technically speaking

Cell-Based Caspase Assays: Analyzing the Data

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Abstract

Caspase assays have been dramatically improved over the past few years. Previously, cell-based caspase assays were relatively insensitive and required the preparation of concentrated cell extracts. Today, caspase assays are extremely sensitive; they can be performed directly in multiwell cell culture plates, and there are both luminescent and fluorescent versions of these homogeneous assays. Along with these improvements, new considerations have arisen that require careful attention when analyzing data. There are several features about cell systems that need to be taken into account when designing an apoptosis paradigm and when analyzing results from homogeneous caspase assays. Several common questions regarding the luminescent Caspase-Glo™ Assays highlight these features.

The sensitivity of the Caspase-Glo™ 3/7 Assay allows detection of caspase activity in serum alone and results in luminescent signal from untreated cells that is well within the linear range of the assay.

Q Why is the luminescent "background" signal in my untreated cells so high?

Apoptosis occurs at a spontaneous rate in all cultured cells. Transformed, immortalized cells have a high rate of proliferation. There is some indication that an associated high rate of apoptosis may be an adaptive mechanism of transformed cells. In a nutrient-restricted environment this may help stabilize the viable cell number (1). Regardless of the mechanism, the signal in the untreated cells is primarily caspase activity, not background as defined as nonspecific signal. It is important to remember that there is specific caspase activity in untreated cells and their activity can be differentiated from the background by running a "no-cell" control. Measuring the signal in culture medium without cells is a more accurate reflection of background independent of the spontaneous apoptosis. A primary source of luminescent signal that is independent of the cells comes from serum. Serum has caspase-3/7-like activity that can be eliminated by caspase-selective inhibitors. The amount of caspase activity in serum or in a typical untreated cell population in a multiwell plate would be below or near the limit of detection by previous caspase assay methods. However, the sensitivity of the Caspase-Glo™ 3/7 Assay[a,b] allows detection of caspase activity in serum and results in luminescent signal from untreated cells that is well within the linear range of the assay. The following example illustrates how the sensitivity of the luminescent Caspase-Glo™ 3/7 Assay makes the signal in untreated cells appear relatively high. A comparison of the Caspase-Glo™ Assay to the Apo-ONE® Assay shows that the luminescent signal in the untreated cells is clearly distinguished from the no-cell control signal, whereas the fluorescent signal in the untreated cells is only slightly above the no-cell control fluorescent signal. Figure 1 shows that untreated cells clearly have caspase-3/7 activity.

Table 1. The Caspase-Glo™ Assay detects significant caspase-3/7 activity in untreated cells. Jurkat cells grown in RPMI-1640 supplemented with 10% fetal bovine serum were treated with anti-Fas (100ng/ml) or left untreated. Cells were plated at 10,000 cells/well in 96-well plates. After a 4-hour anti-Fas treatment, either the Caspase-Glo™ 3/7 Reagent or the Apo-ONE® Homogeneous Caspase-3/7 Reagent was added to both cell populations as well as control wells containing cell culture medium only. After 1 hour, the optimum time for the luminescent assay, readings were taken on a luminometer or fluorometer appropriate for each assay. RLU = relative light units, RFU = relative fluorescent units, S.D. = standard deviation.

	Caspase-Glo™ Assay (RLU)			Apo-ONE® Assay (RFU)		
	Treated	Untreated	No Cells	Treated	Untreated	No Cells
RLU or RFU	143,757.5	17,380	2,136.3	2,804.8	1,565.3	1,419
S.D.	2663.5	719.6	82.3	235.7	30.8	38.1

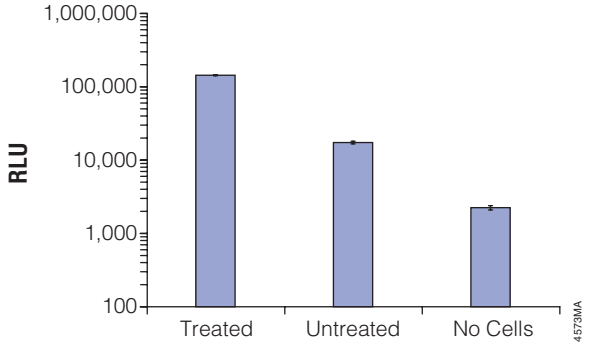

Figure 1. Untreated cells in culture have significant caspase-3/7 activity. A log plot of the Caspase-Glo™ Assay results from Table 1.

Q How do I compare the Caspase-Glo™ Assay to other methods? The signal-to-background ratio appears the same between methods.

In the above Jurkat cell example, different parameters can be analyzed to assess the assays. Both signal-to-background and signal-to-noise ratios are standard means of assessing cell assays and each provides unique information. Signal-to-background ratio is defined as the mean signal/mean background. However, as discussed earlier, background in a cell system can be defined in different ways. In a cell system, signal-to-background ratio would typically be defined as:

$$\frac{\text{(mean signal of the treated cells) – (no-cell control)}}{\text{(mean signal of the untreated cells) – (no-cell control)}}$$

Using the above definition, the signal-to-background ratios for the Jurkat cell data for the Caspase-Glo™ Assay and the Apo-ONE® Assay are 9.3 and 9.5, respectively. This is illustrated in Figure 2. Although the absolute values are different, when the nonspecific background is subtracted, the signal-to-background ratios are the same. This indicates that both methods show an equivalent caspase induction rate. The absolute values will increase over time for the fluorescent assay, which is in contrast to the luminescent assay.

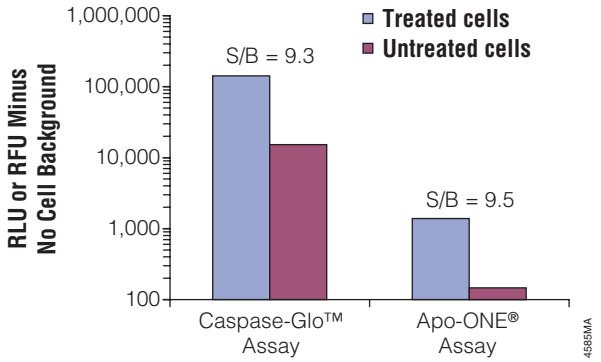

Figure 2. The signal-to-background ratios are the same for the luminescent and fluorescent caspase-3/7 assays. The "no-cell" background was subtracted from both the treated and untreated cell results from Table 1.

The signal-to-background ratio primarily provides information on the rate of induction of caspase activity in the experimental system. The signal-to-background ratio between treated and untreated cells (with the no-cell controls subtracted) should be the same across methods because the caspase induction rate is dependent on the biology of the system and independent of the chemistry of the assays. Signal-to-background ratios are very useful for assesing a cellular paradigm or monitoring the drug effects on caspase activation. The signal-to-background ratio does not provide information about the sensitivity of the assay, nor does it provide any information on the variability in the assay. For comparing sensitivities between assays, the signal-to-noise ratio is more informative. The signal-to-noise ratio is defined as (2):

$$\frac{\text{mean signal – mean background}}{\text{standard deviation of the background}}$$

Signal-to-noise ratios account for the background variability in the assays. By dividing by the standard deviation of the background, the signal-to-noise ratio also serves to normalize data from various instruments with different relative units. Depending on how background is defined in a cell system, the signal-to-noise ratio can be calculated in three ways. All three calculations can provide information about the system. Table 2 shows what these definitions can mean.

The signal-to-noise ratios in examples 2 and 3 of Table 2 compare the treated or untreated cells to the "no-cell" background, respectively. These measure caspase activity relative to nonspecific activity for both cell populations and thus provide a measure of sensitivity. At the 1 hour time point, the luminescent assay has higher signal-to-noise ratios. With longer incubation times, the signal-to-noise ratios for the fluorescent assay would also increase. The limit of detection for a caspase assay can be defined as a signal-to-noise ratio of 3 (i.e., the amount of caspase activity giving a value at least 3 standard deviations above the background). In both examples shown in Table 2, the signal-to-noise ratios indicate that the caspase-3/7 activity in 10,000 untreated cells is above the limit of detection for both assays (Caspase-Glo™ Assay = 185, Apo-One® Assay = 4)

Table 2. Signal-to-Noise Ratio Calculations for the Caspase-Glo™ and Apo-ONE® Assays. Calculations were performed using the data from Table 1.

		Signal/Noise	
		Caspase-Glo™ Assay	Apo-ONE® Assay
1.	$\frac{\text{mean signal of treated cells – mean signal of untreated cells}}{\text{standard deviation of untreated cells}}$	176	40
2.	$\frac{\text{mean signal of treated cells – mean signal of the no-cell control}}{\text{standard deviation of the no-cell control}}$	1,721	36
3.	$\frac{\text{mean signal of untreated cells – mean signal of the no-cell control}}{\text{standard deviation of the no-cell control}}$	185	4

Q How can I improve the signal-to-background or signal-to-noise ratios in my cell system using the Caspase-Glo™ Assay?

There are factors that may affect the above ratios, but it is important to remember that, for the most part, the ratios are defined by the biology of the system. The degree of caspase activation determines the results, and the assay chemistry does not change this. There are three factors that could have a negative impact on the ratios:

1) Saturate the luminescent signal in the treated cells. If the signal reaches the limits of the luminometer, signal-to-background and signal-to-noise ratios will be artificially lowered.

2) If the cells were not healthy, the signal in the untreated cells would be elevated, and the signal-to-background and signal-to-noise ratios would be artificially lowered. It is also true that unhealthy cultures may be induced to undergo apoptosis more readily, so the effect of unhealthy cultures could be quite variable. In general, optimizing the health of the cell cultures during treatment will generate the most consistent results.

3) Reproducibility has a dramatic effect on the signal-to-noise ratios, so making sure that enough replicates are performed to accurately define the variability is important.

There are situations where the experimental conditions can be altered to improve the signal-to-background and signal-to-noise ratios. This leads to a related question.

Q Why am I not seeing a difference between my treated and untreated cells when I know from other methods that there is caspase activity?

Unlike caspase assays using lysates, the Caspase-Glo™ Assays are direct, homogeneous assays, so there is no normalization for protein content or cell number. Parameters of the cell system that may significantly impact the caspase signal include the time of treatment, dose of treatment, and type of drug. Apoptotic cells eventually die and the caspase degrades over time, so the caspase signal may peak and start to fall, decreasing the signal-to-background ratio.

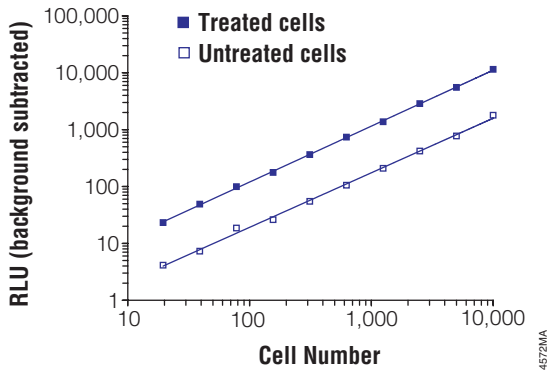

Figure 3. Caspase-3/7 activity is linear with respect to cell number. HeLa cells grown in Opti-MEM® medium supplemented with 1% serum were cultured at 10–10,000 cells/well and induced to undergo apoptosis with the kinase inhibitor, staurosporine (1µM), or treated with a vehicle control. After 4.5 hours of staurosporine treatment, Caspase-Glo™ 3/7 Reagent was added to both cell populations as well as control wells containing cell culture medium only. Luminescence was recorded after 1 hour. The "no-cell" controls were subtracted from both the treated and untreated cell RLU values.

Drugs can have anti-proliferative effects in addition to apoptotic effects. Under these circumstances, the cell numbers per well for the treated cells versus untreated cells may be altered by the time the assay is initiated. Keep in mind that untreated cells have significant spontaneous apoptosis, so as the population increases, the caspase activity also increases. Likewise, if some treated cells have progressed through the apoptotic pathway to death, the caspase activity will decrease. As expected, caspase activity is linear with respect to cell number (Figure 3). The effect of cell number should be considered when an experimental paradigm is being designed. Caspase activity may be masked if the cell numbers in treated and untreated wells are distorted at the time of assay. Ideally, the assay should be initiated at a time point when the caspase activity is at a peak but before the cell number has changed significantly between the treated and untreated cells. With a careful time course and careful dose response curve, it should be possible to identify this time point. Alternatively, it may be necessary to normalize for cell number in parallel wells using an independent method such as cell viability determination assays (i.e., CellTiter-Glo® Assay[a,b], CellTiter-Blue™ Assay, CellTiter 96® AQ$_{ueous}$ One Solution[c] or CytoTox-ONE™ Assay[d]).

How do I calculate the Z´-factor for my cell system using the Caspase-Glo™ Assay, and what will it tell me?

In addition to signal-to-background and signal-to-noise ratios, Z´-factor is widely used to assess the quality of an assay for high-throughput screening. Z´-factor is defined as the following (2):

$$1 - \left[\frac{3\,SD_{positive\ control} + 3\,SD_{negative\ control}}{mean_{positive\ control} - mean_{negative\ control}}\right]$$

For cell caspase assays, the positive control would be the treated cells and the negative control would be the untreated cells. A value > 0.5 is considered an excellent assay. Z´-factor is very informative because it incorporates information about the signal-to-background ratio and accounts for all the variability in the assay (both in the background and in the signal). However, it is important to remember that the Z´-factor is only reflective of the real screen if the conditions for calculating the Z´-factor mimic the real screen. Any conditions that would affect the variability in the assay will affect the Z´-factor.

Conclusions

The sensitivity and direct nature of the new luminescent, homogeneous caspase assays need to be considered when defining a cellular paradigm for apoptosis and when analyzing data. The caspase activity in typical untreated cell populations in single wells of multiwell plates is well within the linear range of the Caspase-Glo™ Assays and is a specific signal. The true background (background coming from noncellular sources) needs to be defined, and this distinction should be clear when analyzing data. Signal-to-background ratios, signal-to-noise ratios and Z´-factor are all useful means of analyzing data and provide distinct information. Because of the direct nature of the assays, there are additional parameters of the cell system that need to be understood to interpret the results accurately. In a dynamic apoptosis system, the time and dose effects of a particular treatment on a given cell population needs to be considered.

References

1. Dedov, V.N., Dedova, I.V. and Nicholson, G.A. (2004) *Cell Cycle 4* (epub ahead of print).
2. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) *J. Biomol. Screen* **4**, 67–73.

Protocols

- *Caspase-Glo*™ 3/7 *Assay Technical Bulletin* #TB323, Promega Corporation (www.promega.com/tbs/tb323/tb323.html)
- *Caspase-Glo*™ 8 *Assay Technical Bulletin* #TB332, Promega Corporation (www.promega.com/tbs/tb332/tb332.html)
- *Caspase-Glo*™ 9 *Assay Technical Bulletin* #TB333, Promega Corporation (www.promega.com/tbs/tb333/tb333.html)
- *Apo-ONE® Homogeneous Caspase-3/7 Assay Technical Bulletin* #TB295, Promega Corporation (www.promega.com/tbs/tb295/tb295.html)

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