IMPROVE THE QUALITY AND SPECIFICITY OF CELL-BASED CASPASE-GLO® 8 AND 9 ASSAYS WITH A PROTEASE INHIBITOR

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Here we describe the use of a protease inhibitor to decrease nonspecific background and dramatically improve data variability in caspase-8 or -9 cell-based assays.

Introduction

Peptide substrate specificities for many of the known caspases were historically determined using positional scanning synthetic combinatorial library techniques (1). In these groundbreaking experiments, fluorogenic tetrapeptide sequences, each ending as an aspartate-aminomethyl-coumarin (Asp-AMC), were manipulated using 20 different amino acids at each of the remaining P2-P4 positions. After incubation with a variety of recombinant caspases, the authors were able to determine optimal sequences for caspase 1–9 substrates. Relevance and applicability of these optimal sequences still exist today, for they are still used widely and correlate well with many known macromolecular substrate sequences for caspases such as poly(ADP-ribose)polymerase, DNA-dependent protein kinase and 70kDa U1 small ribonucleoprotein.

Nonspecific background can be reduced by 90% in these assays without negatively affecting the caspase activity.

Ac-LETD-AMC and Ac-LEHD-AMC are clearly the optimal fluorogenic substrates for recombinant caspase-8 and -9, respectively; thus aminoluciferin versions were incorporated into the Caspase-Glo® 8 and 9 Assays[a–c]. Buffer conditions were optimized for caspase activity and luminescent signal, and a strong lytic detergent was included to allow use on cultured cells. Direct comparison of these aminoluciferin substrates to their aminomethylcoumarin counterparts using recombinant enzymes indicated both the Caspase-Glo® 8 and 9 Assays were approximately 100 times more sensitive than comparable fluorescent assays (2). This gain in sensitivity allowed single-reagent-addition and homogeneous detection of caspase activities in cell-based assays using standard concentrations of cells.

Cell-based protease assays, however, are complex situations where many competing sources of proteases exist. Specificity and activity of a particular enzyme using an “artificial” substrate such as LEHD can be in part targeted by the choice of the substrate sequence and assay buffer formulation (pH, salts, stabilizing components, and detergents), but they can never be complete and absolute.

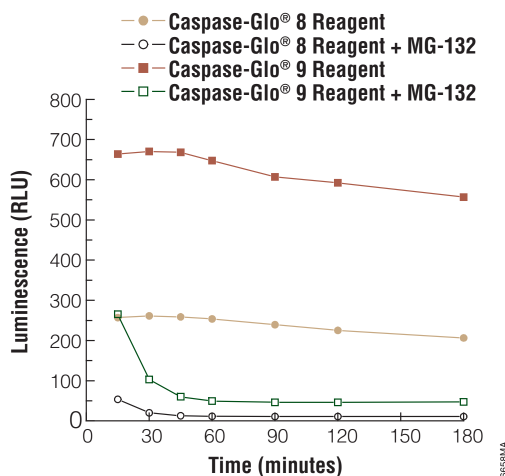

Figure 1. Caspase-Glo® Reagents supplemented with MG-132 reduce nonspecific luminescence in cells. Caspase-Glo® 8 and 9 signal kinetics and inhibition of nonspecific cellular background were determined using U266 cells. Untreated cells were cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate. Cells were plated at 25,000/well in 96-well plates. Following a 3-hour equilibration in a humidified 37°C, 5% CO_2 incubator, Caspase-Glo® 8 or 9 Reagents were prepared with MG-132 inhibitor (+) or with an equivalent amount of DMSO vehicle (–) and equilibrated to 22°C for 30 minutes before use, during which time the assay plate was also equilibrated to 22°C. Reagents were added and mixed by plate shaking, and luminescence was recorded over time using a Dynex MLX® plate luminometer, with the plate returned to a 22°C water bath immediately after each reading. Complete culture medium without cells served as the background control, and this background luminescence was subtracted from each set of cell data.

All of the caspases have a strong specificity for cleavage after an aspartic acid residue. When using LETD or LEHD sequences as caspase substrates in cell-based assays, there is the potential for cross-reactivity from the caspase-like activity of the proteasome. The proteasome is present in the cytosol and nucleus of all eukaryotic cells. Activity of the 20S proteasome is ATP-independent and can cleave short peptide substrates such as LETD and LEHD (and other caspase substrates with P1 aspartic acid residues) independent of ubiquitin targeting. We now know that when using the luminescent Caspase-Glo® 8 or 9 assays for cell-based experiments a significant amount of nonspecific background signal can be inhibited by using Caspase-Glo® Reagent supplemented with the protease inhibitor, MG-132.

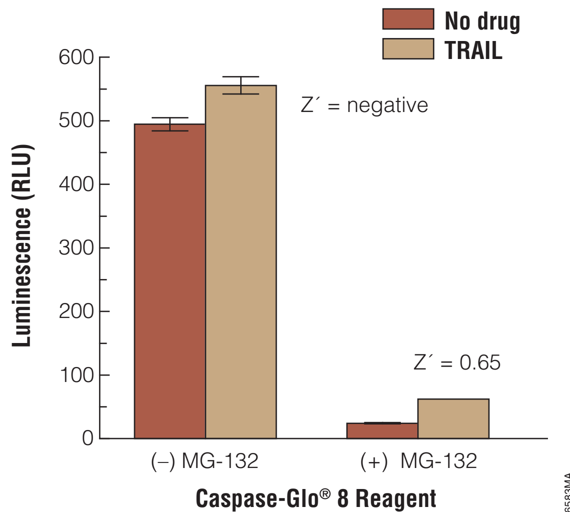

Figure 2. Improving the quality and confidence of Caspase-Glo® 8 data. U937 cells (15,000 cells per well cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) were plated in 90µl per well in a 96-well plate. Cells were cultured overnight at 37ºC, 5% CO_2. Soluble recombinant TRAIL (TNF-related apoptosis-inducing ligand; Biomol) was diluted to 10µg/ml in culture medium; 10µl was added to the wells of a portion of the plate, and 10µl of medium alone was added to the wells of another portion of the plate. Apoptosis was induced for 5 hours at 37ºC, 5% CO_2. The plate was allowed to equilibrate to 22ºC for 30 minutes before adding 100µl per well of Caspase-Glo® 8 Reagent with or without MG-132 Inhibitor. Luminescence was recorded using a Dynex MLX® luminometer 1 hour after adding reagent. To demonstrate improvement in assay quality, Z´-factor values were determined with and without addition of MG-132 Inhibitor. For each reagent, 16 wells contained cells treated with medium alone, and 16 wells contained cells treated with TRAIL. The Z´-factor value for reagent without MG-132 was negative. When reagent was supplemented with MG-132, there was a significant reduction of background, and the Z´-factor value improved to 0.65.

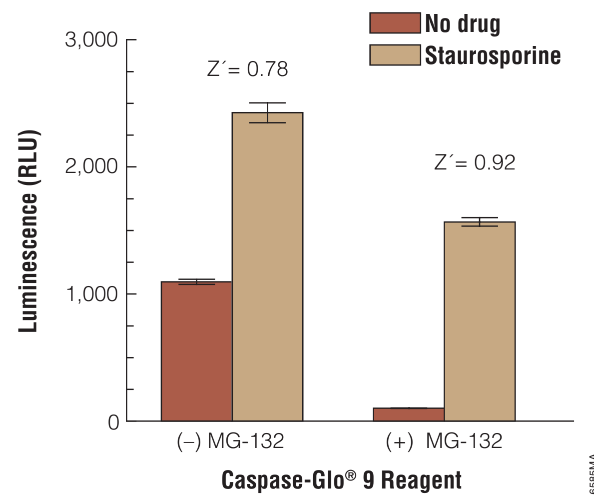

Figure 3. Improving the quality and confidence of Caspase-Glo® 9 data. Jurkat cells (25,000 cells per well cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) were plated in 90µl per well in a 96-well plate. Cells were equilibrated at 37ºC, 5% CO_2 for 2 hours. Staurosporine was diluted to 50µM in culture medium; 10µl per well was added to half of the plate, and 10µl per well vehicle (DMSO in culture medium) was added to the other half of the plate. Apoptosis was induced for 3 hours at 37ºC, 5% CO_2. The plate was allowed to equilibrate to 22ºC for 30 minutes before addition of 100µl per well of Caspase-Glo® 9 Reagent with or without MG-132 Inhibitor. Luminescence was recorded using a Dynex MLX® luminometer 45 minutes after adding reagent. To demonstrate improvement in assay quality, Z´-factor values were determined with and without addition of MG-132 Inhibitor. For each reagent, 24 wells contained cells treated with vehicle alone, and 24 wells contained cells treated with staurosporine. The Z´-factor value for reagent without MG-132 was 0.78. When reagent was supplemented with MG-132 Inhibitor, there was a significant reduction in background, and the Z´-factor value improved to 0.92.

Maintaining desired caspase activity while reducing nonspecific protease activity and background enhances the quality and confidence of assay results.

Optional Use of MG-132

The peptide aldehyde MG-132 (Z-Leu-Leu-Leu-CHO) is a potent reversible inhibitor of the proteasome as well as calpains and cathepsins (3,4) and is now included as a separate component in the Caspase-Glo® 8 and 9 Assays. Researchers now have the option of reducing nonspecific background in cell-based assays simply by supplementing their Caspase-Glo® 8 or 9 Reagent with this inhibitor. The data depicted in Figure 1 show that in healthy, untreated cells, more than 90% of the luminescent signal can be inhibited by using reagent containing the MG-132 Inhibitor. Following addition of reagent containing MG-132 Inhibitor, proteasome activity is rapidly inhibited with a subsequent drop in light output. Peak inhibition and a steady luminescent signal is typically achieved 30–60 minutes after adding Caspase-Glo® Reagent, depending on cell culture conditions.

Quantitative Improvement in Cell-Based Assay Quality

When making comparisons between assays, high-quality assays have large dynamic ranges with little data variability. As a consequence of the significant overall reduction of nonspecific background, Z´-factor values generally will improve when using Caspase-Glo® Reagents supplemented with MG-132 (Figures 2 and 3). Z´-factor coefficients are sensitive to changes in data variability and/or signal dynamic range (5). Here, the signal dynamic range (RLU difference between untreated cells and cells treated to undergo apoptosis) remains relatively constant with or without MG-132 present in the reagent. Caspase-Glo® 8 or 9 Reagents supplemented with MG-132 have no impact on assays using recombinant caspase-8 or -9 (data not shown). However, variations associated with summation of the positive and negative controls are reduced, thus the calculated Z´-factor increases.

Improve Caspase-8 and -9 Assay Data

APOPTOSIS

Summary

Results with the Caspase-Glo® 8 and 9 Assays in cell-based experiments can be improved by supplementing each reagent with the protease inhibitor MG-132. Nonspecific background can be reduced by 90% in these assays without negatively affecting the caspase activity. Assay quality, using Z´-factor values as a numerical indicator, typically will improve when using the inhibitor, primarily due to a reduction in data variability. These improvements can be particularly significant when investigating potentially modest initiator caspase responses.

References

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Protocols

Caspase-Glo® 8 Assay Technical Bulletin #TB332 (www.promega.com/tbs/tb332/tb332.html)

Caspase-Glo® 9 Assay Technical Bulletin #TB333 (www.promega.com/tbs/tb333/tb333.html)

Ordering Information

Product	Size	Cat.#
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
	100ml	G8212

For Laboratory Use.

[a]U.S. Pat. No. 7,148,030 and other patents pending.

[b]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

[c]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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