SHORT PROTOCOL No. 03 I November 2014

Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega®) automated on the ep*Motion*® 5075t

Introduction

Cell-based assays (CBA), measuring complex cellular mechanisms, are widely used in research and pharmaceutical industry for drug identification or characterization. Apoptosis assay, as Cell Viability and Cytotoxicity assays, is a CBA presenting high relevance on the market. The Apo-ONE Homogeneous Caspase-3/7 assay from Promega is a fluorometric method measuring the activities of caspase-3 and caspase-7 playing a key effector role in apoptosis in mammalian cells. This assay can easily be configured for use on the ep*Motion* 5075t. Working with cells implies the necessity to work under clean conditions. The new version of the ep*Motion* 5075t including Hepa filters and UV lights (CleanCap option) allows this type of work. This method could be used for adherent or suspension culture cells. The use of the ep*Motion* 5075t allows handling of multiple 96-well plates in parallel.

This protocol describes the methods used to evaluate a dose response curve of staurosporine toxic compound on adherent Jurkat cells after 5-hour incubation. The workflow is divided into three different steps. On the first day, the cells are seeded in 96-well plates. On the second day, the toxic compound solution is prepared and added to the cells present in the 96-well plate then the CBA assay itself is performed. The cell seeding, the compound dilutions, the compound addition and the assays are performed on the ep*Motion* 5075t. These three steps correspond to three methods on the ep*Motion*. The run time for the entire procedure is 42 minutes for three 96-well plates processed in parallel.

In accordance to the experimental design of the user (Cell amount and type, compound concentration, incubation time, plate design, and plate number), the procedure needs to be adapted. An optimization of the liquid class parameters might be required if another compound is tested.

Material and Methods

Required equipment

> ep*Motion* 5075t (Eppendorf, order no.: 5075 000.302)
> CleanCap option (Eppendorf, order no.: 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)
> Reservoir rack for ep*Motion* (Eppendorf, order no.: 5075 754.002)
> Galaxy® 170R CO_2 incubator (Eppendorf, order no.: CO170R-230-0200)
> PlateReader AF2200 (Eppendorf, order no.: 6141 000.002)

Required 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
(Eppendorf, order no.: 0030 126.505)
> ep*Motion* Reservoir 100 mL
(Eppendorf, order no.: 0030 126.513)
> 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)
> 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)
> UMONIUM38® Medical Spray
(Laboratoire Huckert's International)
> Cell Imaging Plate (Eppendorf, order no.: 0030 741.013)
> Apo-ONE Homogeneous Caspase-3/7 Assay kit
(Promega, order no.: G7790, G7791, G7792)
> Staurosporine from *Streptomyces sp*.
(Sigma-Aldrich®, order no.: S4400)
> Dimethyl sulfoxide
(DMSO, Sigma-Aldrich, order no.: D8414)
> Human T Cell leukemia Jurkat cell line (DSMZ,
order no.: ACC 282) cultivated in RPMI 1640 medium
supplemented with 2 mM L-glutamine,
10 % FBS and 1 % Penicillin-Streptomycin
> RPMI 1640 Medium
(Life Technologies®, order no.: 21875-034)
> L-Glutamine 200 mM
(Life Technologies, order no.: 25030-024)
> FBS Superior, standardized Fetal Bovine Serum
(Biochrom AG, order no.: S 0615)
> 1 % Penicillin-Streptomycin
(Life Technologies, order no.: 15140-122)

Methods

Method Name	approx. Runtime (3x96-well plates)
1. Cell seeding.dws	5 min.
2. STS 100 curve.dws	28 min
3. Apo-ONE Caspase 3-7 assay.dws	9 min

This protocol is programmed to process three 96-well plates in parallel. The complete workflow is divided into three ep*Motion* methods. For the two first methods, the ep*Motion* surfaces and tools are cleaned using a disinfection solution (UMONIUM38; Laboratoire Huckert's International). The UV-lights and HEPA filters are started one hour before using the ep*Motion*. 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).
The methods are developed for the new ep*Motion* 5075t and cannot be transferred on the ep*Motion* 5075 TMX without new programming.

Method 1

Before starting the method 1, a solution of Jurkat cells cultivated in RPMI 1640 Medium supplemented with 10 % FBS, 1 % Penicillin-Streptomycin and 2 nM of L-glutamine at a concentration of 10,000 cells per 90 µL is prepared in a sterile tube and transferred in a sterile autoclaved ep*Motion* reservoir of 100 mL on the ep*Motion*. Culture medium (RPMI 1640 Medium supplemented with 2 mM L-glutamine, 10 % FBS and 1 % Penicillin-Streptomycin) without cells, used as blank, is transferred in a sterile ep*Motion* reservoir of 30 mL on the ep*Motion*. These steps need to be performed under sterile cell culture conditions.

The method 1 comprises the dispensing of 90 µL of cells per well in columns 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 plates and the plates are placed into the CO_2 incubator at 37 °C for 24 hours.

Worktable layout

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)

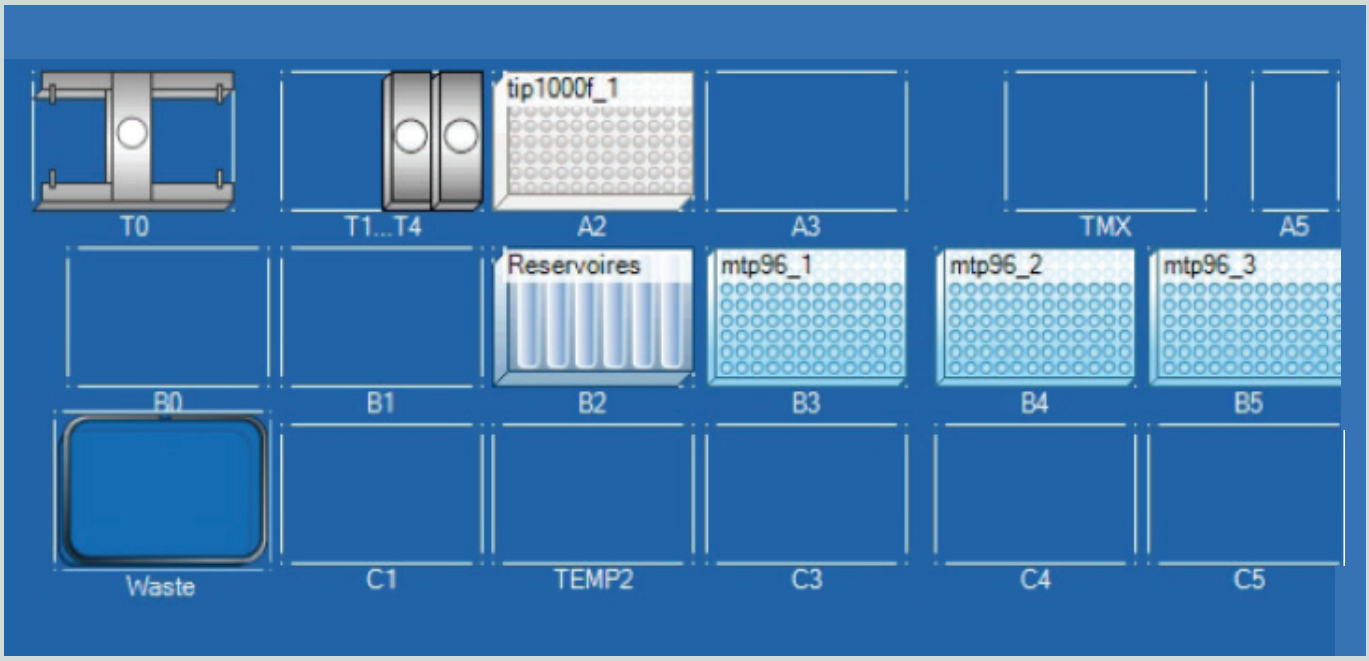

Figure 1: Worktable layout for method 1

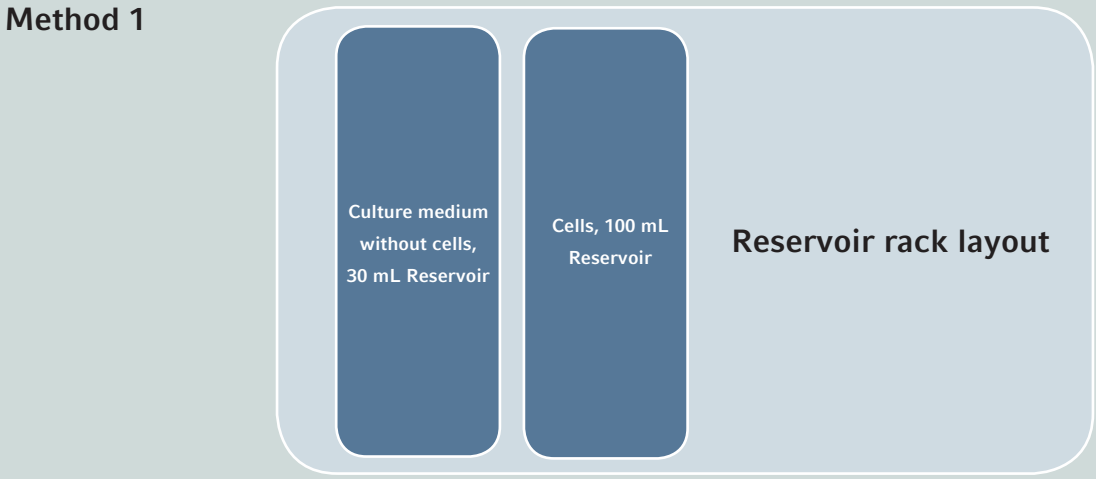

Figure 2: Reservoir rack layout for method 1

Method 2

The method 2 includes 2 parts separated by a "Stop – User intervention" command.
In order to avoid that the cells stay on the worktable at room temperature during the first part of the method, the 96-well plates seeded with cells are not positioned on the worktable for the first part of the method. Due to the plates' absence, during the check of the labware position, a message will appear on the computer screen informing that nothing could be found at the position of the 96-well plates (B3, B4 and B5). The procedure can be carried on by choosing the "ignore" selection. The "Stop command" at the end of the first part allows the user to place the 96-well plates on the worktable for the second part of this method.

The first part consists in the preparation of staurosporine (STS) concentration curve solutions (0 µM, 3 nM, 5 nM, 10 nM, 30 nM, 50 nM, 100 nM, 300 nM, 500 nM, 1 µM, 3 µM, 5 µM, 10 µM, 15 µM, 20 µM and 100 µM) in a Deepwell plate from a STS stock solution at 10.7 mM. In order to generate a concentration curve with the same amount of vehicle solution into each well, the dilution curve is performed using two different dilutions steps. The first dilution is performed into DMSO 99.9 % vehicle solution and the second dilution into culture medium without FBS 10x concentrate. Culture medium without STS and DMSO is used as negative control to measure the effect of DMSO on cells and is added into column 5 of 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 part includes the dispensing of the STS concentration curve solutions from the Deepwell plate to the 96-well plates. 10 µL is added per well. In a first time, 10 µL of culture medium without STS and DMSO was added in column 12 and 11 of the plates. Secondly, 10 µL of STS solution is added in the plates. All STS solutions are dispensed in one plate first and the operations are then repeated for the second and third plates. The final concentration of the vehicle solution is composed of 0.93 % DMSO. The plates are transferred on the ThermoMixer® and mixed at 500 rpm for 30 sec. At the end of the method, the lid is replaced on the plates manually and the plates are placed back in the CO_2 incubator at 37 °C for 5 hours.

These steps need to be performed under sterile cell culture conditions.

Worktable Layout

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

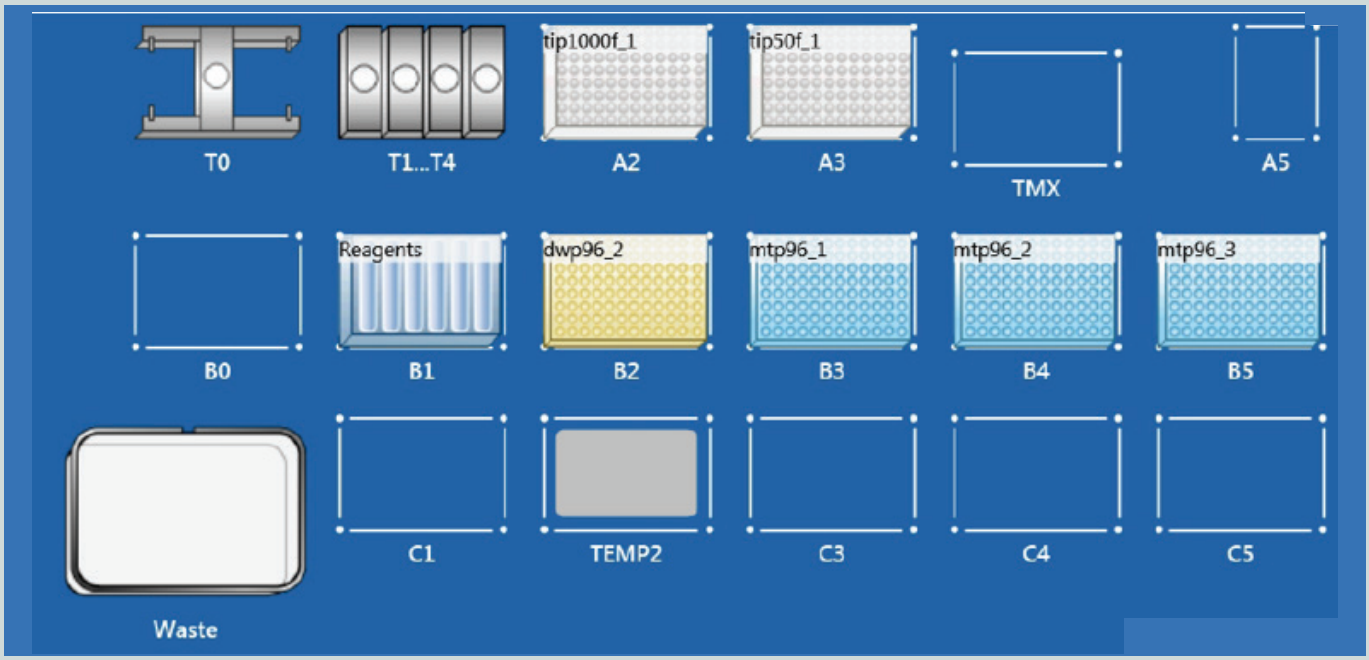

Figure 3: Worktable layout for method 2

Method 2

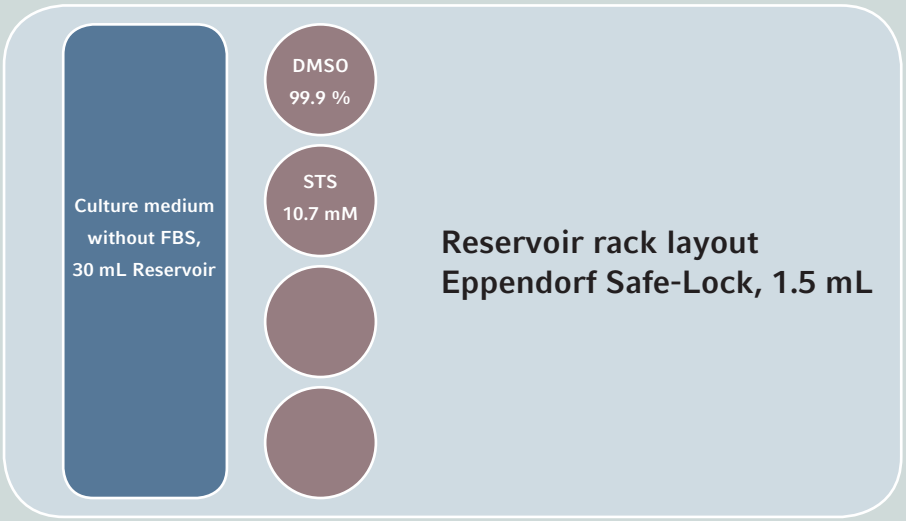

Figure 4: Reservoir rack layout for method 2

96-well Cell Imaging Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 µM	0 µM	0 µM	0 µM	0 µM	500 nM	500 nM	500 nM	500 nM	500 nM	Negative Control	Blank
B	3 nM	3 nM	3 nM	3 nM	3 nM	1 µM	1 µM	1 µM	1 µM	1 µM		
C	5 nM	5 nM	5 nM	5 nM	5 nM	3 µM	3 µM	3 µM	3 µM	3 µM		
D	10 nM	10 nM	10 nM	10 nM	10 nM	5 µM	5 µM	5 µM	5 µM	5 µM		
E	30 nM	30 nM	30 nM	30 nM	30 nM	10 µM	10 µM	10 µM	10 µM	10 µM		
F	50 nM	50 nM	50 nM	50 nM	50 nM	15 µM	15 µM	15 µM	15 µM	15 µM		
G	100 nM	100 nM	100 nM	100 nM	100 nM	20 µM	20 µM	20 µM	20 µM	20 µM		
H	300 nM	300 nM	300 nM	300 nM	300 nM	100 µM	100 µM	100 µM	100 µM	100 µM		

Method 3

After 5 hours of 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 firstly dispensed into the blank and negative controls. The plates are transferred on 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.

Worktable Layout

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)

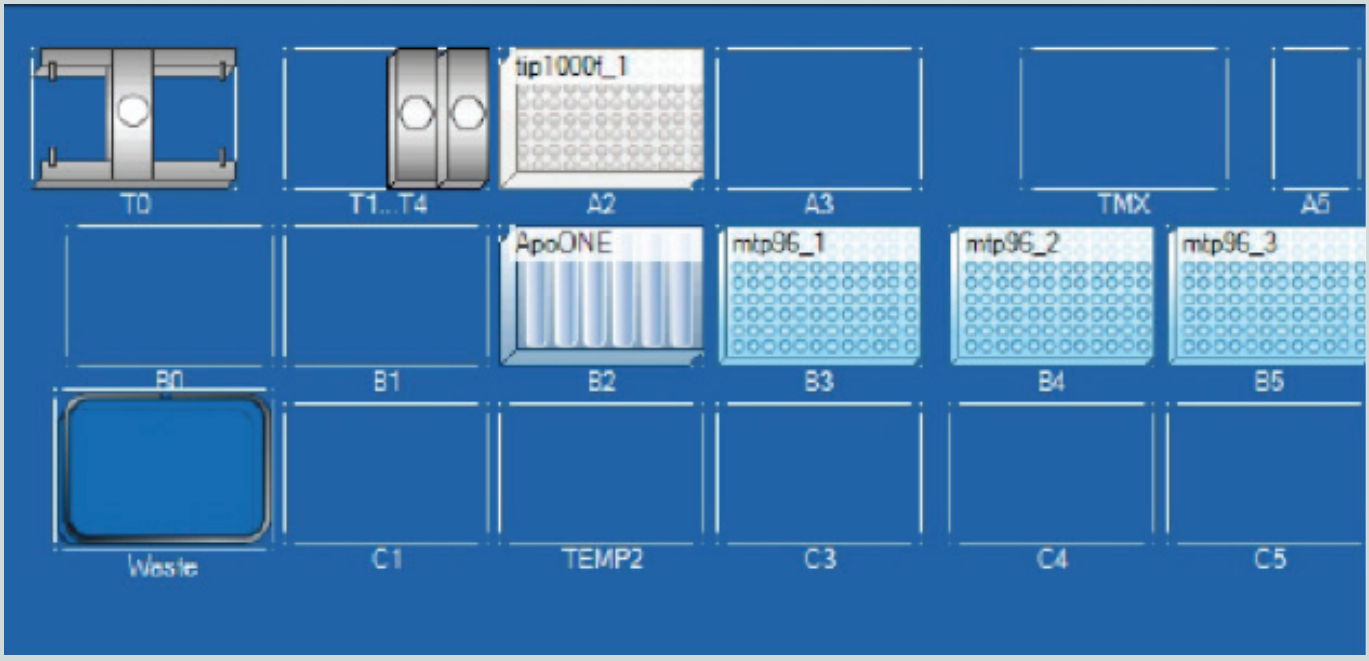

Figure 5: Worktable layout for method 3

Method 3

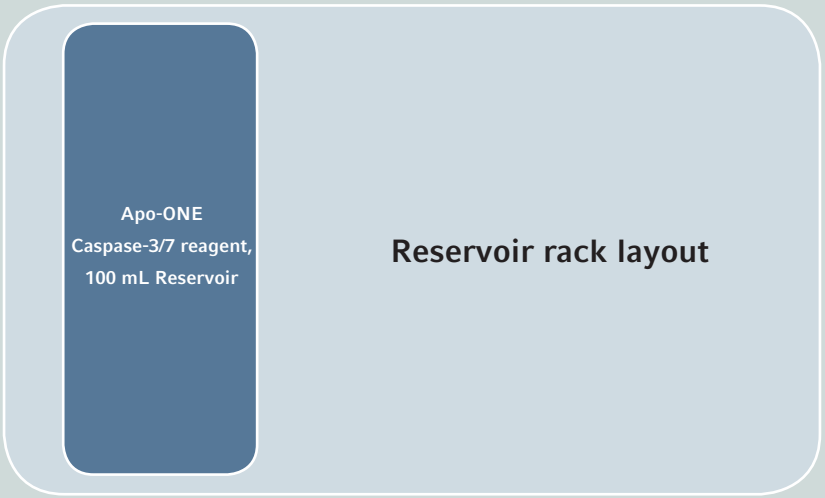

Figure 6: Reservoir rack layout for method 3

Results

At the end of the complete process, the plate reading of the 96-well plates is performed. After quick shake of the plates for 10 sec in the PlateReader AF2200 instrument, the fluorescence is measured in each well using an excitation wavelength of 485 nm and emission wavelength of 535 nm. The same gain was used to measure the fluorescence between plate replicates. The toxic effect of staurosporine on Jurkat cells using this short protocol is illustrated on figure 7. A fluorescence increase is observed due to the higher caspase-3/7 enzymatic activity present for high staurosporine concentration.

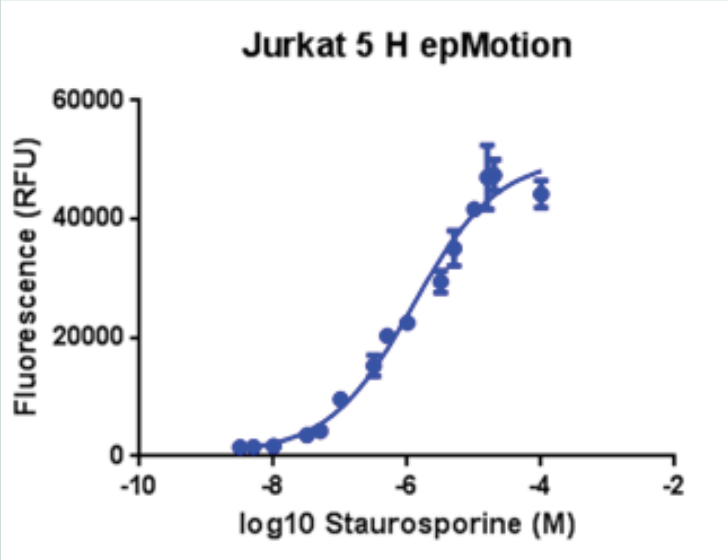

Figure 7: Caspase-3/7 measurement using Apo-ONE Homogeneous Caspase–3/7 Assay. Staurosporine dose curve response on Jurkat cells after 5-hour incubation was performed. Five replicates per concentration were performed and three plates were processed in parallel on the epMotion.

Ordering Information

Description	Order no. international
ep*Motion*® 5075t	5075 000.302
CleanCap option	5075 751.607
Gripper	5282 000.018
TS50 pipetting tool	5280 000.010
TM50-8 pipetting tool	5280 000.215
TM1000-8 pipetting tool	5280 000.258
Reservoir rack for ep*Motion*®	5075 754.002
Galaxy® 170R CO2 incubator	CO170R-230-0200
PlateReader AF2200	6141 000.002
epT.I.P.S.® Motion 50 µL Filter, sterile	0030 015.215
epT.I.P.S.® Motion 1000 µL Filter, sterile	0030 015.258
ep*Motion*® reservoir 30 mL	0030 126.505
ep*Motion*® reservoir 100 mL	0030 126.513
Reservoir rack module TC, Eppendorf Safe-Lock	5075 799.081
Safe-Lock Tubes, 1.5 mL	0030 120.086
Deepwell Plate 96/1000 µL	0030 502.205
Cell Imaging Plate	0030 741.013

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