TECHNICAL MANUAL

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay

Instructions for use of Products
JA1011, JA1012, JA1000 and JA1001

9/17
TM507

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay

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1. Description

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay[a–d] is a live-cell (non-lytic) real-time (kinetic) assay that measures the exposure of phosphatidylserine (PS) on the outer leaflet of the cell membrane during the apoptotic process (Figure 1). Measurement of PS exposure is a reliable, well validated means of assessing apoptosis (1). In the RealTime-Glo™ Assay, annexin V binding is detected with a simple luminescence signal, and necrosis is detected with a fluorescence signal. Assay signals are detected with a plate-based multimode reader.

The Detection Reagent used in the RealTime-Glo™ Annexin V Apoptosis Assay contains near-equimolar ratios of two annexin V fusion proteins (Annexin V-LgBiT and Annexin V-SmBiT) containing complementary subunits of NanoBiT® Luciferase. The reagent also contains a time-released luciferase substrate that provides a constant source of substrate over experimental exposure periods. Because the Annexin V-LgBiT and Annexin V-SmBiT luciferase subunits have only modest affinity for each other, luminescence remains low until PS exposure brings the subunits into complementing proximity. The Annexin V Apoptosis and Necrosis Assay (Cat.# JA1011, JA1012) also includes a cell-impermeant, profluorescent DNA dye, which detects necrosis. The Detection Reagent is well tolerated by a wide variety of cultured cells and can be applied at dosing or prior to treatment for real-time measurement of the dose and time-dependent magnitude of apoptotic progression.

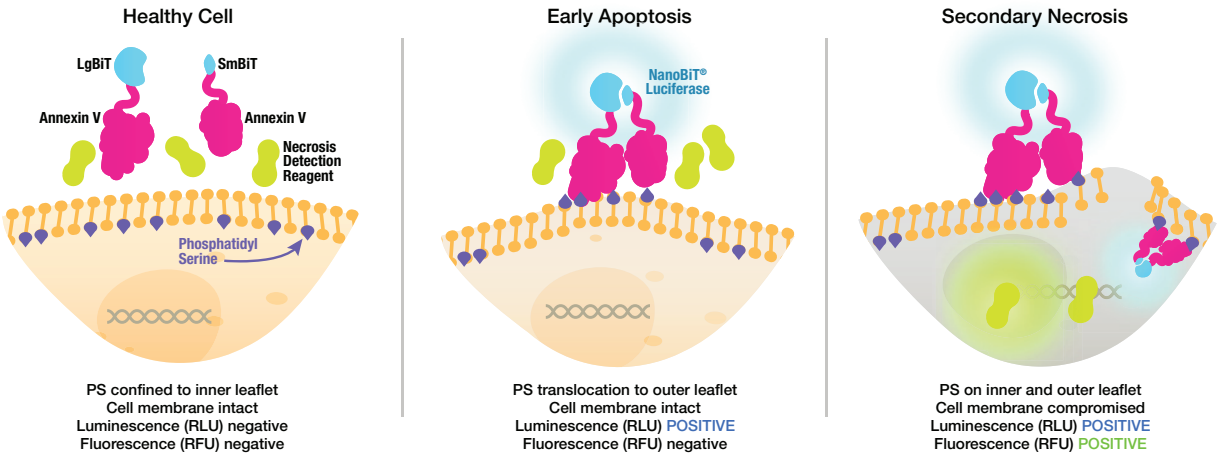

Figure 1. Real-time bioluminescence detection of PS exposure on apoptotic cells. In the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, time-dependent increases in luminescence that occur before increases in fluorescence (due to loss of membrane integrity) reflect the apoptotic process. Increases in both luminescence and fluorescence signals are consistent with secondary necrosis following apoptosis or other non-apoptotic mechanisms.

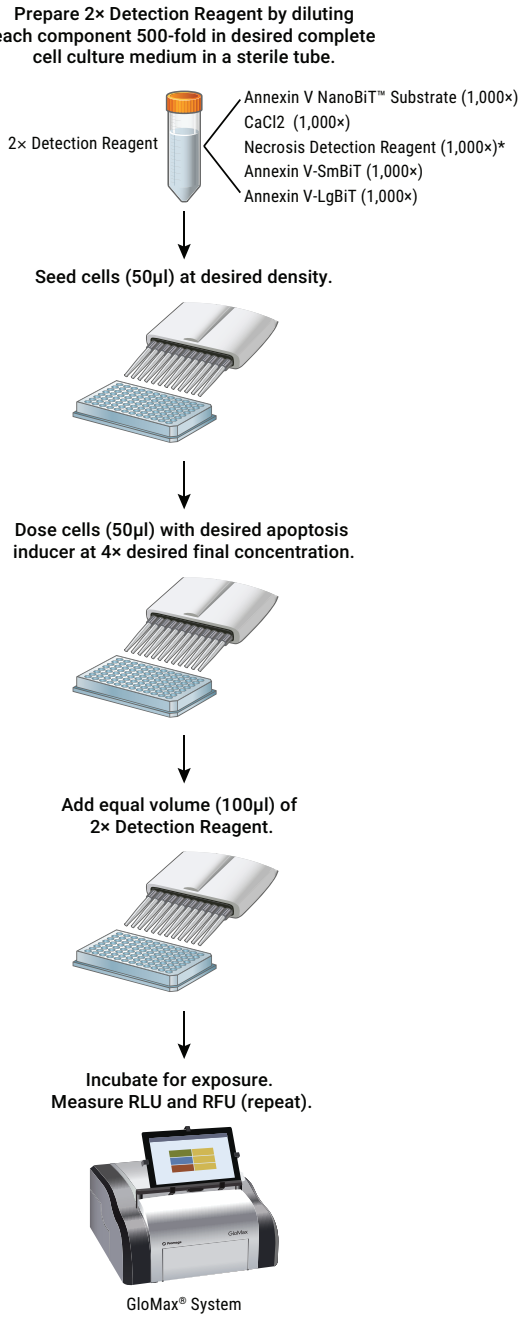

Figure 2. Overview of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay 96-well plate protocol. RLU = Relative Luminescence Units. RFU = Relative Fluorescence Units. *Necrosis Detection Reagent is not provided with the RealTime-Glo™ Annexin V Apoptosis Assay (Cat.# JA1000, JA1001).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	**100 assays**	**JA1011**

Contains sufficient reagents to perform 100 assays in a 96-well plate format or 400 assays in a 384-well plate format. Includes:

- 25µl Annexin V-LgBiT (1,000X)
- 25µl Annexin V-SmBiT (1,000X)
- 250µl $CaCl_2$ (1,000X)
- 25µl Annexin V NanoBiT® Substrate (1,000X)
- 25µl Necrosis Detection Reagent (1,000X)

PRODUCT	SIZE	CAT.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	**1,000 assays**	**JA1012**

Contains sufficient reagents to perform 1,000 assays in a 96-well plate format or 4,000 assays in a 384-well plate format. Includes:

- 250µl Annexin V-LgBiT (1,000X)
- 250µl Annexin V-SmBiT (1,000X)
- 250µl $CaCl_2$ (1,000X)
- 250µl Annexin V NanoBiT® Substrate (1,000X)
- 250µl Necrosis Detection Reagent (1,000X)

PRODUCT	SIZE	CAT. #
RealTime-Glo™ Annexin V Apoptosis Assay	**100 assays**	**JA1000**

Contains sufficient reagents to perform 100 assays in a 96-well plate format or 400 assays in a 384-well plate format. Includes:

- 25µl Annexin V-LgBiT (1,000X)
- 25µl Annexin V-SmBiT (1,000X)
- 250µl $CaCl_2$ (1,000X)
- 25µl Annexin V NanoBiT® Substrate (1,000X)

PRODUCT	SIZE	CAT. #
RealTime-Glo™ Annexin V Apoptosis Assay	**1,000 assays**	**JA1001**

Contains sufficient reagents to perform 1,000 assays in a 96-well plate format or 4,000 assays in a 384-well plate format. Includes:

- 250µl Annexin V-LgBiT (1,000X)
- 250µl Annexin V-SmBiT (1,000X)
- 250µl $CaCl_2$ (1,000X)
- 250µl Annexin V NanoBiT® Substrate (1,000X)

Storage Conditions: Store all assay components at –30°C to –10°C. Store Annexin V NanoBiT® Substrate and Necrosis Detection Reagent protected from light.

Stability of 1,000X Kit Components

Thaw the 1,000X Annexin V NanoBiT® Substrate at room temperature. Minimize freeze-thaw cycles. The 1,000X Annexin V-LgBiT and Annexin V-SmBiT fusion proteins are supplied stored in 50% glycerol and should be placed on ice prior to preparing the 2X Detection Reagent. If the kit components will be used to create 2X Detection Reagent over multiple days, it is best to thaw the components once and aliquot into multiple single-use volumes. Return unused aliquots to −30°C to −10°C. Avoid long-term ambient light exposure of the Annexin V NanoBiT® Substrate and the Necrosis Detection Reagent.

Stability of Prepared 2X Detection Reagent

We recommend preparing only the amount of 2X Detection Reagent required for immediate 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. Do not store the prepared 2X Detection Reagent for longer than 18 hours.

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Formats

Assay formats with and without Necrosis Detection Reagent are available:

The **RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay**[a–d] (Cat.# JA1011 and JA1012) includes Necrosis Detection Reagent, a cell-impermeant profluorescent DNA dye. Upon loss of membrane integrity, the dye enters the cell and binds to DNA, generating a fluorescent signal. The two measurements allow the mechanism of action of PS exposure to be revealed.

The **RealTime-Glo™ Annexin V Apoptosis Assay**[a–c] (Cat.# JA1000 and JA1001) does not include Necrosis Detection Reagent. Although the assay can be conducted without a necrosis detection probe, the mechanism of action of PS exposure will not be revealed. Therefore, when using Cat.# JA1000 or JA1001, we recommend incorporating an asymmetric cyanine dye such as CellTox™ Green (Cat.# G8742) or structurally related molecules, for full assay functionality and data clarity. We discourage the use of propidium idodide, ethidium homodimer or members of the Hoeschst series due to suboptimal performance in plate-based fluorometry applications and their known toxicity to cells.

3. Before You Begin

Please read the entire protocol (Sections 3 and 4) to become familiar with the components and the assay procedure before beginning your experiments.

3.A. Instrumentation and Multiwell Plate Recommendations

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Cat.# JA1011, JA1012) is a multiplexed assay that requires a multimode plate reading instrument capable of measuring luminescence and "green spectrum" fluorescence. Fluorescence from the assay is optimally measured in the 485±20nm excitation range and collected in the 525±30nm emission range. This can be achieved by using a pre-programmed setting on the GloMax® Discover (Cat.# GM3000) or by using the settings suggested by other instrument manufacturers.

All experiments should be conducted using sterile, white, tissue culture-treated 96-well multiwell plates (e.g., Costar® 3917 solid white bottom or Costar® 3903 clear bottom) or 384-well multiwell plates (e.g., Corning® 3570) to minimize fluorescence and luminescence cross-talk between wells. In general, solid white bottom plates will produce higher luminescent and fluorescent intensity values and less cross-talk between assay wells than white plates with clear bottoms. However, white, clear-bottom plates also provide acceptable performance.

3.B. Materials to Be Supplied by the User

- sterile, white, tissue culture-treated 96-well multiwell plates (e.g., Costar® 3917 solid white bottom or Costar® 3903 white clear bottom) or 384-well multiwell plates (e.g., Corning® 3570).
- multimode plate reader capable of measuring luminescence and green fluorescence ($485nm_{Ex}/525–530nm_{Em}$).
- sterile multichannel trough for serial dilution of compounds.
- sterile troughs for addition of other components.
- single-channel and multichannel pipettes with appropriate sterile pipette tips.
- sterile conical tubes.
- positive control compound known to induce apoptosis in a well characterized cell line.
- vehicle (e.g., DMSO) for untreated cell controls.
- plate shaker

3.C. Solubility Considerations for the 2X Detection Reagent

The Annexin V NanoBiT® Substrate is soluble in most common growth and maintenance media, but solubility is greatly enhanced in the presence of serum supplements (e.g., 10% v/v fetal bovine serum (FBS) or 10% v/v horse serum). We recommend adding the Annexin V NanoBiT® Substrate to prewarmed complete medium (normal growth medium + 10% FBS) to make the 2X Detection Reagent, and then vortexing the solution immediately until the substrate has fully dissolved. If defined or serum-free growth medium is necessary, we highly recommend use of protein supplements (cytokines, growth factors, albumin, etc.) to maintain substrate solubility.

The annexin V-PS binding interaction is Ca^{2+}-dependent. Although most common growth and maintenance media are formulated to contain $CaCl_2$ at levels sufficient to support annexin V-PS binding, the RealTime-Glo™ Annexin V Apoptosis and Necrosis Detection Assays benefit from the addition of the provided Ca^{2+} in the reagent. The solubility of additional Ca^{2+} is greatly enhanced in prewarmed media in the presence of FBS.

3.D. Cell Tolerance of Detection Reagent

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Detection Reagent produces no measurable cytotoxicity when co-incubated with a variety of common attachment-dependent and suspension cell lines for up to 72 hours. Concentrations of the assay reagent components have been optimized to remain biologically inert and maximize luminescence intensity and signal persistence during real-time studies of apoptosis. However, all users should be aware of the possibility of unique cell lineage sensitivity to the annexin detection reagents over exposure periods (2–4). To confirm reagent tolerability, we recommend setting up a comparison of untreated and treated controls in the presence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent and of Necrosis Detection Reagent only during the intended exposure period. If the annexin reagent does produce measurable additive toxicity, it should only minimally affect the kinetics of test compound apoptotic induction or influence the progression to secondary necrosis. Cytotoxic effects produced by the annexin reagent can be eliminated or minimized by reducing the concentration of the Annexin V NanoBiT® Substrate; however, this will reduce the overall luminescence intensity and signal sustainability over time.

Note for users purchasing the RealTime-Glo™ Annexin V Apoptosis Assay (Cat.# JA1000, JA1001) and adding their own choice of Necrosis Detection Reagent: CellTox™ Green Reagent (Cat.# G8741, G8742) has been validated as a non-toxic, necrosis detection probe and can be incorporated into the 2X Detection Reagent without detriment. The tolerability (and live cell exclusion) of other fluorescent necrosis detection probes is questionable (unpublished in-house data) and should be addressed by the user prior to incorporation into the Detection Reagent.

3.E. Recommendations for Collecting Data in Real Time

Luminescence and fluorescence data can be gathered in real time by three principle methods, all of which require a multimode instrument capable of reading both luminescence and fluorescence.

1. **Kinetic mode using a multimode instrument equipped with temperature and atmospheric (CO_2) controls:** Cells can be maintained in complete medium of choice. Luminescent and fluorescent measures may be repeatedly collected at any time interval during the prescribed exposure provided that the read times do not exceed the interval. “Edge-effects” and/or excessive condensation on the inner surface of plate lids should be considered when using non-humidified environments in multi-day exposures.
2. **Kinetic mode using a multimode instrument with temperature control only:** Many cell types can be successfully maintained in CO_2-independent media formulations without physiological detriment. Use of CO_2-independent medium obviates the need for atmosperic control and allows for real-time measurement using only temperature control. Individual cell line tolerability to CO_2-independent medium, and relative responsiveness to cytotoxic stimuli, should be verified before performing the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay. “Edge-effects” and/or excessive condensation on the inner surface of plate lids should be considered when using non-humidified environments in multi-day exposures.
3. **Multiple measures at scheduled intervals:** Assay plates should be incubated in a humidified tissue culture incubator with the plate lid on. The assay plate should be shuttled in and out of the incubator to read luminescence and fluorescence at the desired time points. Minimize the time the assay plate is out of the incubator to reduce thermal variation associated with test cells and reagents, and to minimize risk of contamination.

4. Protocol

4.A. Recommended Controls

- **No-cell controls:** For a 96-well format, add 100µl of complete culture medium and 100µl 2X Detection Reagent to replicate wells. These control wells will define background luminescence and fluorescence generated from the reagent in medium alone in the absence of cells, and may guide instrument gain settings. The average of the background luminescence and fluorescence values from the no-cell controls should be subtracted from all of the test sample wells to normalize test data. No-cell luminescence values will be minimal in most cases, but fluorescence values may be substantial due to chosen photomultiplier gain settings.
- **No-compound controls (vehicle only controls):** For a 96-well format, add 50µl of complete culture medium and matched vehicle excipient to 50µl of plated cells. Next, add 100µl of 2X Detection Reagent to each no-compound control well. These wells establish a vehicle-matched (typically DMSO), untreated response during the exposure. All test samples should be compared to the no-compound controls at each time point during the exposure.
- **Positive control compound:** For a 96-well format, add 50µl of a compound known to induce apoptosis (or to produce necrosis) at a known concentration in a chosen model system to 50µl of plated cells. Next, add 100µl 2X Detection Reagent to each positive control well. These wells are particularly useful in demonstrating positive reagent performance and establishing relative magnitude of response while surveying unknown compounds during discovery. The wells may also be used to establish instrument gain settings.

4.B. Preparation of the 2X Detection Reagent

Notes:

We recommend preparing only the volume of 2X Detection Reagent required for immediate 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. Do not store the prepared 2X Detection Reagent for longer than 18 hours.

Immediately before preparing the 2X Detection Reagent, thaw the Annexin V NanoBiT® Substrate, $CaCl_2$ and Necrosis Detection Reagent at room temperature and place the Annexin V-SmBiT and Annexin V-LgBiT on ice. Briefly centrifuge all kit components after thawing to facilitate maximum recovery. Add components in the order described below.

Users purchasing the RealTime-Glo™ Annexin V Apoptosis Assay (Cat.# JA1000, JA1001) should add their own choice of Necrosis Detection Reagent at Step 4, or omit the Necrosis Detection Reagent, as desired.

1. Determine the final volume of 2X Detection Reagent required for all control and test wells. Allow for unrecoverable void volumes in tubes and reagent reservoirs. Typically, 12ml of 2X Detection Reagent is sufficient for a full 96-well plate.
2. Aliquot the desired volume of complete culture medium to a sterile mixing vessel with cap. Add 1,000X Annexin NanoBiT® Substrate to the prewarmed complete medium at a 500-fold dilution (24µl of substrate per 12ml for a full 96-well plate). Vortex immediately.

   Note: (Optional) If performing the assay in a multimode plate reader with kinetic capability and temperature control, but without CO_2 control, prepare your reagent in prewarmed CO_2-independent medium + 10% FBS. Use CO_2-independent medium + 10% FBS for the subsequent steps.
3. Add 1,000X $CaCl_2$ to the prewarmed medium containing Annexin V NanoBiT® Substrate at a 500-fold dilution (24µl per 12ml for a full 96-well plate). Vortex immediately.
4. Add 1,000X Necrosis Detection Reagent at a 500-fold dilution (24µl per 12ml for a full 96-well plate). Vortex to ensure homogeneity.
5. Add 1,000X Annexin V-SmBiT and 1,000X Annexin V-LgBiT at a 500-fold dilution to the medium containing substrate, $CaCl_2$, and Necrosis Detection Reagent as described in Steps 2–4, above (24µl of each protein per 12ml for a full 96-well plate). Mix by careful inversion. Do not vortex or create bubbles!

   Note: It is often advantageous to multiplex the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay with other orthogonal cell health assays in the same sample well. In most instances, the additional volume introduced by multiplexing these endpoint chemistries can be easily accommodated using the 2X Detection Reagent format (see Section 6.C). For low-volume-plate applications where same-well multiplex volumes will exceed well capacity, please refer to Section 6.B, which describes an alternative protocol for the addition of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay components.

4.C. Assay Protocol

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay 2X Detection Reagents are scalable to 384-well plate format as long as the 1:1 ratio between cultured cells and 2X Detection Reagent is maintained. The following sample protocol provides guidance for recommended volumes to use with a standard 96-well assay plate. For a 384-well format, we recommend reducing the volume and cell number to approximately 20–25% of that used in the 96-well format.

Plate Layout: 10,000 cells/well (96-well plate); 2,500 cells/well, (384-well plate)												
	10-point, twofold serial dilution of compound →										0	0
	1	2	3	4	5	6	7	8	9	10	11	12
A	Control Compound (known apoptosis inducer)										Cells Only No Compound	No Cells No Compound
B												
C												
D												
E	Test Compound										Cells Only No Compound	No Cells No Compound
F												
G												
H												

Figure 3. Example plate layout.

1. Harvest cells (attachment-dependent or attachment-independent) and resuspend the cell pellet to 200,000 cells/ml in prewarmed complete medium.

   Notes:

   1. **(Optional)** If performing the assay in a multimode plate reader with kinetic capability and temperature control, but without CO_2 control, resuspend the cell pellet in prewarmed CO_2-independent media + 10% FBS. Use CO_2-independent media + 10% FBS for the subsequent steps.

   2. **(Optional)** In uncommon instances where endpoint, same-well, cell health assay chemistries (such as CellTiter-Glo® Cell Viability Assay or Caspase-Glo® 3/7 Assay) will be added **and** the volume of the well is not large enough, it may be necessary to add the 2X Detection Reagent components directly to cells, rather than a separate 2X Detection Reagent addition. Please refer to this deviation from standard protocol in the Appendix, Section 6.B.

2. Add 50µl of 200,000 cells/ml to wells A1–H11 in a 96-well plate (10,000 cells/well).

3. Add 50µl of complete cell culture medium (no cells) to column 12 for a 96-well plate. This column will be the no-cells, no-compound control.

   Note: If cells are attachment-dependent, return the assay plate (with the lid on) to a humidified tissue culture incubator to allow cells to reattach and recover for a minimum of 3–4 hours. Alternatively, cells may be plated the night before dosing to allow the cells to reattach overnight. This equilibration/reattachment period is unnecessary for cells that grow in suspension.

4. Add 0.5ml of complete medium to channels 2–12 of two sterile 12-channel troughs. To separate 1ml aliquots of complete medium, add control or test compound to 4X the desired starting concentration (for example, if the desired starting concentration in the dilution series is 10µM, prepare a 40µM solution). It may be necessary to make an intermediate dilution of the compound in DMSO in order to dilute down to the desired 4X starting concentration. Add the 1ml of 4X control or test compound in complete medium to channel 1 in the appropriate trough. For both compounds, perform a 10-point, twofold serial dilution from channel 1 to channel 10 by transferring 0.5ml from channel 1 to channel 2, mixing up and down, then transferring 0.5ml from channel 2 to channel 3, etc. Leave channels 11 and 12 as the "no-compound controls" (matched vehicle only in complete medium).

5. Using the appropriate 12-channel trough and a 12-channel multichannel pipette, transfer 50µl of the appropriate 4X concentrated, 10-point, twofold serial dilution to the appropriate replicate wells in the 96-well assay plate containing cells. (See the plate layout in Figure 3.) Add the control compound to replicate wells in rows A–D and test compound to replicate wells in rows E–H. Each well should now contain 100µl. Be sure to follow the plate layout in Figure 3 and add the compound titration series from high to low concentration as listed (highest concentration to lowest concentration from column 1 to column 10). Columns 11 and 12 should contain no compound (vehicle controls).

6. Prepare 2X Detection Reagent as stated in Section 4.B.

Note: After the 2X Detection Reagent is created, proceed immediately to Step 7 in order to start the assay time course (t=0).

7. Add 100µl of the 2X Detection Reagent in complete medium to all wells in the 96-well assay plate. All wells should now contain a final assay volume of 200µl for a 96-well plate. Shake the assay plate on a plate shaker for approximately 30 seconds at 500–700rpm to mix.

8. Incubate and record luminescence and fluorescence measurements by one of the three methods described in Section 3.E.

4.D. Data Analysis

1. Calculate the average luminescence signal from column 12 (no-cell, no-compound background controls; a measure of intrinsic reagent luminescence). Subtract this signal from all sample luminescence values to obtain net relative luminescent units.

   Note: No-cell background signals in the luminescence and fluorescence channels can be significant relative to experimental signals in non-photomultiplier (PMT)-based instruments or in instances where PMT settings have not been optimized (or cannot be changed) and should be mathematically reconciled. Refer to your instrument instruction manual for guidance.

2. Calculate the average fluorescence signal from column 12 (no-cell, no-compound background control; a measure of intrinsic reagent fluorescence), and subtract this value from every sample well in the fluorescence channel to obtain net fluorescence units.

3. 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 (GraphPad Prism®, SigmaPlot®, etc.). It is also instructive to graph net luminescence and net fluorescence signals versus time at a specific compound concentration.

4.E. Data Interpretation

Apoptosis Inducers

Compounds that induce apoptosis will produce time- and dose-dependent increases in luminescence (Annexin V fusion protein binding to exposed PS), which precede temporal increases in fluorescence due to secondary necrosis (loss of membrane integrity). This kinetic difference in the emergence of the signals is the hallmark of the apoptotic phenotype.

Note: In cell models that use less than 500 cells/well and/or produce relatively minor phenotypic changes (<5% cell death), the intrinsic sensitivity of the luminescence signal may make annexin-binding luminescence measurable without detectable increases in fluorescence. Data interpretation may be skewed toward an apoptotic determination due to inherent sensitivity differences. In these instances, the use of orthogonal methods for defining mechanism of action are strongly encouraged (see Section 6.C).

Non-Apoptotic Inducers of Cell Death

Compounds that produce time- and dose-dependent increases in luminescence concurrently with increases in fluorescence are not consistent with apoptosis and likely involve alternative forms of programmed cell death. Orthogonal assays for apoptosis markers, such as caspase activation, can be multiplexed in the same well to confirm the occurrence of apoptosis (see Section 6.C).

4.F. Example Data

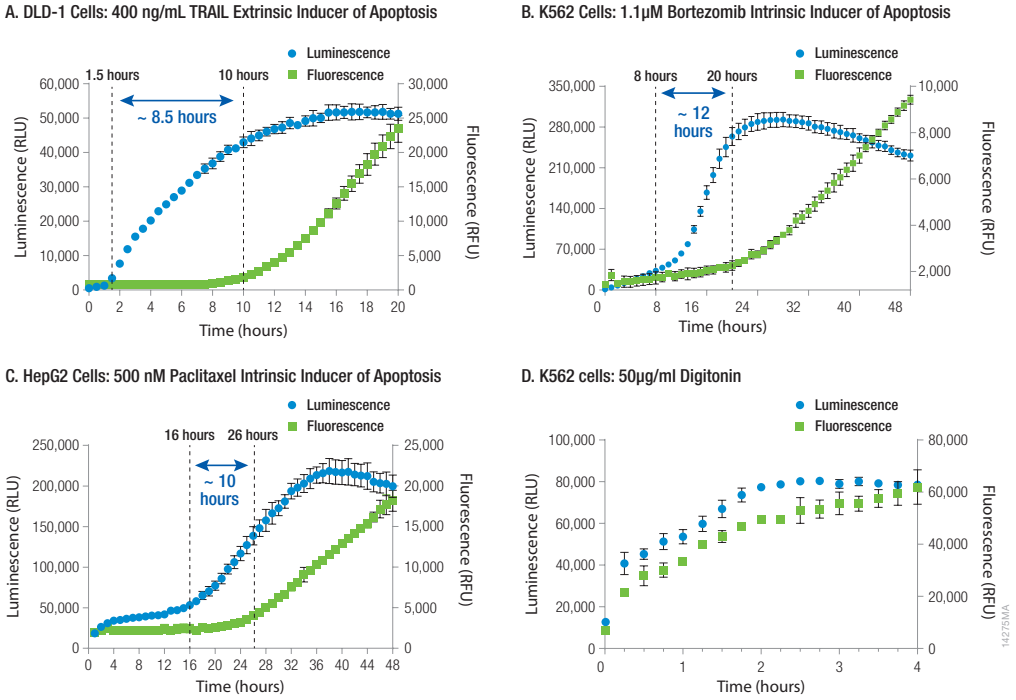

Figure 4. Sequentially repeated measures of luminescence (RLU) and fluorescence (RFU) resolve the kinetic effects of different cytotoxic stimuli and help establish mechanism of action. Panel A. DLD-1 cells treated with 400ng/ml rhTRAIL. **Panel B.** K562 cells treated with 1.1µM bortezomib. **Panel C.** 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.

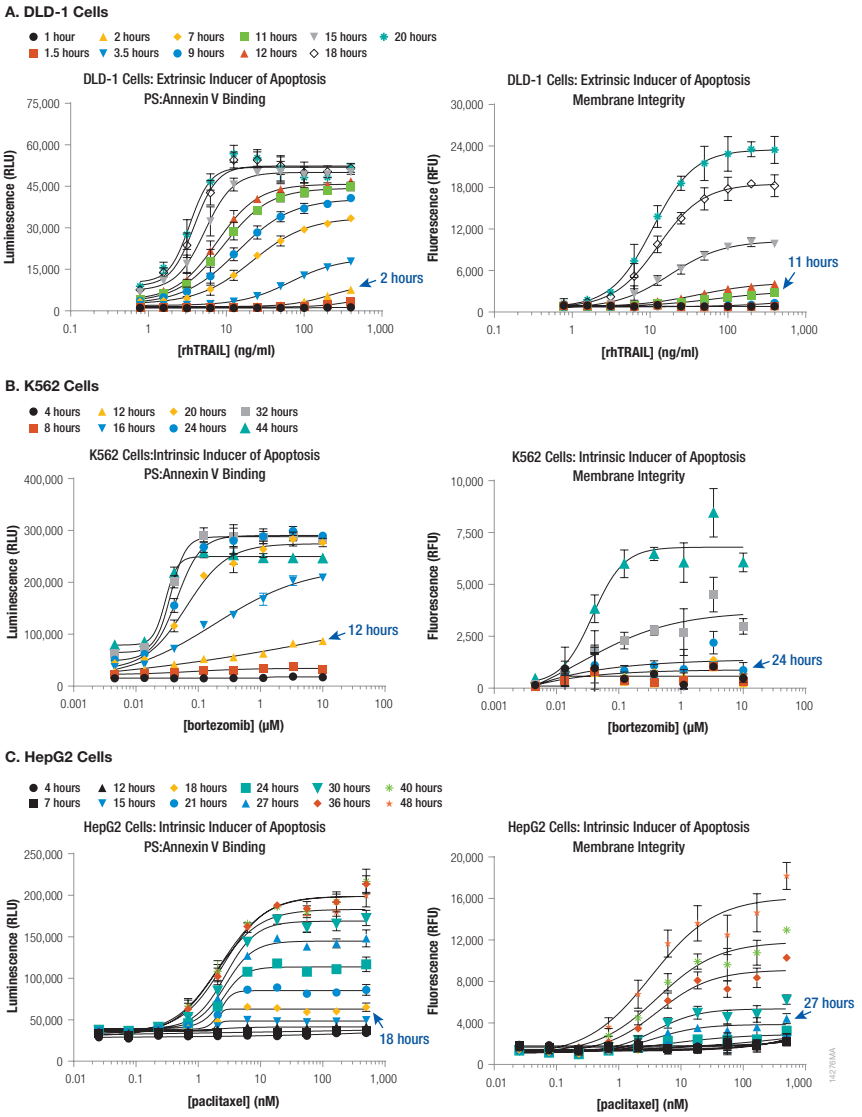

Figure 5. Repeated measures of luminescence and fluorescence during compound exposure define magnitude of response and kinetics of potency. DLD-1 (**Panel A**), K562 (**Panel B**) and HepG2 (**Panel C**) were exposed to serial dilutions of rhTRAIL, bortezomib and paclitaxel, respectively, in the presence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. Plates were incubated in a plate reader equipped with an atmospheric control unit at 37°C/5% CO_2. RLU (Left panels, PS:Annexin V binding) and RFU ($485nm_{Ex}/520–30nm_{Em}$) (Right panels; membrane integrity) were collected at indicated times (**Panel A**) or every 60 minutes over a 48-hour time course (**Panels B and C**). Data represent the mean of 4 readings for each replicate ± SD.

5. References

1. Martin, S., *et al.* (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182**, 1545–56.
2. Gidon-Jeangirard, C., *et al.* (1999) Annexin V delays apoptosis while exerting an external constraint preventing the release of CD4+ and PrPc+ membrane particles in a human T lymphocyte model. *J. Immunol.* **162** (10) 5712–18.
3. Monceau, V., *et al.* (2004) Externalization of endogenous annexin A5 participates in apoptosis of rat cardiomyocytes. *Cardiovsc. Res.* **64 (3)**, 496–506.
4. van Genderen, H.O., *et al.* (2008) Extracellular annexin A5: Functions of phosphatidylserine-binding and two dimensional crystallization. *Biochimica et Biophysica Acta* **1783**, 953–63.

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6. Appendix

6.A. Factors Influencing Real-Time Signal Intensity and Sustainability

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is configured to produce signal intensities proportional to the magnitude of the PS- exposure for up to 48 hours. Careful consideration of standard optical constraints and attention to potential kinetic differences in PS exposure can improve luminescent and fluorescent detection efficiencies.

Optical Considerations

Phenol red in growth or maintenance medium acts as an optical quencher of both fluorescence and luminescence, reducing light output and assay dynamic range. Although typically unnecessary, reducing or eliminating phenol red may improve assay performance.

Tissue culture-treated solid white or white, clear-bottom plates should be used with the assay. Solid white plates will produce higher intensity signals versus clear-bottom plates, and may improve dynamic range. Likewise, assay plate lids reduce unintended environmental contamination and evaporation, but often reduce both excitation and emission efficiency. Although signal intensity can be improved by removing the lid during data collection, such exposure times should be minimized. Removal of plate lids may not be required, depending on the model of plate reader used.

Kinetics of PS Exposure

PS exposure as a result of primary necrosis, immune cell or antibody-directed cytolytic function, apoptosis or alternative cell death mechanisms may occur at any time between initiating the exposure and 72 hours. The 2X Detection Reagent contains a time-released luciferase substrate that is dependent upon inherent cellular esterase activity inside viable cells. In most cell types, steady-state generation of the luciferase substrate (from the prosubstrate) occurs at about 2 hours and persists at a consistent rate for at least 48 hours. For an optimal luminescent signal response with early inducers of PS exposure, we recommend that the 2X Detection Reagent be introduced to test cells 2 hours prior to treatment for induction of apoptosis.

The magnitude and kinetics of the induction stimulus may also affect luminescent signal persistence. Extremely effective inducers that provoke cell death in short time frames (<3–6 hours) may substantially limit the luminescent signal intensity at 48 hours. For inducers that mediate PS exposure near or after 48 hours, it may be necessary to normalize the data collected at each time point to the untreated control luminescence.

In rare instances with sensitive cell types, calcium added in the 2X Detection Reagent may provoke transient PS exposure that does not lead to cell death. This transient exposure may complicate data interpretation. Exogenous calcium can be omitted from the 2X Detection Reagent to resolve this effect.

6.B. Alternative Protocol for Restrictive Volumes

This alternative protocol describes how to deliver components of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay when the size of the wells is not large enough to hold the recommended volumes.

Necessary adjustments must be made for initial cell density to accommodate proliferation and normal cell health maintenance over 48-hour exposures. Reduced volume environments are more susceptible to metabolic depletion of nutrients, metabolic accumulation of waste, evaporation and artifactual edge-effects.

1. Determine the final volume of cells to add to control and test wells. Allow for unrecoverable void volumes in tubes and reagent reservoirs. Typically, 6ml is sufficient for a full 96-well plate.
2. Aliquot the desired volume of cells at 100,000–200,000 cells/ml (125,000–250,000 cells/ml for 384-well plate) in complete medium to a sterile mixing vessel with cap. Add 1,000X Annexin V NanoBiT® Substrate to the cells at a 500-fold dilution (12µl per 6ml for a full 96-well plate). Mix by inversion. **Do not vortex**.
3. Add 1,000X $CaCl_2$ to the cells containing Annexin V NanoBiT® Substrate at a 500-fold dilution (12µl per 6ml for a full 96-well plate). Mix by inversion. **Do not vortex**.
4. Add 1,000X Necrosis Detection Reagent at a 500-fold dilution (12µl per 6ml for a full 96 well plate). Mix by inversion. **Do not vortex**.
5. Add 1,000X Annexin V-SmBiT and 1,000X Annexin V-LgBiT at a 500-fold dilution to cells containing substrate, $CaCl_2$ and Necrosis Detection Reagent as described in Steps 2–4, above (12µl of each protein per 6ml for a full 96-well plate). Mix by careful inversion. **Do not vortex or create bubbles**.
6. Add 50µl of the 2X cell/reagent solution to all wells of a 96-well plate (except for column 12).
7. Create a cell-free, fluorescent reagent background control by adding 2µl of Necrosis Detection Reagent to 1.0ml of complete medium, and add 50µl to each well of column 12.
8. Add 0.5ml of complete medium to channels 2–12 of two sterile 12-channel troughs. One will be used for the control and one for the test compound. To separate 1ml aliquots of complete medium, add control or test compound to 2X the desired starting concentration (for example, if the desired starting concentration in the titration series is 10µM, prepare a 20µM solution). It may be necessary to make an intermediate dilution of the compound in DMSO in order to dilute down to the desired 2X starting concentration. Add 1ml of complete medium containing the 2X control or test compound to channel 1 in the appropriate trough. For both compounds, perform a 10-point, twofold serial dilution from channels 1 to 10. Leave channels 11 and 12 as "no compound controls".
9. Using the appropriate 12-channel trough and a 12-channel multichannel pipette, transfer 50µl of the appropriate 2X concentrated, 10-point, twofold serial dilution to the appropriate replicate wells in the 96-well assay plate already containing cells. See the plate layout in Figure 3. Add the control compound to replicate wells in rows A–D and test compound to replicate wells in rows E–H. Each well should now contain 100µl. Be sure to follow the plate layout in Figure 3 and add the serial dilutions from high to low concentration as listed (highest concentration to lowest concentration from column 1 to column 10). Columns 11 and 12 should contain no compound as controls (vehicle controls).
10. Incubate and record luminescence and fluorescence measurements by one of the three methods described in Section 3.E.

6.C. Multiplexing with Other Assays

Complementary and/or orthogonal cell health assay chemistries from Promega can be added in same-well multiplexes with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay. The data obtained from these assays may reveal anti-proliferative effects not related to cytotoxicity or provide further evidence of the putative mechanism of action for a compound. In most cases, these assay chemistries can be applied as described using their standard protocols or with small variations in the volume added per well. This appendix describes three multiplexing protocols as examples of how multiplexed chemistries can be applied: 1) Caspase-Glo® 3/7 Assay; 2) CellTiter-Glo® 2.0 Cell Viability Assay; and 3) CellTiter-Fluor™ Cell Viability Assay.

Note: The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is a live cell (non-lytic) real-time (kinetic) assay. The following examples of multiplexed chemistries are endpoint assays. Therefore, it is imperative to conduct the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay first and kinetically monitor resulting signals for the desired time course prior to applying the following multiplexed chemistries in endpoint fashion.

Multiplex of RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay with Caspase-Glo® 3/7 Assay

The Caspase-Glo® 3/7 Assay (Cat.# G8090, G8091, G8093, G8092) measures caspase-3/7 activity (an orthogonal apoptosis biomarker) in a cell population. The presence of measurable caspase-3/7 activity is considered definitive for the apoptotic phenotype and can be used to clarify ambiguity or provide evidence to support results obtained with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay.

1. Perform the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, and monitor RLU (PS:Annexin binding) and RFU (membrane integrity) as described in Section 4.C. Monitor kinetically for the desired time course. The final assay volume is 200µl/well for a 96-well plate.
2. Prepare the reconstituted Caspase-Glo® 3/7 Reagent as described in the *Caspase-Glo® 3/7 Assay Technical Bulletin* #TB323.
3. Add 100µl of Caspase-Glo® 3/7 Reagent to all sample and control wells in the 96-well assay plate. The final assay volume per well should now be 300µl/well for a 96-well plate.

Note: The addition of 100µl Caspase-Glo® 3/7 Reagent to wells already containing 200µl cells + compound + RealTime-Glo™ Annexin V and Necrosis Detection Reagent deviates from the standard 1:1 protocol recommended in the Caspase-Glo® 3/7 Technical Bulletin. Although the deviation has been validated to produce robust, acceptable results, users can alternatively use the restrictive volume procedure described in Section 6.B of this Technical Manual to achieve a 1:1 ratio.

4. Gently mix the contents of the wells by shaking at 300–500rpm in a plate shaker for 30 seconds. Incubate at room temperature for 30 minutes to 3 hours, depending on the cell culture system.
5. Record luminescence using a luminometer.

Note: Any luminescence associated with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is quenched by the lytic detergents in the Caspase-Glo® 3/7 Reagent. Therefore, any luminescent signal (above untreated cell control) arising from the multiplex is directly attributable to caspase activity for each concentration of compound.

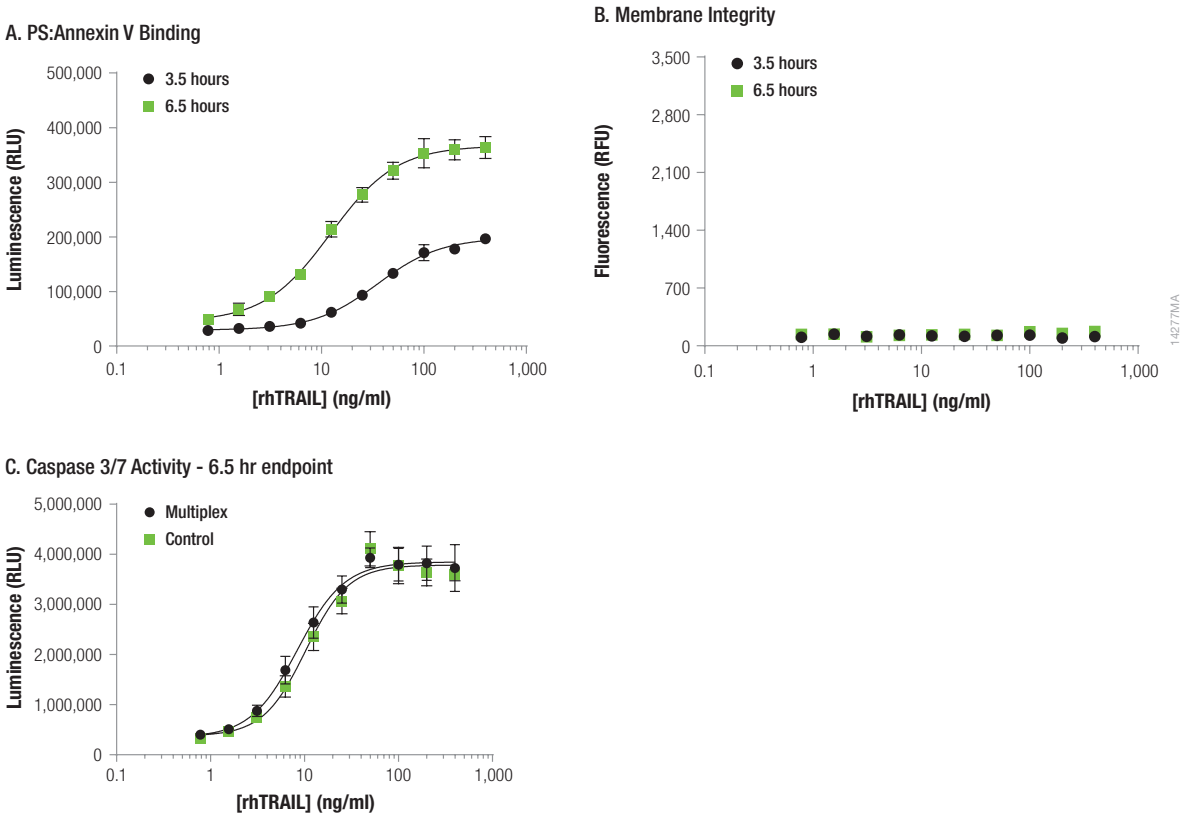

Figure 6. Example multiplex data with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay and Caspase-Glo® 3/7 Assay. DLD-1 cells were exposed to serial dilutions of rhTRAIL in the presence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. The plate was incubated at 37°C/5% CO_2 and luminescence (**Panel A,** PS:Annexin V binding) and fluorescence (485nm$_{Ex}$/520–30nm$_{Em}$) (**Panel B,** membrane integrity) was measured at 3.5 hours (black circles) and 6.5 hours (green squares). Following the 6.5-hour RealTime-Glo™ Annexin V Apoptosis and Necrois Assay read, Caspase-Glo® 3/7 Reagent was added to wells as described in Section 6.C. in a multiplex format (**Panel C**, caspase-3/7 activity, black circles). As a control, parallel wells of DLD-1 cells were exposed to the same serial dilutions of rhTRAIL in the absence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent, and 100µl of Caspase-Glo® 3/7 Reagent was added at 6.5 hours (**Panel C**, caspase-3/7 activity, green squares). The Caspase-Glo® 3/7 Assay data (**Panel C**) demonstrates absolute concordance in signal magnitude and potency, demonstrating chemical compatibility and providing an orthoganol measure of apoptosis directly in the same sample well. Data represent the mean of 4 readings for each replicate ± SD.

6.C. Multiplexing with Other Assays (continued)

Multiplex of RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay with CellTiter-Glo® 2.0 Cell Viability Assay

The CellTiter-Glo® 2.0 Assay (Cat.# G9241, G9242, G9243) detects the ATP content of viable cells via the firefly luciferase reaction. The luminescence generated correlates to viable cell number.

1. Perform the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, and monitor luminescence and fluorescence as described in Section 4.C. Monitor kinetically for the desired time course. The final assay volume per well is 200µl/well for a 96-well plate.
2. Prepare the CellTiter-Glo® 2.0 Reagent as described in the *CellTiter-Glo® 2.0 Assay Technical Manual* #TM403.
3. Add 100µl of CellTiter-Glo® 2.0 Reagent to all sample and control wells in the 96-well assay plate. The final assay volume per well should now be 300µl/well for a 96-well plate.

Note: The addition of 100µl CellTiter-Glo® 2.0 Reagent to wells already containing 200µl cells + compound + RealTime-Glo™ Annexin V and Necrosis Detection Reagent deviates from the standard 1:1 protocol recommended in the CellTiter-Glo® 2.0 Technical Manual. Although the deviation has been validated to produce robust, acceptable results, users can alternatively use the restrictive volume procedure described in Section 6.B of this Technical Manual to achieve a 1:1 ratio.

4. Gently mix the contents of the wells using a plate shaker at 300–500rpm for 2 minutes. Incubate at room temperature for 10 minutes.
5. Record luminescence using a luminometer.

   Note: Luminescence associated with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay is quenched by the lytic detergents in the CellTiter-Glo® 2.0 Reagent. Therefore, any luminescent signal arising from the multiplex is directly attributable to ATP content (viable cells) for each concentration of compound.

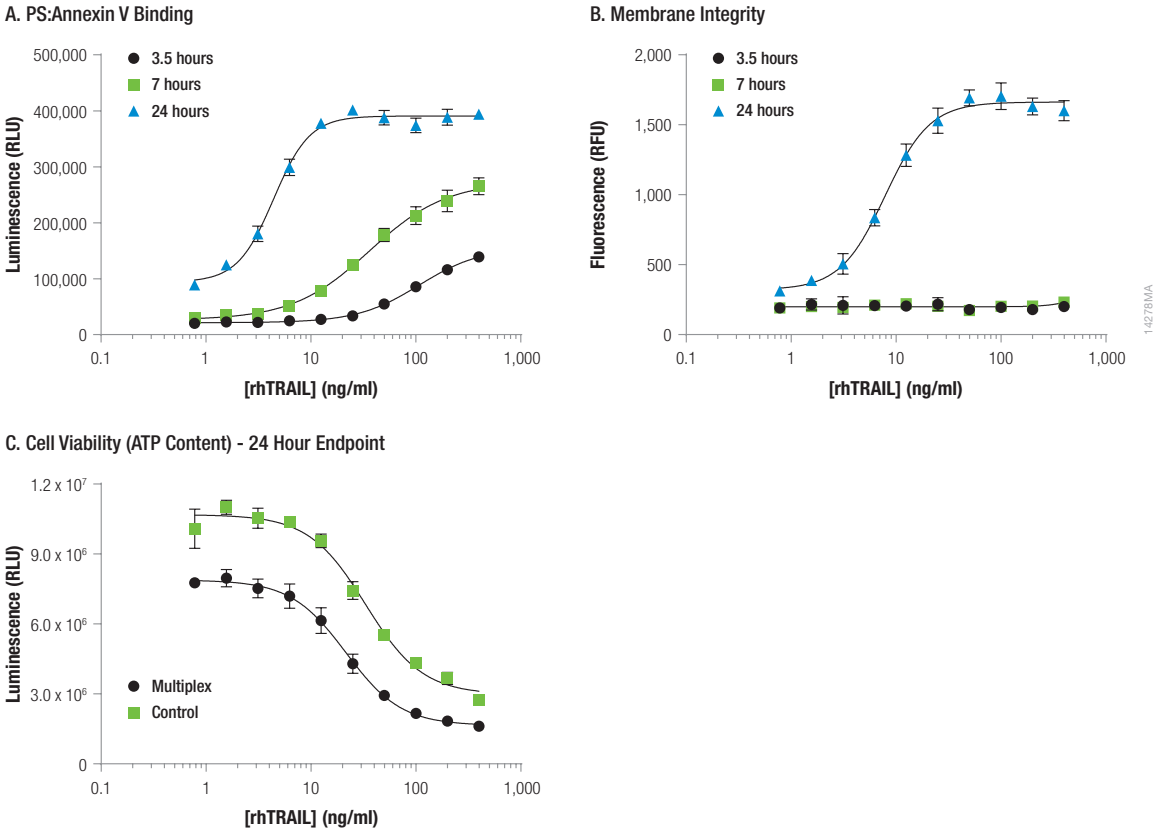

Figure 7. Example multiplex data with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, and CellTiter-Glo® 2.0 Assay. DLD-1 cells were exposed to serial dilutions of rhTRAIL in the presence or absence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. The plate was incubated at 37°C/5% CO_2 and luminescence (**Panel A**, PS:Annexin V binding) and fluorescence ($485nm_{Ex}/520–530nm_{Em}$) (**Panel B**, membrane integrity) was collected kinetically at 3.5 hours (black circles), 6.5 hours (green squares), and 24 hours (blue triangles). Following the 24-hour RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay read, CellTiter-Glo® 2.0 Reagent was added to wells according to Section 6.C in a multiplex format (**Panel C**, cell viability, black circles). As a control, parallel wells of DLD-1 cells were exposed to the same serial dilutions of rhTRAIL in the absence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. CellTiter-Glo® 2.0 Reagent (100µl) was added at 24 hours and RLU recorded following 2 minutes mixing on a plate shaker and 10 minutes incubation at room temperature (**Panel C**, cell viability, green squares). Although the multiplexed and control assay values vary in terms of luminescence magnitude, their potency values are concordant. This concordance demonstrates chemical compatibility of the multiplex and confirms toxicity. Data represent the mean of 4 readings for each replicate ± SD.

6.C. Multiplexing with Other Assays (continued)

Multiplex of RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay with CellTiter-Fluor™ Cell Viability Assay

The CellTiter-Fluor™ Assay (Cat.# G6080, G6081, G6082) is a non-lytic, single-reagent-addition fluorescence assay that measures a conserved and constitutive protease activity within live cells, serving as a marker of cell viability. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (GF-AFC). The fluorescence generated correlates to viable cell number.

1. Perform the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, and monitor luminescence and fluorescence according to Section 4.C. Monitor kinetically for the desired time course.
2. Prepare 5X CellTiter-Fluor™ Reagent as described in the *CellTiter-Fluor™ Cell Viability Assay Technical Bulletin* #TB371. For example, for every 2ml of Assay Buffer, add 10µl of GF-AFC Substrate.
3. Add 40µl of the 5X CellTiter-Fluor™ Reagent to all sample and control wells in the 96-well assay plate.
4. Briefly mix the contents of the wells using a plate shaker at 300–500rpm. Incubate at 37°C for at least 30 minutes, but not longer than 3 hours.
5. Record fluorescence (380–400nm$_{Ex}$/505nm$_{Em}$) using a fluorometer. Adjust the instrument gain appropriately.

   Note: The excitation/emission properties (380–400nm$_{Ex}$/505nm$_{Em}$) of the CellTiter-Fluor™ Reagent (GF-AFC) will not interfere with the excitation/emission properties (485nm$_{Ex}$/520–30nm$_{Em}$) of the Necrosis Detection Reagent component in the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay.

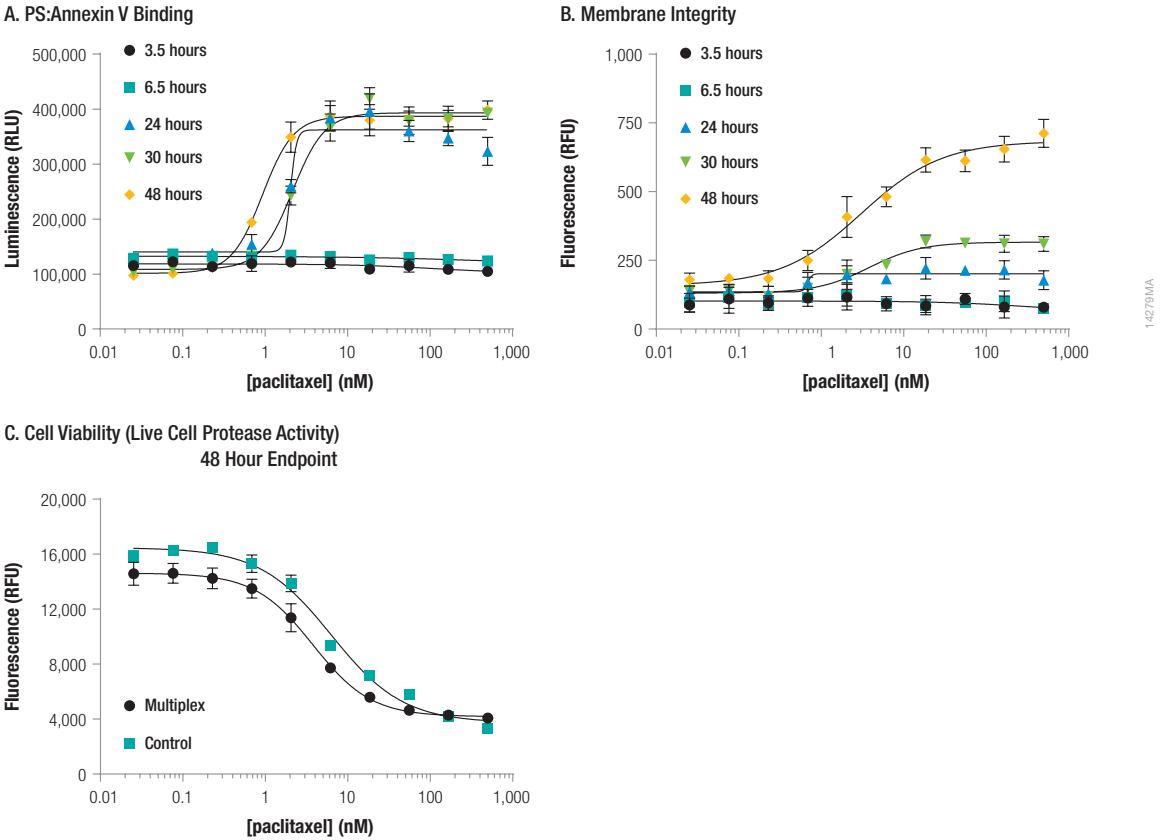

Figure 8. Example multiplex data with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay and CellTiter-Fluor™ Assay. HepG2 cells were exposed to serial dilutions of paclitaxel in the presence or absence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. The plate was incubated at 37°C/5% CO_2 and luminescence (**Panel A**, PS:Annexin V binding) and fluorescence (485nm$_{Ex}$/520–30nm$_{Em}$) (**Panel B**, membrane integrity) was collected kinetically at 3.5 hours (black circles), 6.5 hours (blue squares), 24 hours (blue triangles), 30 hours (green triangles), and 48 hours (orange diamonds). Following the 48-hour RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay read, 5X CellTiter-Fluor™ Reagent was added to wells according to Section 6.C. in a multiplex format (**Panel C**, cell viability, black circles). As a control, parallel wells of HepG2 cells were exposed to the same serial dilutions of paclitaxel in the absence of the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Reagent. 5X CellTiter-Fluor™ Reagent (40µl) was added in endpoint fashion at 48 hours (**Panel C**, cell viability, blue squares). The signal magnitude and potency values from the multiplex and control assays are nearly concordant, suggesting chemical compatibility and utility of the CellTiter-Fluor™ multiplex. Data represent the mean of 4 readings for each replicate ± SD.

6.D. Related Products

RealTime Assays

Product	Size	Cat.#
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
	10 × 100 reactions	G9712
	1,000 reactions	G9713

Product	Size	Cat.#
CellTox Green™ Cytotoxicity Assay	10ml	G8741
	50ml	G8742
	100ml	G8743

Caspase Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	10 × 10ml	G8093
	100ml	G8092
Caspase-Glo® 2	10ml	G0940
Caspase-Glo® 6	10ml	G0970
Caspase-Glo® 8*	10ml	G8201
Caspase-Glo® 9*	10ml	G8211

*Additional sizes available.

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® 2.0 Assay	10ml	G9241
	100ml	G9242
	500ml	G9243
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
	5 × 10ml	G6081
	2 × 50ml	G6082

Multimode Plate Readers

Product	Cat.#
GloMax® Discover System	GM3000
GloMax® Explorer System	GM3500

[a]Licensed under U.S. Pat. No. 6,949,350.

[b]Patents Pending.

[c]U.S. Pat. No. 8,809,529, European Pat. No. 2635582B1, and other patents and patents pending.

[d]U.S. Pat. Nos. 8,598,198 and 9,458,499 and other patents and patents pending.

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