

Apo-ONE[®] Homogeneous Caspase-3/7 Assay: A Flexible Conduit Uniting the Drug Discovery and Development Processes

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Abstract

Modulation of the apoptotic cell death process continues to be the focus of intensive pharmaceutical research targeted at the elimination of human infirmities as diverse as reperfusion ischemia, neurodegeneration, and cancers. Exciting advances in automation and screening technologies have drastically improved assay throughput, but have rarely provided an integrated means to identify new chemical entity actives as well as characterize their biological attributes in a convenient format. Herein, we detail the implementation of our Apo-ONE[®] Homogeneous Caspase-3/7 Assay in apoptotic screen development with adherent and suspension cells, in a mock HTS screen for actives, in a secondary screen for candidate compound potency, and finally in an *in vitro* toxicology testing application. These successful demonstrations with the Biomek[®] 2000 automated module, emphasize the assay's broad flexibility and sensitivity in the drug discovery and development process.

Homogeneous Method Apo-ONE[™] Assay Protocol

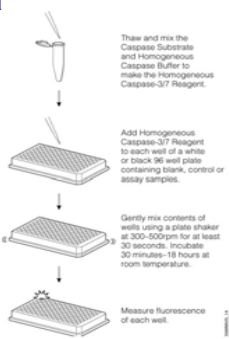
1. Induce cells, or add candidate compound directly to microtiter plate.
2. Add Apo-ONE[™] Homogeneous Caspase-3/7 Assay reagent, measure

Z-(DEVD)₂Rhodamine 110



Caspase 3
or 7

= Z-DEVD +
Z-DEVD +



Apo-ONE[®] Buffer Mediates Immediate Cellular Lysis/Permeabilization

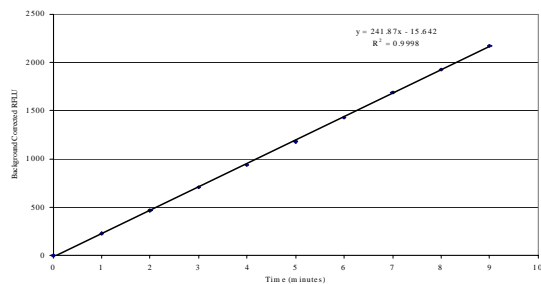


Figure 1. Resulting linear accumulation of fluorescent product generated by addition of Apo-ONE[™] Reagent to 5000 Jurkat cells (96 well format) induced with Anti-Fas mAb.

Apo-ONE[®] Buffer Supports Optimal Activity for Caspases 3 and 7

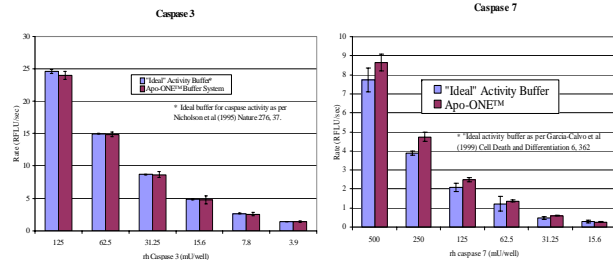


Figure 2. The Apo-ONE[®] buffer supports optimal caspase 3 and 7 activity when compared to dedicated activity buffers described in the literature. Purified rh active Caspase 3 or 7 was diluted in "optimal activity buffers" or Apo-ONE[®] buffer with substrate, and (Z-DEVD)₂R110 cleavage velocity monitored in kinetic fashion at Ex 485nm Em 520nm.

Rhodamine 110 Offers Considerable Fluorescent Advantage Over Coumarin-based Compounds

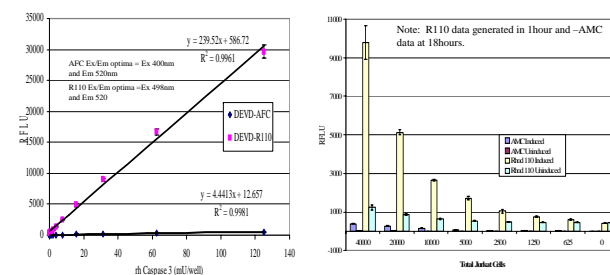


Figure 3. Z-DEVD-R110, Ac-DEVD-AFC, and Z-DEVD-AFC were compared as substrates for rh caspase 3 or induced caspase 3 and 7. Liberated fluor was measured at optimal Ex/Em wavelengths and compared. The intense rhodamine fluor product allows for quick (typically less than 1 hr of incubation) determinations.

Practical Sensitivity of the Apo-ONE[®] Assay is Bolstered by Fluorescent Properties of R110

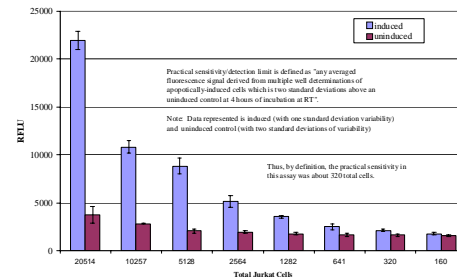
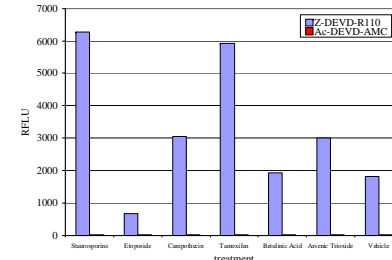


Figure 4. Jurkat cells were two-fold serially diluted, then induced with anti-Fas mAb for a period of 5 hours in 96 well plates. Apo-ONE[™] reagent was added and allowed to incubate for 4 hours. Parallel apoptotic induction verification was conducted with FItc-VAD-fmk and found to be ~50%, indicating an Apo-ONE[™] Sensitivity approaching 160 apoptotic cells.

An Example (Mock) Compound Screen Utilizing the Biomek[®] Automation Platform



Note: After 1 hour, statistical stratification of compounds was possible using the Apo-ONE[™] kit whereas no appreciable signal was generated using Ac-DEVD-AMC.

Figure 5. HeLa cells were seeded at 10,000 cells/well in 96 plates. Compounds known in the literature to induce apoptosis were added to 1uM final concentration and allowed to incubate for 5 hours. Apo-ONE[™] Assay reagent with either Z-DEVD-R110 or -AMC was delivered in 100ul volumes via the Biomek[®] 2000 and allowed to incubate for 1 hour before fluorescence measurement.

Format Flexibility: 384 Well Configuration with Adherent Cells

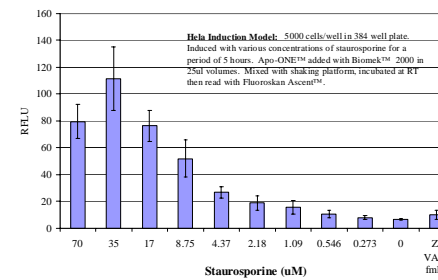


Figure 6. 5000 HeLa cells were seeded into each well of a 384 well plate. Staurosporine was serially diluted and added to the wells and allowed to induce apoptosis for 5 hours. 25ul volumes of Apo-ONE[®] Assay reagent were delivered with the Biomek[®] 2000 automation platform, mixed with orbital shaking platform briefly, and fluorescence measured at 1 hour.

Apo-ONE[™] Caspase Activity Can Be Correlated With Cell Number by Hoechst 33258 Multiplexing

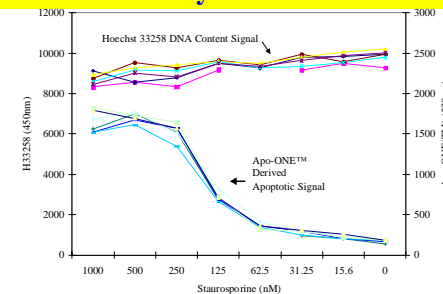


Figure 7. Jurkat cells were seeded into 96 well plates at 10,000 cells/well and induced with varying concentrations of staurosporine. Hoechst 33258 was added, allowed to incubate briefly and then bound dye measured with a fluorometer (Ex 350 Em 450). The cells were incubated an additional 4 hours and Apo-ONE[®] reagent added. After 1 hour, apoptotic caspase 3/7 activity was determined for each dilution.

Apo-ONE[®] Activity Can Be Correlated With alamarBlue[™] Cytotoxicity by Multiplexing

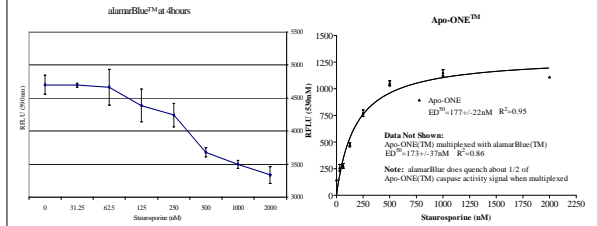


Figure 8. Jurkat cells were seeded into 96 well plates at a density of 10,000 cells/well. Serial dosages of staurosporine were added as described previously. alamarBlue[™] was added to each well in 10% volumes as described by the manufacturer. After 5 hours of induction, the plate was read at 590nm. Apo-ONE[®] was added directly to the plate in equal volumes, in addition to a control plate and read at 10hr post-incubation.

High Z-Factors* With Apo-ONE[®] Assay Translate into Easier HTS Implementation

Cell Number	Mean	Std Dev.	*Z-Factor
20,000	16185	186.6	0.96
10,000	8215	509	0.79
5,000	4857	96	0.92
2,500	2789	95	0.85
1,250	1664	62	0.76
625	1255	55.8	0.57
312	1015	20.5	0.59
0	829	4.4	N/A

Table 1. Various concentrations of Jurkat cells were exposed to 200nM staurosporine for a period of 5 hours in a 96 well plate (100ul volume). Apo-ONE[®] assay reagent was added and allowed to incubate an additional hour prior to measurement. Note: Any Z-factor greater than 0.5 is considered an excellent assay. *Zhang, et al. (1999) J. Biomolecular Screening 4:67-73.

Summary Apo-ONE[®] Assay Attributes

- 1) Simple and fast (one reagent addition directly into cultured cells and medium)
- 2) Sensitive (apoptosis detection in as few as hundreds of cells)
- 3) Versatile (adherent, suspension, primary cell culture, and purified enzyme preps)
- 4) Scalable for HTS (96, 384, etc)

Questions? Comments?

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