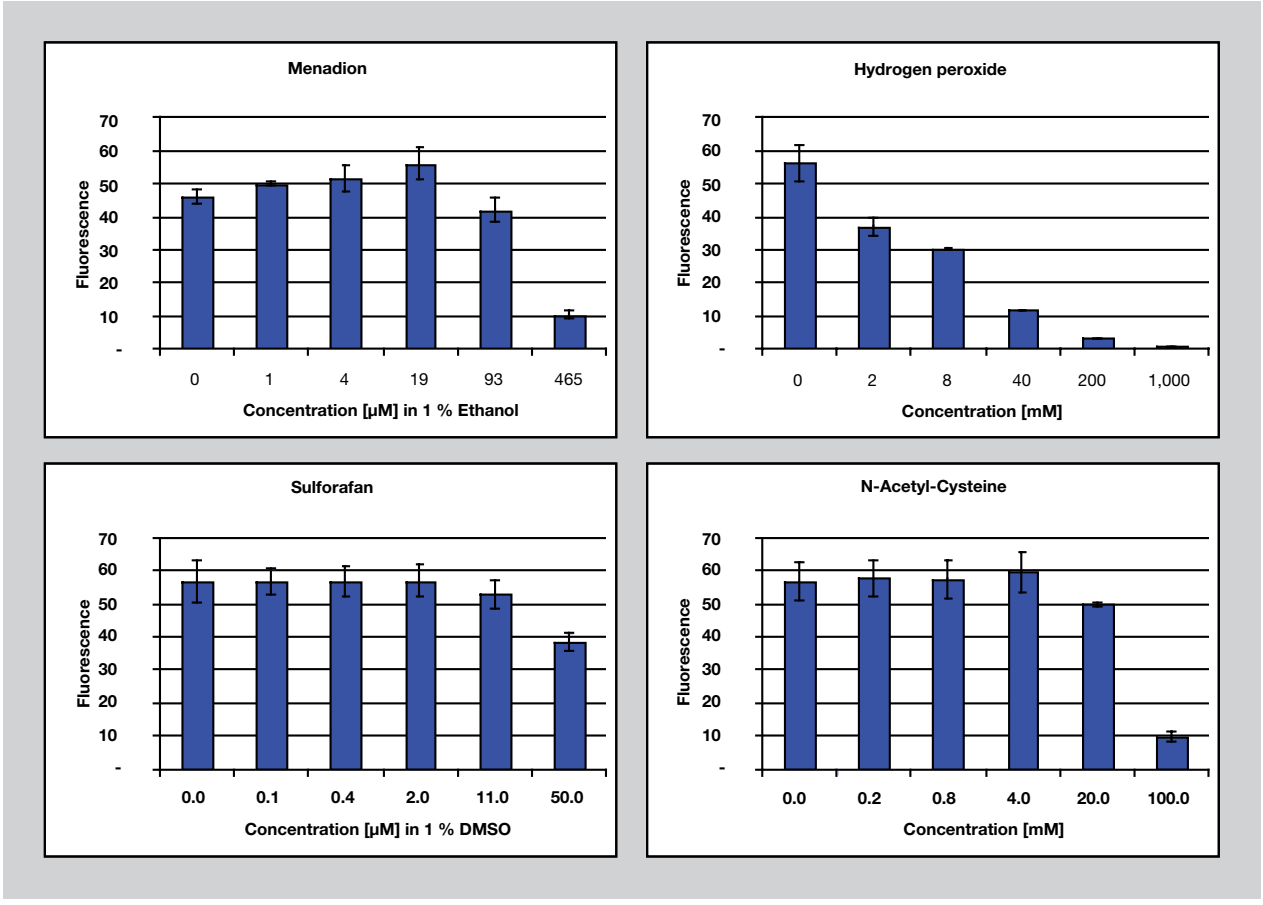

Figure 6: Measured fluorescence as a direct indicator for viablity of treated cells. The bars show averages and standard deviations of three independent cytotoxicity measurements.

Quality

Assessment of cytotoxicity was highly comparable across all four tested substances. The example of hydrogen peroxide, as shown in figure 7, demonstrates that relative cell viability values obtained with semi-automatic and manual procedures are similar.

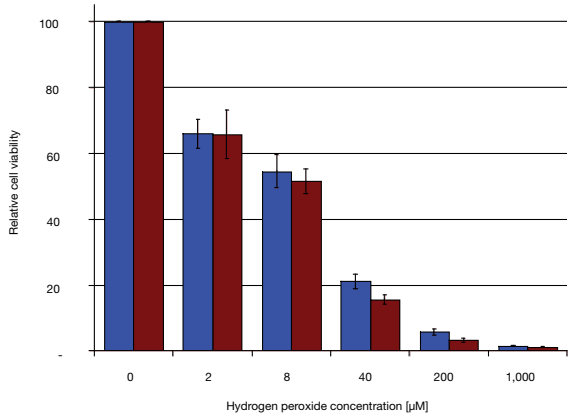

Figure 7: Comparison of viability as determined using semi-automatic ep*Motion* (blue) and manual procedures (red).

ep*Motion* performs pipetting steps with higher precision, as demonstrated by the differences in distribution of the four measured values, respectively. In order to clarify further, all measured groups, which displayed fluorescence values between 40 and 60 units (median range), were compared with one another. The difference between the highest and the lowest fluorescence value was determined. Whereas the manually generated values differed by approximately 8.5 fluorescence units, the values obtained with the ep*Motion* differed by an average of only 5.3 fluorescence units. This difference is highly significant (two-sided t-test, $p<0.001$).

Comparison of time requirements

An additional goal of automation, apart from better reproducibility of results, is time efficiency. Utilization of the protocols outlined above results in the following work times (Table 1):

Table 1: Comparison of time requirements for the different steps.

	ep*Motion*	manual
One time programming and trial runs for the ep*Motion*	approx. 8 h	n/a
Seeding of cells	< 10 min	< 10 min
Treatment of cells	1 h 15 min	1 h 30 min
Addition of dye	< 10 min	< 10 min

As illustrated in table 1, the times required for the performance of a cytotoxicity assay with either method are very similar. The use of the ep*Motion* is an especially reasonable choice when several substances are to be evaluated. In these cases time spent programming the instrument is easily saved at a later time. Simultaneous preparation of numerous cell culture plates in the ep*Motion* 5075 would render the program even more efficient compared to manual handling; dramatically improved time efficiency is to be expected.

Conclusions

Cytotoxicity assays are often employed in the course of cell culture protocols which include the addition of foreign substances. The semi-automatic protocol described in this report illustrates the capabilities of the ep*Motion* 5070/5075 with regards to cell seeding, treatment and dye-addition. Comparisons with a manual protocol validate the cytotoxicity results obtained with the ep*Motion*. Furthermore, use of the ep*Motion* resulted in a marked decrease in scattering of measured values. Taken together, all results indicate that semi-automatic performance of cytotoxicity assays containing large numbers of substances or combinations of substances will benefit from the ep*Motion* with regards to both precision and time efficiency.

References

[1] Operation manual for ep*Motion* 5070 CB; www.eppendorf.com
[2] Eppendorf Application Note 185; www.eppendorf.com

Eppendorf Ordering Information

Product	Order no. International	Order no. North America
ep*Motion*® 5070 CB	5070 000.700	960000021
ep*Motion*® 5075 LH	5075 000.008	960020006
Dispensing tool TM 1000-8	5280 000.258	960001061
Dispensing tool TS_1000	5280 000.053	960001036
epTips Motion 1000 µl Filter	0030 003.993	960050100
Reservoir rack	5075 754.002	960002148
30 ml Reservoir	0030 126.505	960051009
Reservoir rack module Ø 12 mm	5075 799.103	960002630
Thermorack for Safelock Tubes	5075 769.000	960002067
SafeLock Tubes 1.5 ml	0030 120.086	022363204
Height adapter 85mm	5075 751.003	960002105
Multipette® stream	4986 000.017	022460803
Research® pro	3114 000.140	022461419

Other Ordering Information

Product	Vendor	Order no.
NIH/3T3 cells	ATCC	CRL-1658
Cell culture medium 0.9 x DMEM	Biochrom	T043
Cell culture medium 0.1 x FBS	Biochrom	S0115
Penicillin/Streptomycin	Biochrom	A2213
Cell culture plate 96-well	TPP	92696
Round bottom tube 5 ml	TPP	95007
8-channel-pipetting unit	Th. Geyer	9.777 016
Hydrogen peroxide	Roth	8070.1
Ethanol, HPLC grade (99.8 %)	Roth	5054.1
Dimethylsulfoxide (DMSO)	Roth	4720.1
Sulforafan	Sigma	S4441
N-Acetyl-L-Cysteine (NAC)	Sigma	A8199
Menadion	Sigma	M5625
Alamar Blue™	AbD Serotec	BUF012B

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