Real-Time Assessment of Apoptosis and Necrosis

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- **CLARIOstar® with ACU provides a system where real-time measurements can be performed in a controlled environment**
- **The multiplexed RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent detects phosphatidylserine exposure events associated with apoptosis while also reporting loss of membrane integrity resulting from necrosis**

Introduction

Although cell death is an important, normal consequence of multicellular homeostasis it also has an unfortunate role in many human diseases. Discovery of targeted cytotoxic and cytoprotective agents may therefore have important implications in treating cancer, neurodegeneration and a litany of other diseases. In order to fully appreciate the dysregulation in a disease model, and responses to experimental stimuli, it is important establish the mechanism of action of cell death and define response kinetics. Traditional experimental approaches for addressing mode of cell death are often time consuming, labor intensive, costly and inadequate.

Here we describe the monitoring of apoptosis and necrosis in real time. Promega's new RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay provides the simplicity of an add-mix-measure assay, in a live-cell, real-time format. While the CLARIOstar with atmospheric control unit (ACU) provides the sensitive detection of duplexed luminescent and fluorescent signals, all while maintaining the appropriate [O_2] and [CO_2] environment for your cells. This combination will allow you to walk away once the test is set up and ensures that you will not miss important cellular responses regardless of when changes happen.

Assay Principle

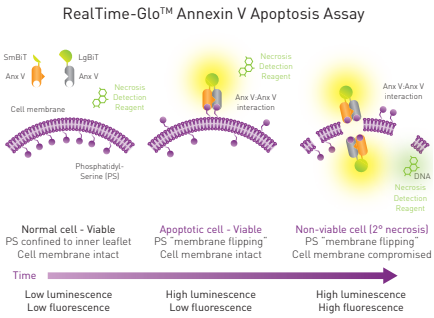

Fig. 1: **RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay Principle.** Two Annexin V – fusion proteins harboring binary subunits of a luciferase enzyme (NanoBiT™) are introduced into a sample and produce no luminescence with healthy cells. Upon binding to PS the complemented enzyme is now capable of producing a light signal. Changes in membrane integrity are monitored with the pro-fluorescent Necrosis Detection Reagent probe.

Translocation of phosphatidylserine (PS) from the inner to outer membrane leaflet is considered a hallmark of healthy cells transitioning to apoptosis[1]. Multiple assays take advantage of Annexin V binding to PS to identify cells beginning apoptosis.

The RealTime-Glo™ Annexin V Apoptosis and Necrosis assay utilizes annexin-fusion proteins that contain binary subunits of a luminescent enzyme[2] (NanoBiT™) which are drawn into complementing proximity only due to their affinity for PS. In the presence of a time-released substrate, the complemented enzymes report real-time PS exposure. The Necrosis Detection Reagent reports changes in membrane integrity as a result of necrosis. Together, these real-time measures establish the mechanism of action (apoptosis, primary necrosis, or alternative programs) for cell death.

Materials & Methods

- RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega, Cat #JA1011)
- CLARIOstar® with ACU (BMG LABTECH)
- White, clear or opaque bottom, 96-well plates (Costar)
- K562 cells from ATCC®
- Negatively-selected Natural killer (NK) cells from Lonza
- Other reagents from commercially available sources

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay reagent was prepared as indicated in the technical manual and added at the time of dosing or treatment.

For the dose response experiments, an 8 point dilution series of bortezomib with a maximum concentration of 10 µM was applied to K562 cells pre-seeded at 10,000 cells per well. A vehicle treatment and cell-free blank were also included.

For NK cell experiments, NK cells were stimulated with IL-15 then added to K562 cells in the indicated ratios. NK or K562 cells alone served as exposure controls. Measurement and incubation were accomplished with the CLARIOstar microplate reader using a script and the following settings.

Instrument settings

RealTime-Glo™ Annexin V Apoptosis detection		
Optic settings	Luminescence, bottom optic	
	Filters	No filters
	Gain	3500
General settings	Measurement interval time	1.0 s

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Necrosis detection		
Optic settings	Fluorescence intensity, top optic	
	Filters	Excitation 482-16 Diochroic LP504 Emission 530-40
	Gain	Adjusted prior to run
General settings	Settling time	0.2 s
	No. of flashes	40
Kinetics and atmospheric control		
Kinetic settings	Number of cycles and cycle times are indicated individually for each experiment	
Incubation	37 °C	
ACU	5 % CO_2 and monitoring of oxygen	

Results & Discussion

The CLARIOstar with ACU enabled the real-time duplexed detection of a luminescent and fluorescent signal every hour throughout the 48 hour exposure to bortezomib (49 cycles). In figure 2 the PS-exposure response (luminescence) precedes the necrotic response (fluorescence) indicating the expected apoptotic mechanism for this therapeutic proteasome inhibitor.

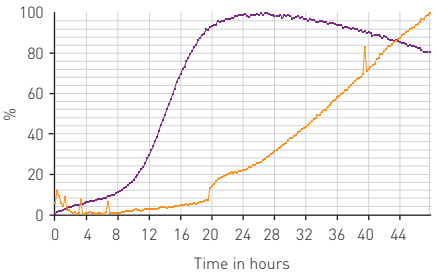

Fig. 2: Simultaneous measurement of PS positivity (purple) and membrane integrity (orange).

Figure 3 illustrates time-dependent dose response curves that can be generated from a single experiment. The results reinforce the relationship between dose and time with respect to response magnitude. Similar results were generated to examine the change in membrane integrity as a result of the secondary necrotic response (data not shown).

The final example explored the innate ability of stimulated natural killer (NK) cells to target, engage and induce apoptosis in a K562 cancer cell population. Specifically, kinetic readings of the admixed cells were taken every 5 minutes over a two hour time course (25 cycles). The data indicated that PS-exposure and cell death proceeded in a NK cell number dependent manner with a maximal response to a high ratio of NK cells obtained at 2 hours (data not shown).

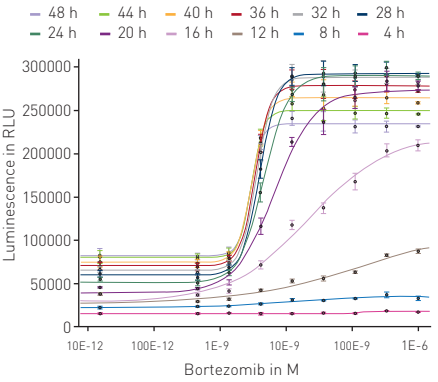

Fig. 3: **Time and dose-dependent on set of apoptosis.** Dose-response curves were generated at the indicated time points for treatment with bortezomib (n = 4). PS exposure as a result of apoptosis is measurable beginning at 12hr and progresses with increasing potentcy until 24hr. Luminescence is maintained thereafter, offering a persistent view of the totality of the apoptotic response

Figure 4 displays the correlation between increasing the ratio of NK cells to target cells and an increase in luminescent signal indicative of apoptosis.

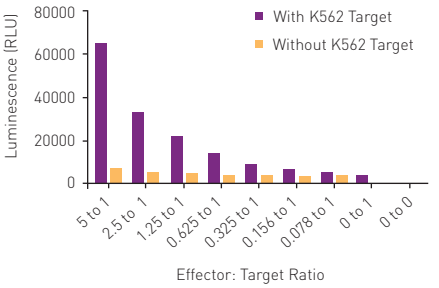

Fig. 4: **Natural Killer Activity Assay.** The RealTime-Glo™ Annexin V Apoptosis Assay result indicate a significant killing capability even at low effector: target ratio, but a clear enhancement as the ratio increases.

Conclusion

The results show a combination of a novel assay methodology and instrumentation that allows the user to truly walk away and return to outstanding results. The real-time nature of the results allows you to capture information that would require extensive effort to be achieved using previous apoptosis assay techniques.

References

1. S.J. Martin et al. (1995) *J. Exp. Med.* **182**, 1545-1556.
2. A. S. Dixon et al. (2016) *ACS Chem. Biol.* **11**, 400-408.