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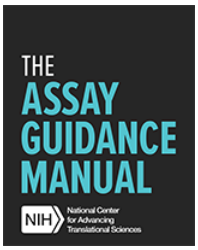

Apoptosis Marker Assays for HTS

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Abstract

A variety of assays are available to detect apoptosis; however, few are conveniently adapted for high-throughput screening (HTS) using a multimode plate reader. The markers most commonly used for *in vitro* detection of apoptosis include caspase-3/7 activity and phosphatidylserine (PS) exposure on the outer leaflet of the cell membrane. Caspase-3/7 activity is routinely measured using consensus tetrapeptide substrates in lytic cell-based assays to generate a fluorescent or luminescent signal measured with a plate reader. PS exposure historically has been measured using flow cytometry to detect binding of fluorescently-tagged annexin V; however, sample throughput and washing steps to remove unbound fluorescent probe have limited broad adoption of the flow cytometric approach for HTS. Recently, recombinant annexin V fusion proteins engineered to contain subunits of a shrimp-derived luciferase have been used to develop a no-wash enzyme complementation approach for detecting PS exposure using a multimode plate reader. This homogeneous annexin V-binding assay approach has broadened the availability of assays compatible with ultraHTS.

Abbreviations

AFC	aminofluorocoumarin
AMC	aminomethylcoumarin
BiT	binary technology
caspase	cysteine-aspartic protease
DEVD	Asp-Glu-Val-Asp
dUTP	2′-Deoxyuridine, 5′-Triphosphate
ELISA	enzyme-linked immunosorbent assay

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Table continued from previous page.

FITC	fluorescein isothiocyanate
FMK	fluoromethylketone
HTS	high throughput screening
LgBiT	Large BiT
LOPAC	library of pharmaceutically active compounds
PARP	poly ADP ribose polymerase
pNA	*p*-nitroaniline
PS	phosphatidylserine
R110	rhodamine 110
RFU	relative fluorescence units
RLU	relative luminescence units
SmBiT	Small BiT
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

This chapter is not meant to be a thorough review of cell death or apoptosis detection assays, but will describe the basic methods for the measurement of caspase activity and phosphatidylserine (PS) exposure that can be used in a high-throughput screening (HTS) format to detect markers consistent with apoptosis (1).

Apoptosis is a form of programmed cell death that is critical for carrying out normal developmental and physiological processes. Deregulated apoptosis is associated with many disease states. Too much cell death occurs in many degenerative diseases and too little cell death occurs in some kinds of cancer when the apoptotic process has become inhibited or non-functional leading to continued net proliferation of tumor cells. Much has been learned about the complex signaling events in the extrinsic and intrinsic pathways that modulate apoptosis. There is much interest in attempting to further understand the process of apoptosis by investigating individual members of the signaling pathways as potential targets for therapeutic development. Phenotypic screening campaigns to identify compounds that induce apoptosis often precede efforts to identify the individual target interactions. Despite the interest in individual target interaction, there remains a need to efficiently screen large chemical libraries by measuring markers to detect apoptosis.

Historically, apoptosis was detected based on changes in cell morphology. Some of those hallmark changes include: membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation. The process of apoptosis also results in discrete patterns of DNA fragmentation. Assays developed for detection of DNA fragmentation as a marker of late stage apoptosis included the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and ELISA methods. The TUNEL assay is a method for detecting DNA fragmentation by labeling the 3′- hydroxyl termini in the double-strand DNA breaks generated during apoptosis and the ELISA approach relies on antibody detection of DNA fragments. However, both of those approaches are not amenable to HTS because they require multi-step procedures including wash steps.

Caspase Activity Detection

Most apoptosis involves the activation of a cascade of cysteine-aspartic proteases (caspases) that mediate upstream signaling events ultimately leading to the activation of executioner caspases involved with destroying the cell.

Executioner caspases are involved with the terminal proteolytic events of the apoptotic process. Those events include breaking down the cytoskeleton that leads to morphological changes observed during apoptosis and inactivating repair enzymes such as poly ADP ribose polymerase (PARP) which was among the first substrates recognized to be cleaved by "caspase" (2-4).

With few exceptions, if a cell has active executioner caspase activity, it is beyond the point of no return in the apoptotic process. Thus, measuring executioner caspase-3/7 activity has become the most popular assay performed in an HTS format using a standard multimode plate-reader to detect the process of apoptosis.

Early in apoptosis research, the protease now called caspase-3 was shown to cut PARP at the C-terminal end of a DEVD amino acid sequence generating ~p89 and p24 fragments and resulting in inactivation of the DNA damage repair properties of PARP. This caspase activity is sometimes referred to as DEVDase activity.

Research into the cleavage site specificities of the various members of the caspase family of proteases lead to identification of four amino acid recognition sequences useful as selective substrates (5). Those four amino acid long substrates are typically *N*-terminally blocked with a protecting group to prevent aminopeptidase activity from liberating the reporting group, thus increasing substrate selectivity. The most commonly used artificial substrate for detecting caspase-3 or 7 activity include DEVD linked via a peptide bond to either a chromophore (*p*-nitroaniline, pNA), a fluorophore (aminomethylcoumarin (AMC), aminofluorocoumarin (AFC), or rhodamine 110; R110) or aminoluciferin as a reporter (6). The inherent reagent background signal and the reporting molecule dynamic response influence detection sensitivity and usefulness for HTS. The choice of the fluorogenic reporting molecule (e.g. AMC, AFC, R110, or a far red emitting dye) can affect the overall performance of the protease assay and can also serve to enable opportunities for multiplexing with other fluorescent assays. For example, the coumarin class of fluorophores are excited by light in the UV region (e.g. AMC = 340nmEx / 440nmEm) which is more likely to result in fluorescent interference from compounds in small molecule libraries. The R110 fluorophore contains 2 amine groups, both of which form peptide bonds with the peptide substrate forming $(Z\text{-DEVD})_2$-R110. Although it is generally not an issue for detecting the presence or absence of caspase-3/7 activity, the cleavage of two peptides from a single R110 fluorophore may raise questions during interpretation of results. For additional details regarding fluorescence interference, please refer to the Assay Guidance Manual chapter Interference with Fluorescence and Absorbance by Simeonov and Davis (7). Interference with luminescent assays also needs to be considered. For example, small molecule libraries contain known colored compounds that can result in signal quenching and luciferase inhibitors that may interfere with the detection assay chemistry. For a detailed description of luciferase inhibitors, see the Assay Guidance Manual chapter Interferences with Luciferase Reporter Enzymes by Auld and Inglese (8).

The luminogenic protease substrate is about 20-50-fold more sensitive than the fluorogenic versions (and far more sensitive than the colorimetric pNA format) which has enabled miniaturization of caspase-3/7 assays to high density plate formats used for HTS. Figure 1 was copied from the Technical Manual of the Caspase-Glo-3/7 Assay and shows the general scheme for the luminogenic assay. Caspase-3/7 activity cleaves at the *C*-terminal side of aspartic acid to release aminoluciferin that is subsequently used as a substrate for firefly luciferase to generate photons. The light is quantified as relative luminescence units (RLU) using a standard plate reading luminometer.

The caspase assay can be applied to cells grown as a monolayer, in suspension, or as a 3D culture model system. The luminescent assay detection sensitivity is adequate for using small numbers of cells in 96-, 384- or 1536-well plate formats. Figure 2 shows Panel B of Figure 6 from O'Brien et al., 2005 (6) illustrating the detection sensitivity of the luminescent assay is about 20-fold better than a fluorescent approach for Jurkat cells. The general assay performance and relative detection sensitivity for each cell line and well format should be empirically determined and validated in the user's lab.

Several results of "Cellular Toxicity (caspase-3)" from National Center for Advancing Translational Sciences (NCATS) labs validating the luminescent caspase-3/7 assay approach have been posted to the PubChem database. The uHTS approach in the 1536 well format using several different cell lines (HepG2, Jurkat, HUV-EC-C, SHSY5Y, BJ, MRC-5, Mesangial, SK-N-SH, FSKstim, H-4-II-E, HEK293, N2a,NIH 3T3, Renal Proximal Tubule) has been reported as AID654-AID667 (e.g. for HepG2 https://pubchem.ncbi.nlm.nih.gov/bioassay/654). The PubChem database also contains results from a cell-based screening bioassay of 325,733 compounds demonstrating its utility for implementing an automated HTS campaign (Luminescence Cell-Based Primary HTS to Identify Inhibitors of A1 Apoptosis: AID2462, https://pubchem.ncbi.nlm.nih.gov/bioassay/2462). A further example from screening 9667 compounds as part of the U.S. Tox21 Program is PubChem AID1347037 (Caspase-3/7 induction in CHO-K1 cells by small molecules, qHTS assay; https://pubchem.ncbi.nlm.nih.gov/bioassay/1347037).

The luminescent assay detection chemistry is not affected by the presence of routine concentrations of DMSO used as a vehicle to deliver test compounds. Figure 3 (reproduction of Figure 9 from O'Brien et al., 2005) shows results comparing the effects of 0.1%, 1% or 10% DMSO added to assays containing two concentrations of caspase-3. Although there was a slight increase in the background (no caspase, white bars) samples containing 10% DMSO, routine concentrations of DMSO did not have a substantial effect on the results.

Protocol to Detect Caspase-3/7 Activity as a Marker of Apoptosis

A detailed protocol is included in Promega Technical Bulletin #323 (https://www.promega.com/Resources/Protocols/Technical%20Bulletins/101/Caspase%20Glo%2037%20Assay%20Protocol/?fq=caspase-glo%203%207).

1. Choose opaque walled white plates for best results when using luminescent assays. Clear bottom plates are optional and recommended for viewing cells with a microscope during the experiment.
2. Reproducible results among different days, different end users, etc. require a standard operating procedure to produce a consistent source of cells for each experiment (9).
3. Prepare assay plates containing replicates of: blanks (complete medium without cells); negative control (cells treated with vehicle only); and positive controls (wells containing cells treated with a compound known to cause apoptosis in the cell model being used).
4. Dispense a uniform number of cells in each well (except for blanks) and allow the plates to equilibrate prior to addition of test compounds, positive control samples and vehicle controls.

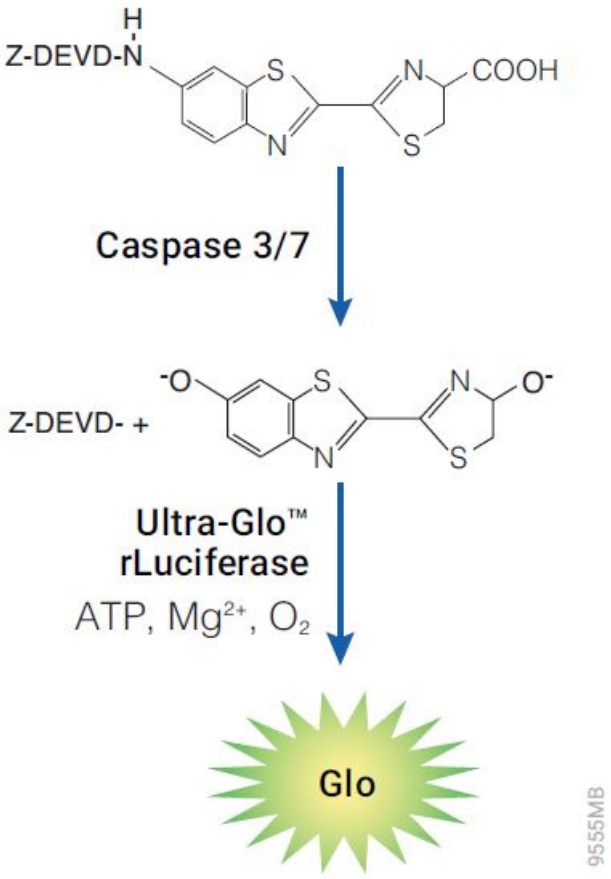

Figure 1. Schematic diagram of the Caspase-Glo 3/7 Assay technology. Following caspase cleavage of the DEVD-proluciferin substrate, a substrate for luciferase (aminoluciferin) is released and in the presence of luciferase, ATP, O_2 and Mg^{2+}, results in the luciferase reaction and the production of light.

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5. Add test compounds and positive control samples dissolved in the same solvent and volume as the vehicle control. **Note:** Some potentially useful positive control compounds, their mode of action and a recommended concentration are included in Table 1.
6. Incubate assay plate using empirically determined conditions for desired time. (**Note**: The process of apoptosis is a series of transient kinetic events. Detection of apoptosis markers can occur in less than 1 hour or more than a day depending on the model system and culture conditions. The optimum incubation time should be determined empirically for each model system. In addition, heterogeneity among individuals in the population of cells can be expected. When using an endpoint assay, choosing a consistent time to record data can be critical to achieve reproducible results.)
7. Prepare the assay reagent by addition of the Buffer to the lyophilized substrate and allow the reagent to equilibrate to room temperature prior to use. Follow detailed instructions provided in the vendor Technical Bulletin. The reconstituted caspase-3/7 reagent requires storage at 4°C to maintain activity. The reagent can be stored at 4°C for up to 3 days with no loss of activity.
8. Remove assay plates containing cells from the incubator and allow plates to equilibrate to room temperature to ensure a consistent temperature among all assay wells. (**Note**: Most luminescent assays are designed to rely on the rate of the luciferase enzyme to produce photons of light and that enzymatic rate is dependent on temperature.)
9. Add a volume of assay reagent equal to the volume of medium in each assay well.

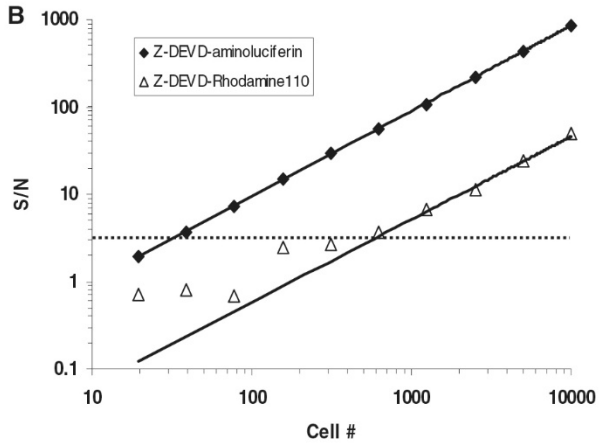

Figure 2. A comparison between bioluminescent and fluorescent caspase assays. The bioluminescent and fluorescent assays were run simultaneously using the same anti-Fas treated Jurkat cells serially diluted in 96-well plates. After addition of the appropriate substrate reagents, readings were taken at 1 h. Results were plotted as S/N. Background RLUs or RFLUs were determined from wells containing culture medium without cells. The limit of detection is defined as the number of cells giving an S/N ratio ≥3 (dotted line). For the fluorescent results, only the points above the limit of detection were used to determine the linear trend because values below the limit of detection deviate significantly from the linear trend.

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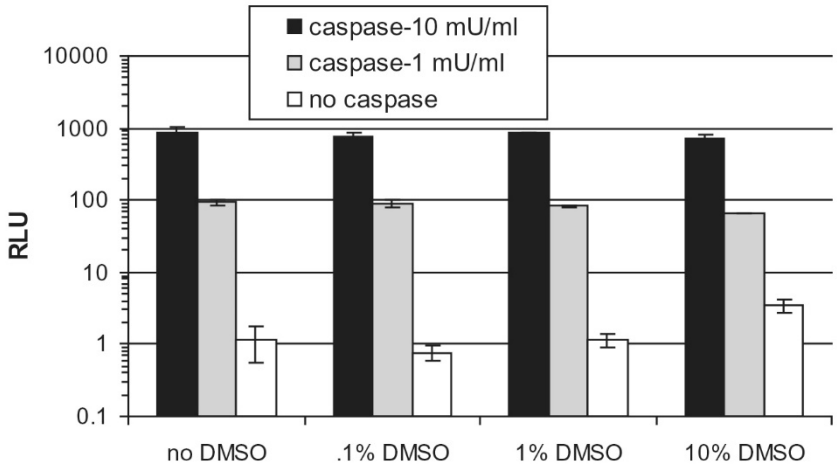

Figure 3. DMSO tolerance of the luminescent caspase assay. DMSO was tested at 0.1%, 1%, and 10% with 2 concentrations of caspase-3 (10mU/mL and 1 mU/mL) for its effects on the luminescent signal. 10 mU of caspase-3 = 1 ng.

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10. Mix the contents of all wells using a plate shaker at 300–500 rpm for 30 seconds. (**Note**: An appropriate plate shaker designed to mix the contents of the wells is necessary. Plate shakers may not be effective at mixing the contents of high density 1536-well plates.)
11. Incubate the plate at room temperature protected from direct light for 30 minutes to 3 hours. (**Note**: The optimal incubation period should be determined empirically and be consistent each time the assay is performed. If the room temperature fluctuates, use a constant-temperature incubator.) (**Note:** Cell culture models of 3D spheroids may require longer incubation in the presence of the detergent-containing assay reagent to completely lyse all the cells.)

12. Measure the luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer. It is recommended to follow the basic guidelines for reporting nonclinical data as described in Dahlin et al., 2019 (10).

The luminescent caspase-3/7 assay also has been demonstrated to be suitable for use with 3D culture models. Figure 4 shows data from measurement of caspase-3/7 activity from HCT116 cell 3D microtissues formed using the hanging drop method, then treated with Panobinostat for various times up to 3 days. Note that the caspase-3/7 activity after 72 hours incubation is lower than the values at 48 hours. That is consistent with cells undergoing apoptosis at an earlier time followed by subsequent loss of caspase-3/7 activity when cells undergo secondary necrosis and release cytoplasmic contents into the culture medium. These time course data highlight the importance of understanding the kinetics of the onset of apoptosis and empirically determining the length of compound treatment prior to using lytic endpoint assays. The use of a kinetic real-time assay approach (described later in this chapter) is helpful for efficiently establishing optimal test compound exposure times prior to implementing a lytic endpoint assay.

Figure 5 shows example results from screening 640 compounds from the LOPAC library to detect inhibitors of the luminogenic caspase-3/7 assay in the presence of 1mU recombinant caspase-3. The data demonstrate adequate performance of the luminescent assay approach for HTS. The Z' factors for controls are: no caspase (Z' = 0.72), 1mU caspase + Z-VAD-FMK inhibitor (Z' = 0.54) and 1mU caspase + Ac-DEVD-CHO inhibitor (Z' = 0.52). The dotted line represents two standard deviations below the mean of all the LOPAC compounds tested. The open triangles that have been circled represent 6 hits below the dotted line in this scenario.

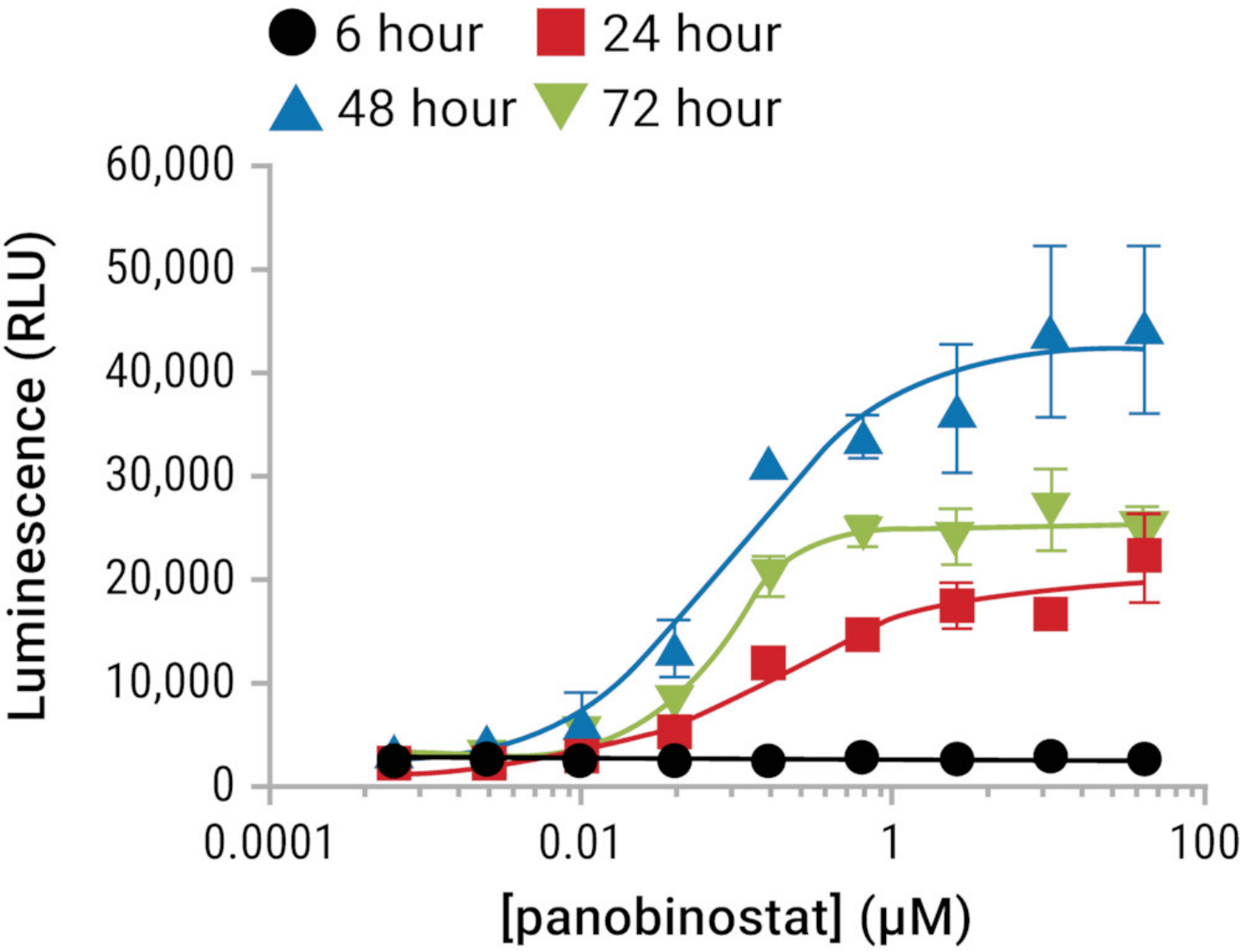

Figure 4. Caspase-3/7 activity resulting from treatment of 3D microtissues with panobinostat in a time course experiment. HCT116 cell microtissues were created using the InSphero GravityPLUS Hanging Drop System, transferred to Gravity Trap plates, then treated with panobinostat for up to 72 hours. At indicated time points, samples were transferred to solid white Corning 96 well plates (Cat. No. 3917), Caspase-Glo 3/7 Reagent was added to the wells and shaken for 30 minutes prior to recording luminescence. The values represent the mean ± SD from 3 technical replicates.

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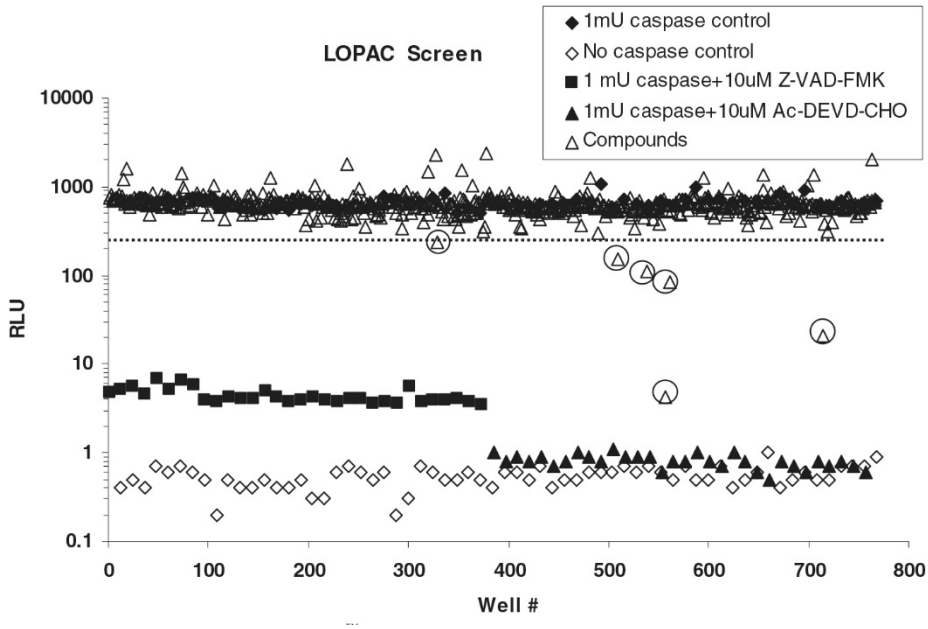

Figure 5. Screen for caspase-3/7 inhibitors using the LOPAC collection. The luminescent caspase assay was tested for compound interference using 640 compounds. The compounds were tested at a final concentration of 10 µM in 1% DMSO. The controls for the screen were caspase (1 mU/well or 1 ng/mL), no caspase, and caspase +Z-VAD-FMK (10 µM) or caspase +Ac-DEVD-CHO (10 µM). Hits were defined as compounds that gave RLU values 2 standard deviations or more below the mean of all samples (dotted line). Circles denote the 6 hits detected in this screen.

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Table 1. Example compounds frequently used as positive controls to induce apoptosis

Positive Control Compound	Mode of Action	Recommended Concentration
Panobinostat	HDAC inhibitor	10µM
Staurosporine	Kinase inhibitor	20µM
Bortezomib	Proteasome inhibitor	10µM
Doxorubicin	DNA intercalator/damager	10µM
Camptothecin	Topoisomerase inhibitor	10µM
Paclitaxel	Microtubule poison	10µM

Detection of PS Exposure as a Marker of Apoptosis

One of the signaling events facilitated by caspases is the activation of lipid scramblases that result in PS exposure on the outer leaflet of the cell membrane. That event is involved with producing the "eat me signal" and recognition of apoptotic cells by phagocytes, ultimately resulting in engulfment and destruction of the apoptotic cells *in vivo*. In contrast, all cells that undergo apoptosis *in vitro* eventually undergo secondary necrosis exhibiting a loss of membrane integrity which must be considered when seeking to selectively measure apoptosis rather than general necrotic cell death.

Measurement of PS exposure on the cell surface prior to loss of membrane integrity by secondary necrosis is a frequently reported method used to assay for detection of apoptosis (11). The protein annexin V is known to bind to PS in the presence of calcium. Fluorescently-labeled annexin V has been used as a tool for detection of apoptosis for over 20 years, primarily using flow cytometry for detection (12). However, the protocol steps for

sample preparation and washing away the unbound fluorescent annexin V as well as comparatively low-throughput of flow cytometry have limited the widespread adoption of annexin V binding for HTS applications.

When cells undergo secondary necrosis and lose membrane integrity, annexin V can enter the cell and bind to PS remaining on membrane components in the cytoplasmic compartment. To distinguish annexin V binding to apoptotic cells versus necrotic cells, a vital DNA binding dye can be used as a control to stain the cells that have lost membrane integrity. The vital dye does not penetrate viable cells or cells in the early stages of apoptosis when membrane integrity remains intact, thus only necrotic cells are stained. Historically, a combination of annexin V-FITC and propidium iodide (which are available from several vendors) have been used as reagents to detect apoptosis and secondary necrosis using flow cytometry.

A homogeneous HTS-compatible assay protocol has recently been developed for detecting PS exposure and secondary necrosis in real-time using a standard multimode plate reader (13). Instead of using annexin conjugated to a fluorophore, the reagent contains two recombinant annexin V fusion proteins containing different subunits of a luciferase (annexin V-LgBiT and annexin V-SmBiT) that was engineered from a marine shrimp (14). Addition of the two annexin V fusion proteins and a luciferase substrate to samples of viable cells does not produce a signal above background (Figure 6). However, when cells undergo apoptosis and expose PS on the outer leaflet of the cell membrane, the two annexin V fusion proteins (containing either a large or small subunit of the NanoBiT luciferase) bind in close proximity, the luciferase domains can interact to reconstitute an active enzyme and produce photons of light. When cells proceed to secondary necrosis, the DNA binding dye (labeled as Necrosis Detection Reagent in Figure 6) can enter the cell and stain the nucleus green.

The kinetic real-time aspect of the assay comes from the fact that the Detection Reagent can be added to the cells once at time zero and incubated up to 48 hours with periodic recording of luminescence and fluorescence to monitor the onset of apoptosis and secondary necrosis, respectively.

Figure 7 illustrates that different cell types and different apoptosis-inducing treatments result in different kinetics for the onset of PS exposure (marker consistent with the process of apoptosis) and loss of membrane integrity (necrosis). The detection reagent was added once at time zero and the assay plate incubated in a multi-mode plate reader equipped with an environmental chamber programmed to record luminescence and fluorescence every hour for two days. The aqua-colored circles represent luminescence indicating PS exposure. The green squares represent fluorescence resulting from loss of membrane integrity (necrosis) and binding of the vital dye to DNA.

Figure 7 Panel A shows a relatively rapid onset of apoptosis (~1.5 hours) in TRAIL-treated DLD-1 cells whereas substantial PS exposure in HepG2 cells treated with paclitaxel does not occur until ~16 hours (Panel C). The gradual increase in signals is the result of heterogeneity among individuals in the population of cells in the sample wells. Figure 7 Panel D illustrates the effects of digitonin which causes loss of membrane integrity without initiating a preceding apoptotic process. The changes in luminescence and fluorescence from the same sample well provide a real-time indication of markers consistent with apoptosis and secondary necrosis. This contrasts with the endpoint caspase-3/7 assay described above that lysis the cells upon addition of assay reagent and restricts multiplexing options.

Figure 8 illustrates that up to 1% DMSO does not substantially change the RLU values or calculated EC_{50} values from the luminescent annexin V assay applied to cells treated with various concentrations of recombinant TRAIL.

Figure 9 illustrates similar kinetics for the appearance of two orthogonal markers of apoptosis, PS exposure and caspace-3/7 activity. Data showing the increase in PS exposure were recorded repeatedly from a single plate using the kinetic real-time assay approach (Panel A). The appearance of caspase-3/7 activity was measured from a set of parallel plates using the luminescent lytic endpoint assay (Panel B).

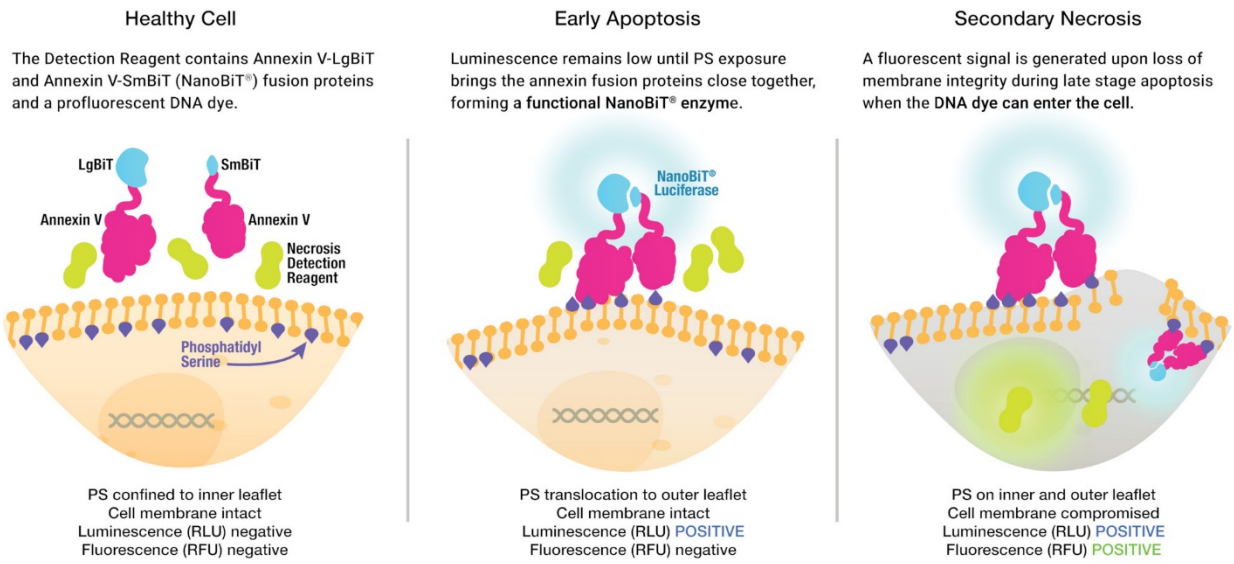

Figure 6. A diagram depicting no luminescent or fluorescent signal from a normal viable cell (left), progressing to an apoptotic cell with an intact cell membrane generating a luminescent signal, but no fluorescent signal (center) and a cell with secondary necrosis generating both luminescent and fluorescent signals (right).

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Protocol to Detect PS Exposure as a Marker of Apoptosis

Detailed directions for use of the RealTime-Glo Annexin V Apoptosis and Necrosis Assay are described in Promega Technical Manual #507 (https://www.promega.com/Resources/Protocols/Technical%20Manuals/500/RealTime%20Glo%20Annexin%20V%20Apoptosis%20and%20Necrosis%20Assay%20Protocol/?fq=realtime%20glo%20annexin%20v). It is recommended to prepare the 2X Detection Reagent immediately before use; however, the 2X Detection Reagent can be stored at room temperature, 4°C or 37°C for up to 18 hours with no detectable loss in functionality.

Choose opaque walled white plates for best results when using luminescent assays. Clear bottom plates are optional for viewing cells with a microscope during the experiment. The assay detection sensitivity is adequate for using a 96 or 384 well plate format. The recommended volumes mentioned below are for 384 well plates.

1. Harvest cells (suspension or anchorage-dependent) and resuspend the cell pellet to 200,000 cells/ml in prewarmed complete medium.
2. Add 12.5µl of a 200,000 cells/ml suspension to appropriate wells of 384-well plates (2500 cells/well) or 12.5µl of complete cell culture medium to a set of wells for no cells, no-compound controls. **Note**: If cells are attachment-dependent, return the assay plate (with the lid on) to a humidified tissue culture incubator to allow cells to attach and recover for a minimum of 3–4 hours. Alternatively, cells may be plated the night before dosing to allow the cells to attach overnight. This equilibration/attachment period is unnecessary for cells that grow in suspension.
3. Add 12.5µl of appropriate dilutions of test compounds, positive control compounds or medium only (no cell vehicle controls) to chosen wells. **Note:** All wells should now contain 25µl.
4. Prepare 2X Detection Reagent according to vendor recommendations. The reconstituted annexin reagent is stable for up to 18 hours at ambient temperature which is sufficient for a standard 8 hour shift.
5. Add 25µl of the 2X Detection Reagent in complete medium to all wells. All wells should now contain a final assay volume of 50µl for a 384-well plate.
6. Shake the assay plate on a plate shaker for approximately 30 seconds at 500–700rpm to mix.

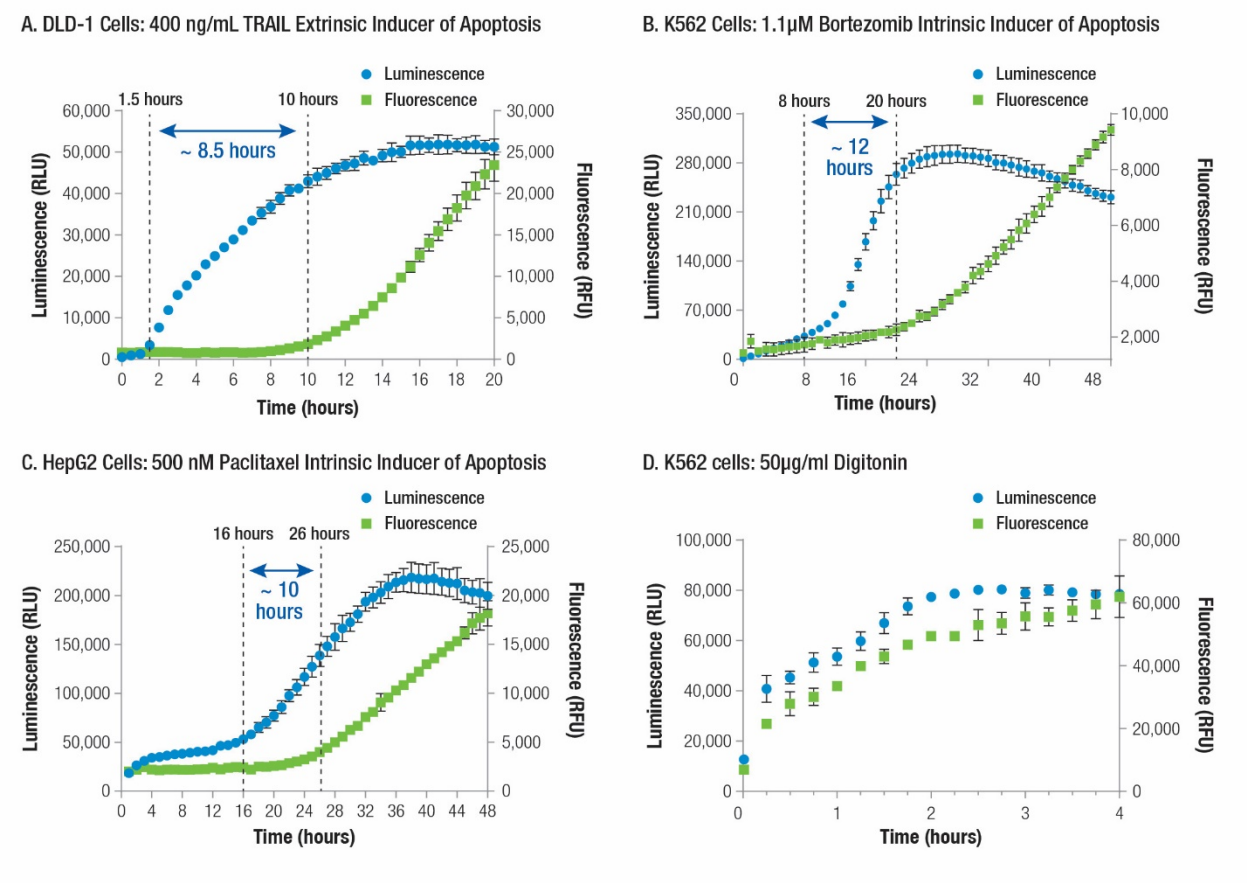

Figure 7. Sequentially repeated measures of luminescence (RLU) and fluorescence (RFU) resolve the kinetic effects of different cytotoxic stimuli and help establish mechanism of action. Panel A. DLD-1 cells treated with 400ng/ml rhTRAIL. Panel B. K562 cells treated with 1.1µM bortezomib. Panel C. HepG2 cells treated with 500nM paclitaxel. Panel D. K562 cells treated with 50µg/ml digitonin. Background-subtracted luminescence (blue circles) and fluorescence (green squares) readings are shown. For Panels A–C, there is a significant time delay between the emergence of PS:Annexin V binding and the loss of membrane integrity, results that are indicative of an apoptotic phenotype leading to secondary necrosis. In Panel D, luminescence and fluorescence signals emerge concurrently, suggesting a non-apoptotic phenotype. Data represent the mean of 4 readings for each replicate ± SD.

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7. Incubate plate using standard cell culture conditions and record luminescence and fluorescence repeatedly from the same samples at desired times.

Data Calculation

Subtract the average luminescence signal from the no-cell, no-compound background controls (a measure of intrinsic reagent and instrument luminescence) from all sample luminescence values to obtain net RLU. **Note:** Although it will depend on the cell culture model system, if cultures are handled properly, there should be less than 5% apoptotic cells in vehicle treated controls.

Subtract the average fluorescence signal from the no-cell, no-compound background controls (a measure of intrinsic reagent and instrument fluorescence) from all sample fluorescence values to obtain net relative fluorescence units.

Graph net luminescence (resulting from PS:Annexin V binding) and net fluorescence (resulting from changes in membrane integrity) versus compound concentration (include the untreated control in the analysis when possible) for each time point using commercially available curve-fit software (e.g. GraphPad Prism, SigmaPlot,

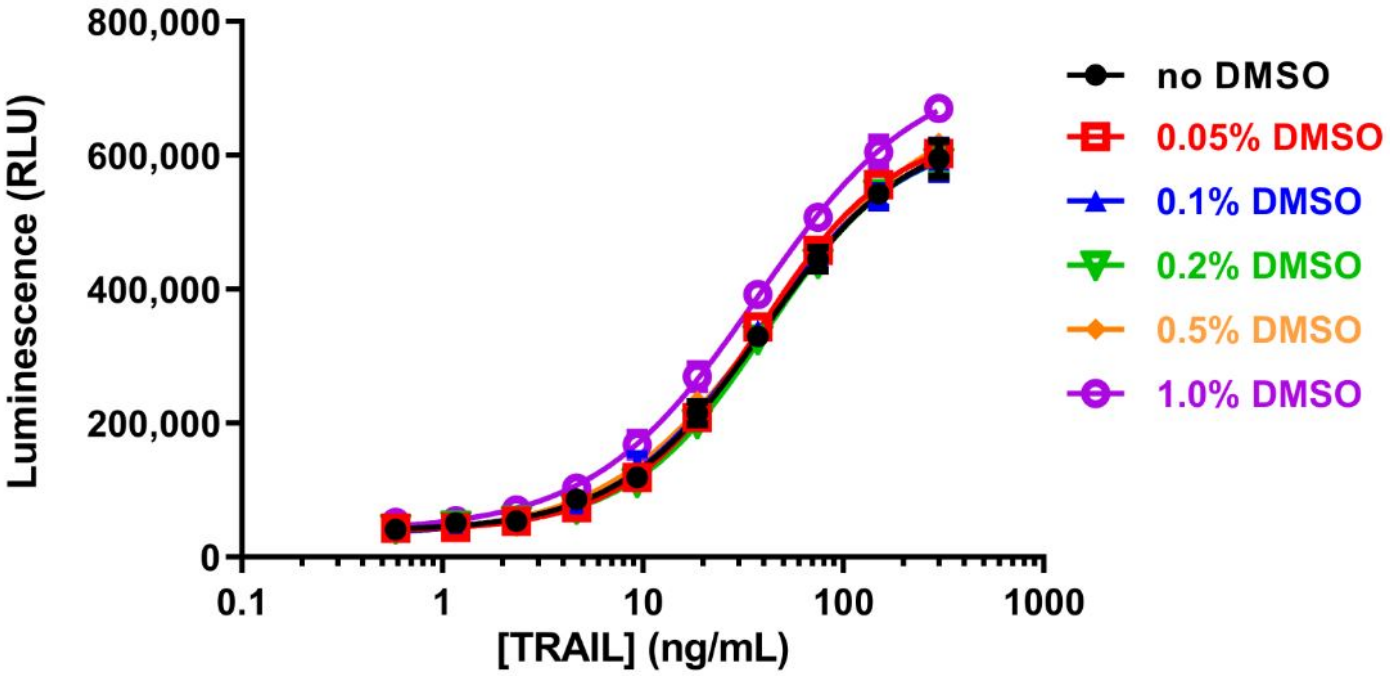

Figure 8. Various concentrations of TRAIL were added to 10,000 DLD-1 cells/well cultured in McCoy's 5A medium + 10% FBS. Samples were incubated for 6.5 hours in the presence of the listed concentrations of DMSO. The RealTime-Glo Annexin V Apoptosis and Necrosis Assay reagent was added and luminescence recorded. The plotted values represent the mean ± SD of four technical replicates.

etc.). It is instructive to graph net luminescence and net fluorescence signals versus time at a specific compound concentration.

The luminogenic annexin V-PS binding assay can be multiplexed with the luminogenic caspase-3/7 assay to measure two different orthogonal markers of apoptosis in the same sample of cells. This multiplex is possible because of the different assay chemistries of the luciferases from different species. For this multiplex to be successful, data from the real-time annexin V-PS assay must be recorded first. The caspase-3/7 assay detection reagent will disrupt the luminescent signal from the shrimp-derived NanoBiT luciferase in the annexin V-PS binding assay and provide the necessary substrate to enable generation of the luminescent signal from the firefly-derived luciferase in the caspase-3/7 assay. Figure 10 shows an example of data from multiplexing the two assays. The results for the annexin V assay to detect PS exposure on the cell membrane and the caspase-3/7 assay to detect executioner protease activity are in agreement.

Any change to the contents of the culture medium (such as the presence of reagents for multiplexing) or handling of the sample needs to be considered and the assay protocol validated for any change. For example, if the temperature of the plate is altered by recording values of the first assay, it may change the values of a secondary luciferase assay (because luminescence is reporting the rate of the luciferase enzyme). The statistical advantage of measuring two different signals from the same sample instead of creating and assaying a parallel sample also needs to be considered.

Figure 11 provides evidence that the presence of RealTime-Glo Annexin V Apoptosis and Necrosis Assay Reagent does not have a substantial effect on the signal or performance of the luminescent caspase-3/7 assay. The signal from the luminescent caspase-3/7 assay is similar in samples with or without the presence of the RealTime-Glo Annexin V Apoptosis and Necrosis Assay Reagent.

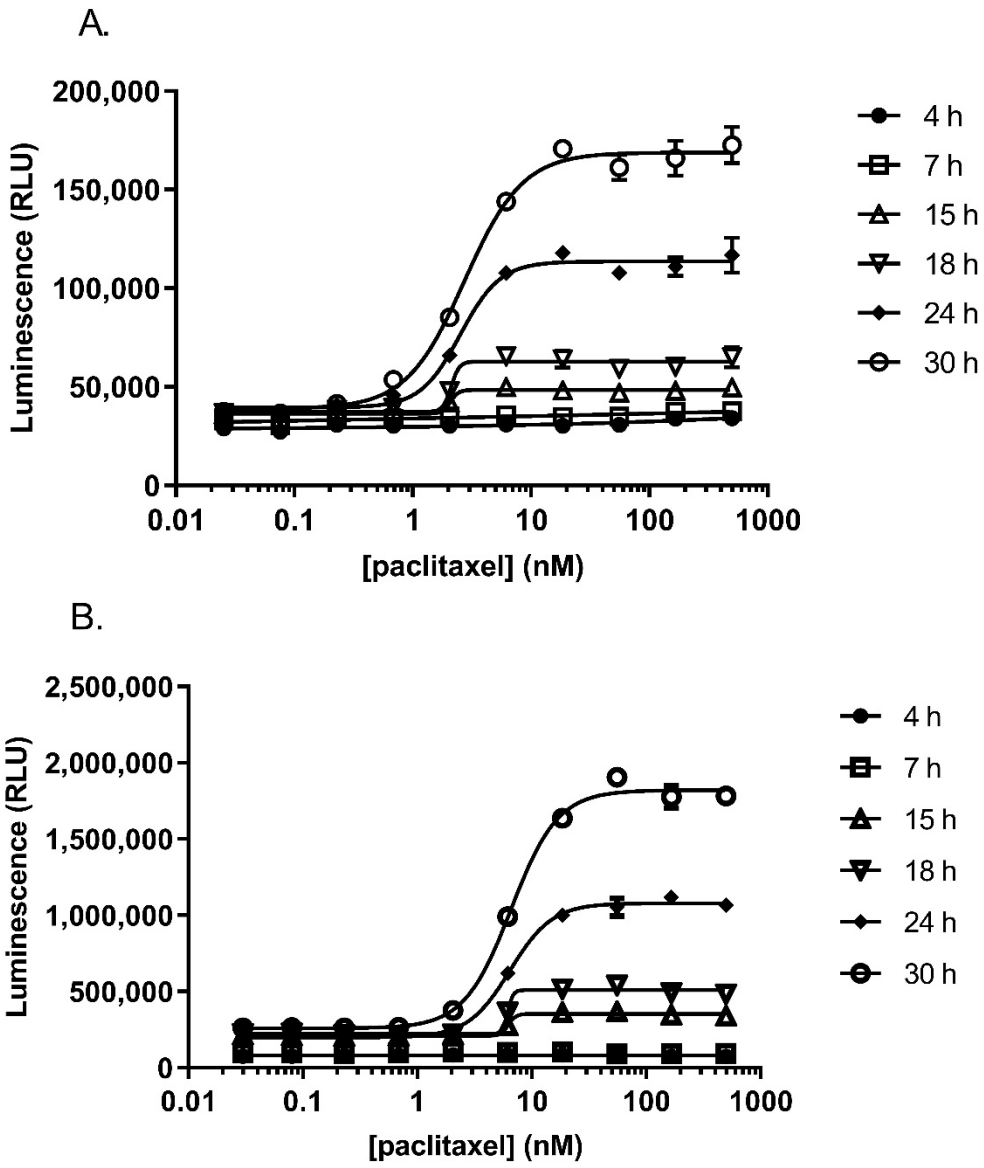

Figure 9. A kinetic comparison between PS exposure (Panel A) and caspase activation (Panel B) after paclitaxel treatment of HepG2 cells. Annexin-binding luminescence was collected by sequential measurements of the same plate over a 30 hour time course (A). Caspase activity data were collected from parallel plates using lytic endpoints (Panel B). The values represent the mean ± SD of four technical replicates for each dose.

Figure 12 shows an example of multiplexing a fluorescent cell viability assay with the luminescent annexin v assay. The fluorescent cell viability assay uses a cell permeable Gly-Phe-AFC protease substrate to measure an aminopeptidase activity selectively present in viable cells. Details of the fluorogenic assay are described in the AGM chapter on cell viability assays (15). (https://www.ncbi.nlm.nih.gov/books/NBK144065/pdf/Bookshelf_NBK144065.pdf). The signal magnitude and potency values from the multiplex and control assays are similar suggesting compatibility of the two reagents in a multiplex format.

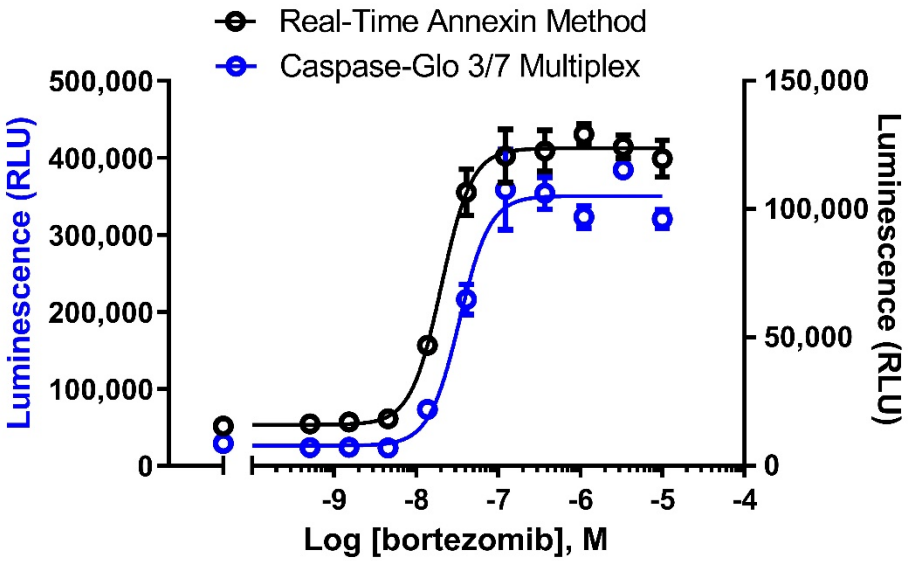

Figure 10. Sequential multiplex of orthogonal assays to measure different markers to confirm the occurrence of apoptosis. K562 cells (10,000/well in 100µl RPMI1640 + 10% FBS) were seeded in assay plates and treated with various concentrations of bortezomib. The RealTime-Glo Annexin V Apoptosis and Necrosis Assay reagent (100µl) was added at time zero and luminescence recorded at 18 hours (black circles). The Caspase-Glo 3/7 Assay reagent was added, the samples were mixed and incubated at room temperature for 30 minutes and luminescence recorded (blue circles). The values represent the mean ± SD of four technical replicates.

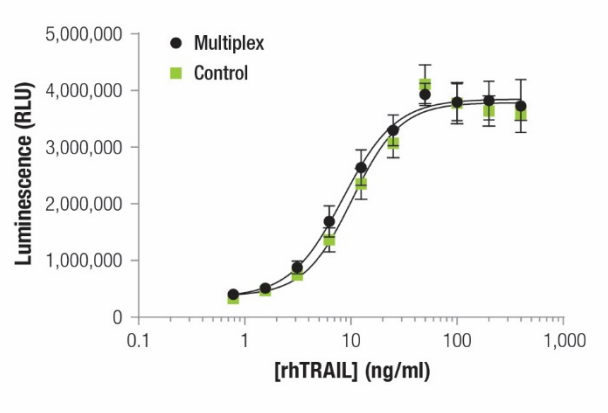

Figure 11. The effect of the RealTime-Glo Annexin V Apoptosis and Necrosis Assay reagent on subsequent multiplex using the Caspase-Glo 3/7 Assay. DLD-1 cells were exposed to serial dilutions of rhTRAIL either in the presence (black circles) or absence (green squares) of the RealTime-Glo Annexin V Apoptosis and Necrosis Assay Reagent. The plate was incubated at 37°C/5% CO_2 for 6.5 hours then the Caspase-Glo 3/7 Reagent was added to all wells and the assay performed as described above. Data represent the mean ± SD from four technical replicates. Additional details are shown in TM507.

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Commercial Reagents

Caspase-3/7: Commercial kits with colorimetric and fluorogenic caspase-3/7 substrates containing pNA, AMC, or AFC are available from several vendors; however, they are not listed here because the detection sensitivity is generally inadequate for HTS. Commercial kits with fluorogenic caspase-3/7 substrates containing R110 also are available from several vendors (examples shown below); however, the luminogenic reagent kit is available only from Promega Corporation.

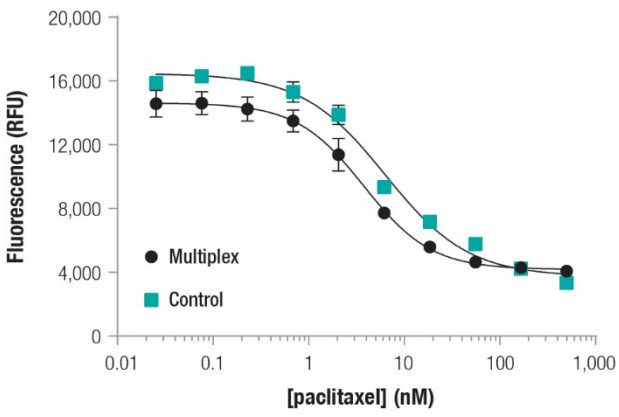

Figure 12. Example multiplex data with the RealTime-Glo Annexin V Apoptosis and Necrosis Assay and CellTiter-Fluor Cell Viability Assay. HepG2 cells were exposed to serial dilutions of paclitaxel in the presence (black circles) or absence (green squares) of the RealTime-Glo Annexin V Apoptosis and Necrosis Assay Reagent. The plate was incubated at 37°C/5% CO_2 for 48-hours prior to adding the CellTiter-Fluor Reagent using the multiplex format and recording fluorescence indicating viable cell number. Data represent the mean ± SD from four technical replicates.

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Fluorogenic

- EnzChek Caspase-3 Assay Kit, Thermo Fisher Catalog Number E13184
- Apo-ONE Homogeneous Caspase-3/7 Assay; Promega Corp. Catalog Number G7791
- EarlyTox Caspase-3/7 R110 Assay Kit: Molecular Devices Catalog Number R8347
- NucView 488 Caspase-3 Enzyme Substrate, 1 mM in DMSO. Biotium Catalog Number 10402

Luminogenic

- Caspase-Glo 3/7 Assay, Promega Corporation Cat.# G8092

Phosphatidyl Serine: Annexin V is available conjugated to several different fluorophores (at least seven from Thermo Fisher); however, they are designed for microscopic imaging and flow cytometry and are not recommended for fluorescent plate readers. Many kits also contain a vital dye such as propidium iodide as a control to detect secondary necrosis as described above.

Fluorescent

- Annexin V Alexa Fluor 488 Conjugate, Thermo Fisher Catalog Number A13201
- Annexin V-FITC Apoptosis Detection Kit, Millipore Sigma Catalog Number APOAF-50TST
- Cell Meter APC-Annexin V Binding Apoptosis Assay Kit, ATT Bioquest Catalog Number 22837
- FITC Annexin V Apoptosis Detection Kit with PI, Biolegend Catalog Number 640914
- Annexin V-FITC Apoptosis Staining / Detection Kit, Abcam Catalog Number ab14085

Luminogenic

- RealTime-Glo Annexin V Apoptosis and Necrosis Assay, Promega Corp. Catalog Number JA1012

Conclusion

Although the assays described in this chapter may be “fit-for-purpose” and identify “hits” by detecting an apoptotic marker, we need to keep in mind that no assays are perfect. Confirming the occurrence of apoptosis by using more than one method has historically been a requirement for peer reviewed journals. There are many different marker assays that can serve to confirm the presence of apoptosis. However, care should be taken to not

choose a confirmatory marker that is selective for either intrinsic (e.g. mitochondrial polarization changes and caspase-9 release) or extrinsic pathways (e.g. caspase-8 association with a member of the death receptor family) because diverse members of small molecule libraries may be selective for only one pathway. Observing gross cell morphology or using antibodies to detect neo-epitopes resulting from caspase cleavage of target proteins are options to confirm the occurrence of apoptosis. Detecting cleaved PARP or cleaved caspase can be accomplished using Western blotting or immunocytochemistry approaches (4); however, those assays are labor intensive for large numbers of samples. Assays such as TUNEL or ELISA to detect DNA fragmentation are options to confirm apoptosis; however, they also require many procedural steps and may not be practical for follow-up of 100s of hits. A logical approach to confirm the results of a high-throughput screen for apoptosis modulators is to repeat the original assay in combination with a multiplexed method detecting an orthogonal marker. An example of multiplexing two homogeneous orthogonal assays compatible with HTS is shown in Figure 10.

Apoptosis is a process of programmed cell death that is of interest to the drug development community because members of the signaling cascade leading to cell suicide are potential targets for development of therapeutics to treat degenerative diseases or cancer. Although there are a variety of methods to detect markers indicative of apoptosis, not all methods are easily adaptable for HTS. Easy homogeneous methods to detect markers of apoptosis in a high throughput format are valuable for initial phenotypic screening. The apoptosis marker assays most frequently reported in the literature are caspase-3/7 activity and PS exposure on the surface of cells detected by annexin V binding. Both of those methods do not require prior engineering of cells to express an artificial reporter or sensor molecule. Measuring caspase-3/7 activity in an HTS setting can be done using homogeneous fluorogenic or luminogenic methods, with the latter providing much greater sensitivity enabling miniaturization. Measuring PS exposure historically has relied on flow cytometry; however, recently a homogeneous procedure based on enzyme complementation has been developed that enables detecting the onset of apoptosis in real-time using a plate reading luminometer. The real-time method enables monitoring the progress of apoptosis in heterogeneous populations of cells and can provide useful information for development of assays that measure orthogonal endpoints. This chapter provides a brief description of those two homogeneous HTS assay methods and includes references and links to detailed Technical Manuals describing assay protocols.

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