MULTIPLEXING HOMOGENEOUS CELL-BASED ASSAYS

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Researchers often desire more than one type of data from a cell sample in order to gain a more complete understanding of the biological processes that they are investigating. Here we provide brief protocols for performing a combination of cell viability, cytotoxicity, apoptosis or reporter assays on a single sample.

Introduction

The high-throughput demands of modern biomedical research have affected everything from nucleic acid purification technologies to cell-based assays including reporter analysis. The latest generation of Promega cell-based assays include luminescent and fluorescent chemistries to measure markers of cell viability, cytotoxicity, and apoptosis as well as to perform reporter analysis. Using these tools, researchers can investigate how cells respond to growth factors, cytokines, hormones, mitogens, radiation, effectors and other signaling ligands. In drug discovery, these assays provide efficient means for testing drug candidates for toxicity or efficacy before investing in animal research and clinical trials.

However, researchers often need more than one type of data from a sample, so the ability to multiplex, to analyze more than one parameter from a single sample, is desirable. Promega cell-based assays are homogeneous, that is they can be performed directly in cell culture wells without removing medium or washing cells. This homogeneous format allows researchers to multiplex assays (Table 1). Multiplexing more than one assay from the same culture well can provide internal controls and eliminate the need to repeat work. For example, a researcher can choose to perform a fluorescent assay to measure cytotoxicity or viability while next performing a luminescent caspase activity assay or reporter assay on the same sample[a]. Multiplexing more than one assay from the same culture well can also save time, cell sample, cell culture reagent and rare or expensive test compounds.

In the area of apoptosis Promega offers a range of fluorescent and luminescent methods to measure caspase-3/7, caspase-8 and caspase-9 activities that can be used in parallel, as well as in multiplexing formats, with luminescent or fluorescent cell viability, cytotoxicity and reporter assays. This approach can provide a more complete understanding of how a compound treatment affects cell viability and/or the mechanism of cell death.

The tables that follow provide basic guidelines for multiplexing cell-based assays and are intended as starting points. As with any homogeneous assay, multiplexing assays will require researchers to optimize the assays for any specific experimental system. We strongly recommend running appropriate controls, including performing each assay individually on the samples. Additional background, optimization and control information for each assay is provided in the accompanying technical literature, listed at the end of this article. For a complete review to help you choose the right cell-based assays to address your experimental questions, please see references 1, 2 or 4.

Multiplexing Cell Viability, Cytotoxicity and Apoptosis Assays

The CellTiter-Blue® Cell Viability Assay determines the number of viable cells in culture using resazurin to characterize the reducing potential of the cells. The reduction of resazurin to resorufin is proportional to the number of metabolically active, cells present. The CellTiter-Blue® Assay can be multiplexed with either the Caspase-Glo™ 3/7 Assay[b,c] or the Apo-ONE® Homogeneous Caspase-3/7 Assay (Table 2).

Table 1. Summary of Promega Cell-Based Assays for Multiplexing

Assay	Detection Method	Measures	Multiplex with:
CellTiter-Glo® Luminescent Cell Viability Assay	Luminescent	ATP, viability	EnduRen™ Live Cell Substrate (*Renilla* reporter) CytoTox-ONE™ Homogeneous Membrane Integrity Assay (fluorescent)
CellTiter-Blue® Cell Viability Assay	Fluorescent	Reducing Potential, viability	Caspase-Glo™ 3/7 Assay (luminescent) Apo-ONE® Homogeneous Caspase 3/7 Assay (fluorescent)
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	Fluorescent	LDH Release, cytotoxicity	Caspase-Glo™ 3/7 Assay (luminescent) Apo-ONE® Homogeneous Caspase 3/7 Assay (fluorescent)
Caspase-Glo™ 3/7 Assay	Luminescent	Caspase-3/7 Activity	CellTiter-Blue® Cell Viability Assay (fluorescent) CytoTox-ONE™ Homogeneous Membrane Integrity Assay (fluorescent)
Caspase-Glo™ 8 or Caspase-Glo™ 9 Assay	Luminescent	Caspase-8 or -9 Activity	Apo-ONE® Homogeneous Caspase 3/7 Assay (fluorescent)
Apo-ONE® Homogeneous Caspase 3/7 Assay	Fluorescent	Caspase 3/7 Activity	Caspase-Glo® 8 or Caspase-Glo® 9 Assays (luminescent) CellTiter-Blue® Cell Viability Assay (fluorescent) CytoTox-ONE™ Homogeneous Membrane Integrity Assay (fluorescent) EnduRen™ Live Cell Substrate (*Renilla* reporter)
EnduRen™ Live Cell Substrate	Luminescent	*Renilla* Reporter Gene Activity	CellTiter-Glo® Luminescent Cell Viability Assay (luminescent) Apo-ONE® Homogeneous Caspase 3/7 Assay (fluorescent)

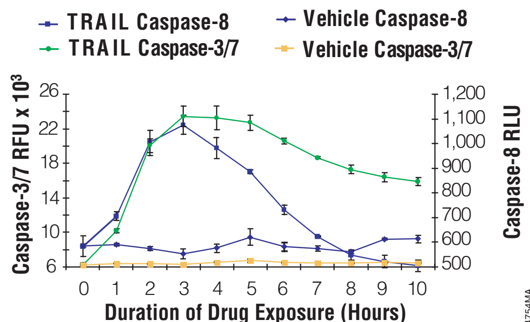

Figure 1. Multiplexing luminescent caspase-8 and fluorescent caspase 3/7 assays. Jurkat cells were seeded at 25,000 cells/well. 50µl of rTRAIL (Chemicon, 100ng/ml final) or a vehicle control (RPMI 1640 with 10% FBS) was added to replicate wells every hour for 10 hours. Caspase-Glo™ 8 Reagent was prepared by combining the assay buffer with the substrate. A fluorescent caspase-3/7 substrate [(Z-DEVD)2-R110] was mixed into the Caspase-Glo™ Reagent at a final concentration of 50µM. The combined Reagent/substrate was added in 100µl volumes, incubated 60 minutes, and then luminescence and fluorescence were measured.

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay[a] measures the release of lactate dehydrogenase (LDH) into the surrounding medium by cells that have lost membrane integrity. The assay estimates the number of nonviable cells present in a mixed population of living and dead cells. The CytoTox-ONE™ Reagent does not damage living cells and can be performed directly in cell culture. Tables 2 and 3 describe how the CytoTox-ONE™ Assay can be multiplexed with either the Caspase-Glo™ 3/7 Assay, the CellTiter-Glo® Luminescent Cell Viability Assay, or the Apo-ONE® Assay.

CellTiter-Glo® Luminescent Cell Viability Assay determines the number of viable cells in culture based on quantitation of ATP present, an indicator of metabolically active cells. It can be multiplexed with either EnduRen™ Live Cell Substrate (Table 4 and Figure 2) or CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Tables 2 and 3).

Multiplexing Apoptosis Assays

The Caspase-Glo™ Assays measure caspase activities using a luminogenic caspase substrate and a proprietary stabilized luciferase in a reagent optimized for specific caspase activity, luciferase activity and cell lysis. Adding the single Caspase-Glo™ Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate. This cleavage liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal. The signal is proportional to caspase activity present. Currently Caspase-Glo™ Assays are available to measure caspase-3/7, caspase-8 or caspase-9 activity. These assays can be multiplexed with cell viability and cytotoxicity assays (Table 2). The Caspase-Glo™ 8 or 9 Assays can be multiplexed with the Apo-ONE® Homogeneous Caspase-3/7 Assay to provide a more complete picture of apoptotic signaling (Figure 1,Table 2).

The Apo-ONE® Homogeneous Caspase-3/7 Assay measures active caspase-3 and -7 by including a profluorescent caspase-3/7 consensus substrate and an optimized bifunctional cell lysis/activity buffer. The buffer lyses cultured mammalian cells and supports optimal caspase-3/7 enzymatic activity. The substrate and buffer are combined to make the Apo-ONE® Caspase-3/7 Reagent that is added directly to samples. Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes, the rhodamine 110 becomes fluorescent when excited at a wavelength of 498nm. The amount of fluorescent product generated is representative of the amount of active caspase-3/7 present in the sample.

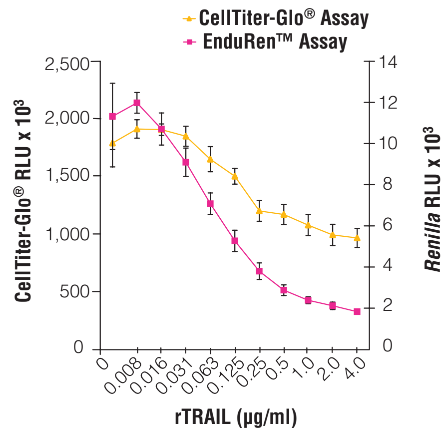

Figure 2. Multiplex of *Renilla* reporter assay with a luminescent cell viability assay. HeLa cells stably expressing a synthetic *Renilla* luciferase gene were plated at 10, 000 cells/well in a 96-well plate. EnduRen™ Live Cell Substrate (Cat.# E6482) was added to all the wells at a 1:1,000 dilution in DMEM + 10% FBS. The TRAIL protein (CalBiochem Cat.# 616375) was added to the indicated wells starting at 4µg/ml with subsequent twofold serial dilutions. Cells were incubated for 16 hours and assayed. *Renilla* expression was measured using a Veritas™ Microplate Luminometer (Cat.# E6521) immediately after incubation. CellTiter-Glo® Reagent was then added at a volume of 1:1 to each well and luminescence read on the Veritas™ Microplate Luminometer.

Multiplexing Reporter Assays with Cell Viability and Apoptosis Assays

The EnduRen™ Live Cell Substrate[c,d,e] is a non-destructive assay that allows repeated *Renilla* reporter gene activity measurements over a period of time or allows users to multiplex with cell viability or apoptosis assays and potentially other lytic assays. Multiplexing a reporter assay with a cell viability assay allows researchers to normalize reporter assay data with respect to cell viability and reduce relative error of results (Figure 2, Table 4). EnduRen™ Live Cell Substrate also provides researchers with a tool to investigate caspase activity and reporter activity in the same sample (Table 4).

Summary

Multiplexing is an efficient way to normalize cell-based data to understand complex cellular processes. The range of homogeneous cell-based assays available from Promega gives researchers the flexibility and tools they need to dissect complex cellular processes. ■

Table 2. Sequential Multiplexing of Promega Homogeneous Cell Viability and Apoptosis Assays (same well).

Assay Goal: Determine cell viability and the mechanism of death. CellTiter-Blue® Assay (viability, fluorescent) and Apo-ONE® Assay (caspase-3/7, fluorescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Add 20µl/well CellTiter-Blue® Reagent during the final 1–2 hours of drug treatment and incubate at 37°C.
3. Record fluorescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB317 to measure cell viability.
4. Add an equal volume (120µl) Apo-ONE® Homogeneous Caspase-3/7 Reagent and incubate for 1–4 hours at room temperature.
5. Record fluorescence ($485_{Ex}//527_{Em}$) as described in Technical Bulletin #TB295 to indicate caspase activity as a marker of apoptosis.

Assay Goal: Determine cell viability and the mechanism of death. CellTiter-Blue® Assay (viability, fluorescent) and Caspase-Glo™ 3/7 Assay (luminescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Add 20µl/well of CellTiter-Blue® Reagent (diluted 1:4 with Dulbecco's PBS) during the final 1–2 hours of drug treatment and incubate at 37°C.
3. Record fluorescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB317 to measure cell viability.
4. Add an equal volume (120µl) of Caspase-Glo™ 3/7 Reagent to each well. and incubate 1 hour at room temperature to achieve luciferase steady state.
5. Record luminescence as described in Technical Bulletin #TB323 to indicate caspase activity as a marker of apoptosis.

Note: Ensure that all of the wells change to an even pink color after incubating with Caspase-Glo™ Reagent. If all of the wells contain the same pink color when luminescence is recorded, the light is quenched evenly throughout the sample, regardless of the initial CellTiter-Blue® activity.

Assay Goal: Determine the mechanism of compound cytotoxicity. CytoTox-ONE™ Assay (LDH release, fluorescent) and Caspase-Glo™ 3/7 Assay (luminescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Reconstitute CytoTox-ONE™ Substrate at **2X** concentration and add **25µl/well.**
3. Shake while incubating 10 minutes at room temperature. Record fluorescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB306 to measure necrotic cells.
4. Add an equal volume (125µl) of Caspase-Glo™ Reagent to each well.
5. Incubate for 1 hour at room temperature to achieve luminescence steady state. Record luminescence as described in Technical Bulletin #TB323.

Note: Ensure that all of the wells change to an even pink color after incubating with Caspase-Glo™ Reagent. If all of the wells contain the same pink color when luminescence is recorded, the light is quenched evenly throughout the sample, regardless of the initial CytoTox-ONE™ activity.

Assay Goal: Determine cytotoxicity and cell viability. CytoTox-ONE™ Assay (LDH release, fluorescent) and CellTiter-Glo® Assay (cell viability, ATP)

1. Culture and treat cells with drug of interest in **75µl** of medium in a 96-well plate (black or white).
2. Reconstitute CytoTox-ONE™ Substrate at **1X** concentration, and add **50µl/well.**
3. Shake gently and incubate 10 minutes at room temepratature. Record fluorescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB306.
4. Reconstitute the CellTiter-Glo® Substrate and add 20mM DTT. Add an equal volume (125µl) to each well.
5. Shake gently and incubate for 1 hour at room temperature. Record luminescence as described in Technical Bulletin #TB288.

Note: Ensure that all of the wells change to an even pink color after incubating with CellTiter-Glo® Reagent. If all of the wells contain the same pink color when luminescence is recorded, the light is quenched evenly throughout the sample, regardless of the initial CytoTox-ONE™ activity.

Assay Goal: Differentiate caspase activities. Caspase-Glo™ 8 or 9 Assay (luminescent) and Apo-ONE® Assay (caspase-3/7 activity, fluorescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Prepare the Caspase-Glo™ 8 or 9 Assay Reagent. Thaw the Apo-ONE® Substrate and add it to the Caspase-Glo™ 8 or 9 Reagent at a dilution of 1:200 (50µl/10ml of Caspase-Glo™ Reagent). The Apo-ONE® Buffer will not be used in this assay. Add an equal volume (100µl) to each well.
3. Incubate 1 hour at room temperature. Record luminescence as described in Technical Bulletin #TB332 or #TB333 to indicate caspase-8 or -9 activity.
4. Record Apo-ONE® Homogeneous Caspase-3/7 Assay fluorescence ($485_{Ex}/527_{Em}$) as described in Technical Bulletin #TB295 to indicate caspase-3 activity.

References

1. Riss, T. *et al.* (2003) *Cell Notes* 6, **6**–12.
2. Niles, A. *et al.* (2004) *Cell Notes* **9**, 11–14.
3. Riss, T. and Moravec, R. (2003) *Promega Notes* **83**, 10–13.
4. Riss, T. and Moravec, R. (2004) *Assay Drug Dev. Techol.* **2**, 51–62.

Protocols

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288 (www.promega.com/tbs/tb288/tb288.html)

CellTiter-Blue® Cell Viability Assay Technical Bulletin #TB317 (www.promega.com/tbs/tb317/tb317.html)

CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin #TB306 (www.promega.com/tbs/tb306/tb306.html)

Apo-ONE® Homogeneous Caspase-3/7 Assay Technical Bulletin #TB295 (www.promega.com/tbs/tb295/tb295.html)

Caspase-Glo™ 3/7 Assay Technical Bulletin #TB323 (www.promega.com/tbs/tb323/tb323.html)

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)

EnduRen™ Live Cell Substrate Technical Manual #TM244 (www.promega.com/tbs/tm244/tm244.html)

Table 3. Multiplexing Promega Homogeneous Cell Viability and Apoptosis Assays (medium and cells separately).

Assay Goal: Determine cytotoxicity and caspase-3/7 activity. CytoTox-ONE™ Assay (LDH release, fluorescent) and Apo-ONE® Assay (caspase-3/7 activity, fluorescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Transfer supernatant to a new plate and add CytoTox-ONE™ Reagent to measure LDH release indicating necrotic cells.
3. Incubate and record flourescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB306.
4. Add Apo-ONE® Homogeneous Caspase-3/7 Reagent to the cells in the original plate and incubate for 1–4 hours at room temperature.
5. Record Apo-ONE® Assay fluorescence ($485_{Ex}/527_{Em}$) as described in Technical Bulletin #TB295.

Assay Goal: Determine cytotoxicity and cell viability. CytoTox-ONE™ Assay (LDH release, fluorescent) and CellTiter-Glo® Assay (viability, luminescent)

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate (black or white).
2. Transfer supernatant to a new plate and add CytoTox-ONE™ Reagent.
3. Incubate and record flourescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB306.
4. Add CellTiter-Glo® Reagent to cells in original sample plate. Incubate 10 minutes and record luminescence as described in Technical Bulletin #TB288.

Table 4. Multiplexing Promega Cell Viability and Reporter Assays

Assay Goal: Normalize reporter gene signal with respect to cell viability. EnduRen™ Live Cell Substrate (*Renilla* luciferase, luminescent) and CellTiter-Glo® Assay (viability, luminescent)

1. Culture and treat cells with the drug of interest in 90µl of medium in a 96-well plate.
2. Dilute the EnduRen™ Live Cell Substrate as directed in Technical Manual #TM244. Add 10µl/well of EnduRen™ Substrate (60µM) and incubate for an additional 2 hours at 37°C, 5% CO_2. You may add the EnduRen™ Substrate before or after experimental treatment depending on cell tolerance to it.
3. Record luminescence to indicate reporter activity.
4. Add an equal volume of CellTiter-Glo® Reagent (100µl/well), mix for 2 minutes on an orbital shaker to induce cell lysis, and incubate and additional 10 minutes at room temperature to stabilize luminescent signal.
5. Record luminescence as described in Technical Bulletin #TB288 to indicate cell viability.

Assay Goal: Determine gene regulation and apoptosis involvement. EnduRen™ Live Cell Substrate (*Renilla* luciferase) and Apo-ONE® Assay (caspase-3/7 activity)

1. Culture and treat cells with the drug of interest in 90µl of medium in a 96-well plate.
2. Dilute the EnduRen™ Live Cell Substrate as directed in Technical Manual #TM244. Add 10µl/well of EnduRen™ Substrate (60µM) and incubate for an additional 2 hours at 37°C, 5% CO_2. You may add the EnduRen™ Substrate before or after experimental treatment depending on cell tolerance to it.
3. Record luminescence.
4. Add an equal volume of Apo-ONE® Reagent (100µl/well) and incubate for 1 hour at room temperature.
5. Record fluorescence ($485_{Ex}/527_{Em}$) as described in Technical Bulletin #TB295.

Ordering Information

Product	Size	Cat.#
Caspase-Glo™ 8 Assay[b,c]*	100ml	G8202
Caspase-Glo™ 8 Assay[b,c]*	100ml	G8212
Caspase-Glo™ 3/7 Assay[b,c]*	100ml	G8092
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml	G7791
CellTiter-Glo® Luminescent Cell Viability Assay[b,c]	10 × 10ml	G7571
CellTiter-Blue® Cell Viability Assay	20ml	G8080
CytoTox-ONE™ Homogeneous Membrane Integrity Assay[a]	1,000–4,000 assays	G7891
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP[a]	1,000–4,000 assays	G7892
EnduRen™ Live Cell Substrate*[a,d,e]	0.34ng	E6482

Available in additional sizes. *For Laboratory Use.

[a]Patent Pending.
[b]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other 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.
[d]Certain applications of this product may require licenses from others.
[e]This product does not convey a license to use recombinant ***Renilla*** luciferase under U.S. Pat. Nos. 5,292,658, 5,418,155 and related patents. Promega sells licensed ***Renilla*** luciferase vectors, which may be used in conjunction with this product.

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