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Apo-ONE® Homogeneous Caspase-3/7 Assay on the Eppendorf® PlateReader AF 2200

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Abstract

The Apo-One® Homogeneous Caspase-3/7 Assay by Promega was analyzed using the Eppendorf PlateReader AF2200. Reproducibility and limit of detection were studied at different cell concentrations under the influence of the apoptosis-triggering substance staurosporine. Fluorescence was measured at 485/535 nm using both »Top-Reading« and »Bottom-Reading«. Measurement from above showed considerably higher auto-fluorescence.

For analysis on the Eppendorf PlateReader AF2200 one of the methods pre-programmed on the instrument was employed. The data generated from the measurements were directly and automatically transferred to, and displayed as, an Excel® file. With this software feature, we could figure out that the need for the user to perform lengthy and elaborate calculations and analyses is eliminated.

Introduction

Cell-based assays enable direct analysis of the influence of certain factors on living cells. Typically, the influences of substances on eukaryotic cells are the subject of study. Such factors may include metabolites, novel active pharmaceutical ingredients, certain chemicals, food components, hygiene products or perfume products, but also radiation and mechanical impact which exert a metabolic effect on the cells. This effect, in turn, can be detected directly or indirectly using specific testing methods.

It is critical that these testing methods take advantage of reaction mechanisms of the cell's metabolism. For example, some apoptosis assays exploit certain enzyme cascades which are required for protein breakdown, such as the caspase apparatus. Once the enzyme cascade has been activated by apoptosis (programmed cell death)-triggering substances, this event may be measured by the addition of a specific substrate. The substrate is converted by the apoptosis enzymes, the process of which becomes evident through a color conversion within the reaction solution. The intensity of this color conversion may be used as a measure of the impact of the test substance on the metabolism, or viability, respectively, of the cell.

In addition to apoptosis assays which are based on programmed cell death, the impact of substances on cell viability, cell toxicity or proliferation (cell division) may be studied. Naturally, the choice of testing method will depend on the substance of interest.

Detection may be conducted via absorbance measurements (colorimetric), fluorescence or luminescence. The choice of detection method is dependent on the technical specifications of the plate reader.

Cell based assays are typically carried out in microtiter plates with 6 to 1536 wells, with the 96 and the 384 well formats used most frequently.

The following article is based on the application of an apoptosis assay by the company Promega® [1] on the Eppendorf PlateReader AF2200. The test is available on the instrument software as a pre-programmed method, based on the information provided by the kit manufacturer. The adherent cell line CHO-1 is used for the measurement.

CHO-1 (»Chinese Hamster Ovary«) is an immortalized cell line from ovaries of the Chinese hamster (*Cricetulus griseus*), which is used in the fields of cell biology and biotechnology for recombinant protein production [2].
For the purpose of this assay, apoptosis is triggered in the CHO cells using staurosporine, a kinase inhibitor initially isolated from *Streptomyces staurosporeus*. Staurosporine has anti-fungal properties. Several protein kinases are inhibited by binding of the substance to the ATP binding sites of the enzymes. Staurosporine is able to permeate the cell membrane, and it inhibits its target enzymes at very low concentrations [3].
In order to demonstrate the functionality of the assay in the Eppendorf PlateReader AF2200, defined cell numbers were treated with a concentration of staurosporine capable of triggering apoptosis.
For the purpose of the assay, the cells were first lysed. Lysis is achieved with the buffer provided, which also guarantees optimal caspase activity. The non-fluorescent substrate (Z-DEVD-R110) for the caspase is added separately. Caspase activities, and therefore apoptosis, are only present if the substrate is cleaved and the fluorescent dye rhodamine 110 is released.
Measurement of relative fluorescence (RFU) is conducted in the Eppendorf PlateReader at 535 nm following light excitation at 485 nm.

Materials and Methods

Materials:

- CHO-K1 cells (DSMZ ACC 110) in F-12 Nutrient Mixture (Ham) Medium (Life Technologies® 21765-029), supplemented with 10 % FBS superior (Biochrom S0615) and Pen/Strep (Life Technologies 15140122)
- Staurosporine (Sigma-Aldrich® S6942)
- Apo-ONE Homogeneous Caspase-3/7 Assay (Promega G7790)
- Cell Imaging Plate, 96-well, black/clear, film bottom (Eppendorf)
- Eppendorf PlateReader AF2200
- Fluorescence filter slide for PlateReader AF2200 (Eppendorf)
- Eppendorf New Brunswick™ CO_2 incubator Galaxy® 48R

Methods: Apo-ONE® Homogeneous Caspase-3/7 Assay:
CHO-K1 cells (90 µL/well) were seeded at different cell densities in a 96 well plate (please refer to plate diagram in figure 1). The suspensions containing different cell densities were prepared by serial dilution (1:2).

	1	2	3	4	5	6	7	8	9	10	11
A											
B		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
C		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
D		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
E		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
F		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
G		Without cells Only reagent (Blank)	Positive control (100 %)	20000	10000	5000	2500	1250	625	312	156
H											

Figure 1: Plate allocation for apoptosis Apo-ONE Assay (s. figure 2).

Column B2-G2 received only reagents and medium. This column is used for measuring the blank. No caspase activity should be detected in these wells. The wells B3-G3 received 40,000 cells. This column serves as the 100 % control (positive control: maximum induction of apoptosis) for the assay. The subsequent columns contain a 1:2 dilution of the previous column, respectively. Thus, cell dilutions down to 156 cells per well are prepared. It is to be shown, especially for the lower cell concentrations, whether small changes in caspase activity may be detected with this measurement system.
Since most plates show higher evaporation rates at the edges than in the center (edge effect), the outermost wells are not occupied. This effect causes the assay and media components to become increasingly concentrated which may have adverse effects on cell physiology, thus distorting measurement results.
The cells are to adhere to the plate for 18 h in the Galaxy 48R CO_2 incubator. Subsequently 10 µL staurosporine are added to all filled wells (3 µM/10 x concentrated). The cells are then incubated for 7 h in the Galaxy 48R CO_2 incubator. 100 µL of Apo-ONE assay reagent are added to each well and the plate is again incubated for 1 h in the CO_2 incubator. The filter pair 485 (EX)/535 (EM) was selected for measurement of fluorescence as an indicator of caspase activity. For the purpose of comparison, measurements are conducted once from above (top reading) and once from below (bottom reading).

Method programming on the PlateReader AF2200
The control software for the PlateReader AF2200 contains pre-programmed methods, including those for a cell viability assay and an apoptosis assay. Immediately following the start-up of the PlateReader software the pre-programmed measurement flow may be opened directly from the method explorer (figure 2).

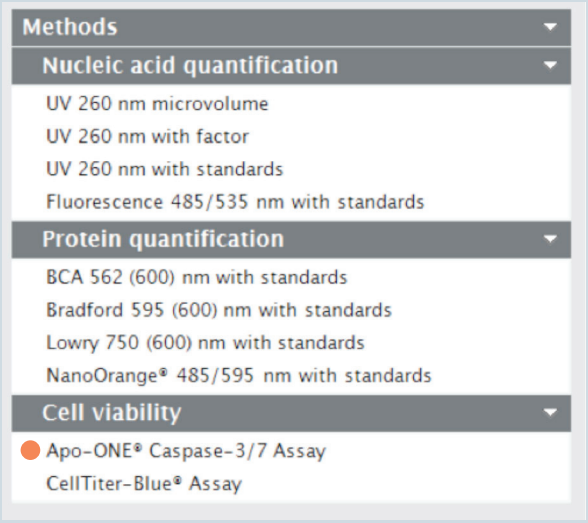

Figure 2: Double-clicking on the method Apo-ONE® Caspase-3/7 assay, or dragging the method into the work area, will open the method directly.

Figure 3 shows a complete overview of the method.

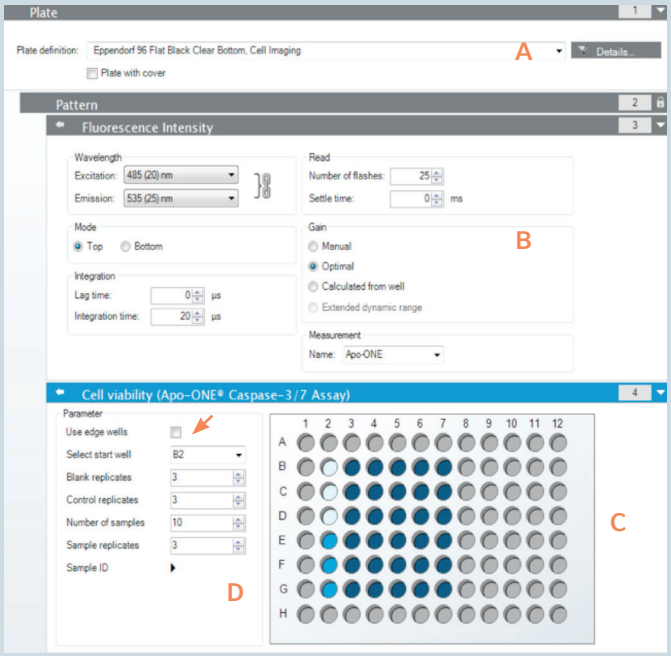

Figure 3: Complete overview of the Apo-ONE method. A) Plate definition, B) Measurement setting, C) Plate allocation, D) Definition of blanks, controls, samples and starting position. Red arrow: Utilization of outer wells may be selected if required. In addition, individual samples may be labeled, if required (sample ID).

The Eppendorf Cell Imaging Plate is automatically pre-programmed upon opening the method (figure 3). Also other plates may also be used from the plate database in the software. However, care should be taken to select a plate which is suitable for fluorescence measurements. Black plates made from polypropylene are an especially good choice, as this type prevents signal overlay (cross talk) between wells. If plates are to be analyzed using »bottom-reading« the plate bottom, like that of the pre-programmed Eppendorf Cell Imaging Plate, must be optically transparent to the wavelengths used. The latter measurement method is recommended for the CHO cells since these are adherent to the bottom of the well, as previously discussed. Thus, lower auto-fluorescence due to the cell culture medium is to be expected. The choice »bottom reading« may be selected under definition of measurement parameters.
Following plate selection, measurement parameters can be optimized. Pre-programmed methods already contain all selections for optimized analysis. In case minor adjustments are still required, e.g. measurement sensitivity, the »gain« (signal amplification in the photo multiplier) may be adjusted. Normally, this parameter is set to »optimum«. In this case, the measurement is guided by the highest signal obtained automatically during pre-measurement of the plate. Usually all wells are measured using the highest sensitivity during this process. If an even higher value is selected manually for »gain«, for instance in order to increase assay sensitivity, one may risk obtaining a signal outside the measurement range for wells with high fluorescence. Furthermore, increased gain simultaneously amplifies the background noise which may lead to misinterpretation of results. For these reasons it is recommended to keep the »gain« setting at »optimum« for standard assays.

Now the reading parameters for the measurement, e.g. well allocation, need to be defined. This is helpful in ensuring optimum use of the plate, and the plate may be used for several measurements. Use of the outer wells is optional if the edge effect is inconsequential for the assay. In order to avoid this effect, the cells may be incubated at room temperature prior to placing the plate into the CO_2 incubator, for example. This procedure should minimize the edge effect [4].
In addition to the starting point of the measurement, the number of wells required for blank and control need to be determined. The pre-programmed methods for analysis of cell-based assays define only one control. For the Apo-ONE assay a positive control with fully induced apoptosis serves as said control, which then allows calculation of percent caspase activity in the experimental samples.
The respective samples, or additional controls, may be defined prior to measurement using »sample ID«.

Results and discussion

As previously mentioned in the introduction, in order to demonstrate the functionality of the assay, caspase activity was measured for different cell numbers subjected to a defined amount of staurosporine. Specifically, caspase activity is based on measurement of the fluorescence emitted by the released rhodamine 110 (see above).
The results of the measurement are displayed automatically in an Excel® file. Raw data (separate sheet) and the measurement parameters are displayed, as well as the plate layout (figure 4).

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11
A											
B		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
C		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
D		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
E		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
F		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
G		Blank	100 % control	ID_1	ID_2	ID_3	ID_4	ID_5	ID_6	ID_7	ID_8
H											

Figure 4: Plate layout taken directly from the PlateReader AF2200 software, displayed within the Excel® data sheet.

The averaged results from all replicates of blank, control and sample measurements are displayed clearly in table format within the Excel® data sheet (table 1). In addition to the blanked fluorescence units the raw data for all measurements (including blank and standard deviation) are shown. Furthermore, the percent Gaussian error propagation for each series of measurements is listed. Caspase activity is shown as percent relative to positive control (fig. 5).

Table 1: Results for Apo-ONE assay in table format

Results

[RFU]	average raw data	SD raw data	SD [%] raw data	average blanked	SD blanked	Gauss [%]	% of control
blank	543	4	0.74	0	4	14.13	0.00
control	33000	4585	13.89	32457	4585	19.98	100.00
ID_1	11200	1100	9.82	10657	1100	14.17	32.83
ID_2	3450	650	18.84	2907	650	14.13	8.96
ID_3	1330	270	20.30	787	270	14.13	2.42
ID_4	810	60	7.41	267	60	14.13	0.82
ID_5	709	64	9.03	166	64	14.13	0.51
ID_6	603	16	2.65	60	16	14.13	0.18
ID_7	580	6	1.03	37	7	14.13	0.11
ID_8	560	5	0.89	17	6	14.13	0.05

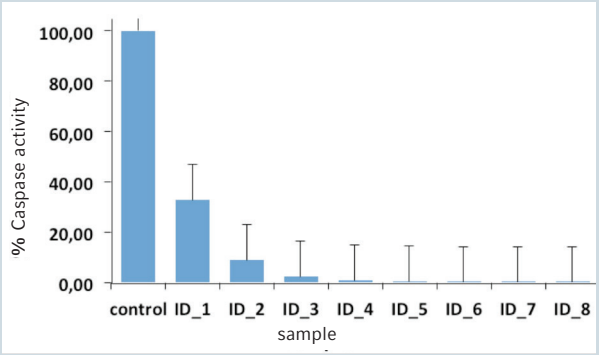

Figure 5: Caspase activity

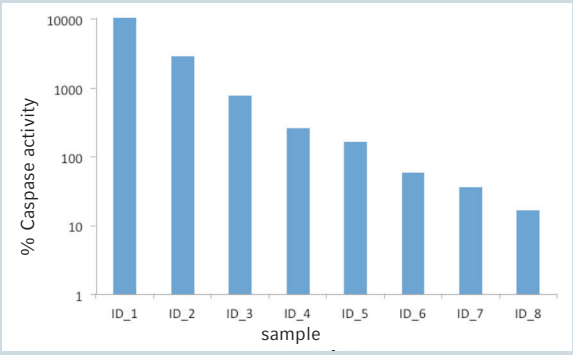

Figure 6: Logarithmic display of caspase activity.

In order to obtain an accurate overview of the activity in samples containing small cell numbers, the fluorescence values relative to control are also displayed in logarithmic format (figure 6).

The logarithmic display clearly shows that even in samples containing few cells, which as such generate low levels of fluorescence, activity can be detected.
Thus, the Apo-ONE assay was able to showcase its functionality on the PlateReader AF2200 across a broad measurement range. This sensitivity is critical for detection of even small effects of a test substance on cell metabolism which, in turn, could lead to further studies using other cell-based methods of analysis.

Conclusion

By virtue of the easily generated plate layout with clear positions for blank, control and samples, the plate allocation for the pre-programmed Apo-ONE assay by Promega is easily adapted on the Eppendorf PlateReader AF 2200. The data generated from the measurements are directly and automatically transferred to, and displayed as, an Excel® file. This eliminates the need for the user to perform lengthy and elaborate calculations and analyses. In cases where further analysis is required, all raw data are available to the user within the Excel® file. Judging from the caspase activities measured, the assay demonstrates very high sensitivity on the PlateReader AF2200 under conditions of low numbers of cells. This feature is especially valuable when detecting minute effects on cell metabolism.
The method described here is ideally suited to test the functionality of an assay using a specific substance potentially capable of triggering apoptosis. Additional concentrations of such substance may then be tested during further optimization of the assay. An additional negative control which includes cells but no caspase substrate and/or no caspase buffer may be included. In both cases, no caspase activity should be detected.

Literature

[1] Promega, Apo-ONE® Homogeneous Caspase-3/7 Assay, Technical Bulletin, 2009
[2] Karthik P. Jayapal, Katie F Wlaschin, Wei-Shou Hu and Miranda G. S. Yap, Wei-Shou Hu, Recombinant Protein Therapeutics from CHO Cells – 20 Years and Counting, Chem. Eng. Prog. 103(7):40–47, 2007
[3] Kocher M, Clementson KJ, Staurosporine both activates and inhibits serine/threonine kinases in human platelets, Biochem. J. 275, 301–306, 1991
[4] Lundholt BK, Scudder KM, Pagliaro L., A simple technique for reducing edge effect in cell-based assays. J Biomol Screen. Oct; 8(5):566–70, 2003

Ordering information

Product name	Order no. international	Order no. North America
Eppendorf PlateReader AF2200, 230 V/50–60 Hz	6141 000.002	–
Eppendorf PlateReader AF2200, 120 V/50–60 Hz	–	6141 000.010
Fluorescence filter slide for PlateReader AF2200, Pre-configured filter slide, optimized for the most common fluorescent dyes in the molecular and cell biology laboratory (360/465, 485/535, 485/595, 535/595)	6141 070.027	6141 070027
Eppendorf Cell Imaging Plate, 96 Wells; 20/individually wrapped; sterile	0030 741.013	0030741013
Galaxy® 48R, 230 V/50/60 Hz, Standard	CO48R-230-0000	–
Galaxy® 48R, 120 V/50/60 Hz, Standard	–	CO48R-120-0000

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