CHOOSING THE RIGHT ASSAY TO MONITOR YOUR SIGNAL TRANSDUCTION PATHWAY

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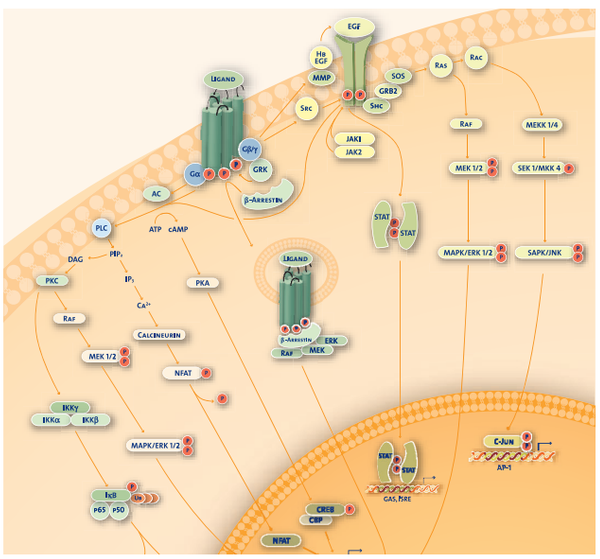
Signal transduction is one of the most widely studied areas in biology. Extracellular information is translated into an intracellular response, often through elaborate networks of interwoven intracellular signaling cascades. This article is designed to help you choose the right Promega assays to monitor your pathway of interest.



The complexity of the cellular environment complicates the analysis of biochemical changes occurring inside cells.

Proliferation, Viability, Cytotoxicity and Apoptosis Assays

Treatments to cells that induce or inhibit signal transduction can ultimately result in cell proliferation, apoptosis, necrosis or senescence. Assays for proliferation and viability range from the direct measurement of cellular ATP to the use of reagents such as MTS or resazurin to determine the overall reducing capacity of the culture. Assays that require only a few minutes to generate a measurable signal (e.g., ATP quantitation using the CellTiter®-Glo Reagent) provide information representing a snapshot in time and have an advantage over assays that may require several hours of incubation to develop a signal (e.g., MTS or resazurin; 1,2). Treatment-induced senescence, which can be missed if you are only using a traditional cytotoxicity assay, can be detected by including a proliferation assay.

Cytotoxicity can be measured using several different methods depending upon the assay sensitivity required and the instrumentation that is available in your laboratory. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released from cells after cell lysis or membrane damage and is used extensively as a marker for cytotoxicity (e.g., CytoTox 96® Assay and CytoTox-ONE™ Homogeneous Membrane Integrity Assay). More recent developments in viability and cytotoxicity monitoring include the use of proteases that are either present in live cells or released into the culture following rupture of the cellular membrane (e.g., MultiTox Technology Platform Assays). These protease biomarker assays can be performed in multiplex to provide relative numbers of live and dead cells within the same population of cells (3).

Promega manufactures kits for colorimetric, fluorescent, or luminescent-based measurements of cell viability, all of which can be performed in a homogeneous format (adding the reagent directly to cultures in 96-well or 384-well plates). Used alone or in tandem with other systems (e.g., CellTiter-Blue® Reagent and the Apo-ONE® Homogeneous Caspase-3/7 Assay; 4,5), these assays provide a more accurate picture of the overall health of a culture.

The activity of caspase-8 and caspase-9 are used to monitor the mechanism of apoptotic induction early in the process. The luminescent Caspase-Glo® 8 or 9 Assays can be used directly with cells in culture or in multiplex with some other cell viability or cytotoxicity assays (6). Later events in apoptosis, including activation of the effector caspases-3 and -7 can be detected with the luminescent Caspase-Glo®-3/7 Assay or the fluorescent Apo-ONE® Homogeneous Caspase-3/7 Assay. Both of these assays can be performed in multiplex with cytotoxicity or viability assays. For instance if you perform the MultiTox-Glo Assay to look at proliferation and cytotoxicity, you can perform the Apo-ONE® Assay on the same sample to ask if any lack of proliferation might be the result of apoptosis (3). Remember however that apoptosis markers, such as caspase expression, may be transient and can be missed if your assays are not performed at the critical time (7).

Assessing Receptor-Mediated Modulation of Pathways: Reporter Assays and Assays for Secondary Messengers

Several methods exist for monitoring receptor-mediated (e.g., GPCR) activation of cellular signaling, including those that use response elements upstream of a primary reporter to measure transcriptional activation and those that directly measure secondary messengers such as cAMP or intracellular calcium.

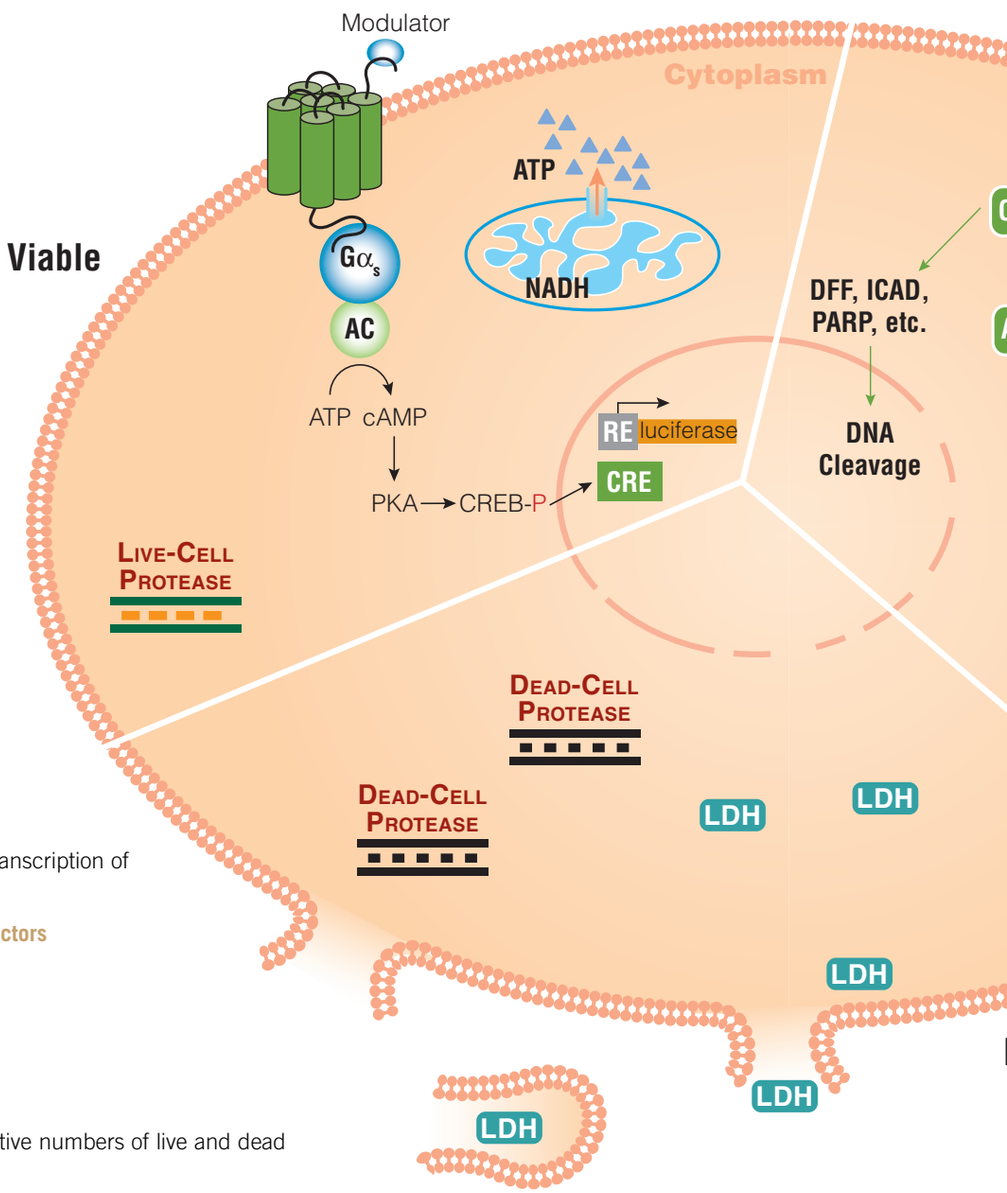
If you are investigating whether a treatment modulates a specific promoter or other gene regulatory sequence but do not necessarily know the signaling pathways that are involved, genetic reporter assays will allow you to see modulation of gene expression by a regulatory sequence of interest (8). Additionally, libraries of regulatory sequences can be screened using genetic

Does your treatment induce proliferation?

CellTiter-Glo® Luminescent Cell Viability Assay

Does your treatment modulate cAMP levels?

cAMP-Glo™ Assay



Do you need to measure activity of a purified kinase?

ProFluor® PKA or Src-Family Kinase Assay

SignaTECT® Kinase Assay Systems

Kinase-Glo® Luminescent Kinase Assay Platform

Do you need to distinguish ATP competitive versus noncompetitive inhibitors? Or does your kinase have a high K_m for ATP?

Kinase-Glo® Luminescent Kinase Assay Platform

Are you interested in what signals upregulate transcription of a target gene?

Check out Promega pGL4 Luciferase Reporter Vectors

Do you need to know the relative numbers of live and dead cells within a population?

MultiTox-Glo Multiplex Cytotoxicity Assay
MultiTox-Fluor Multiplex Cytotoxicity Assay

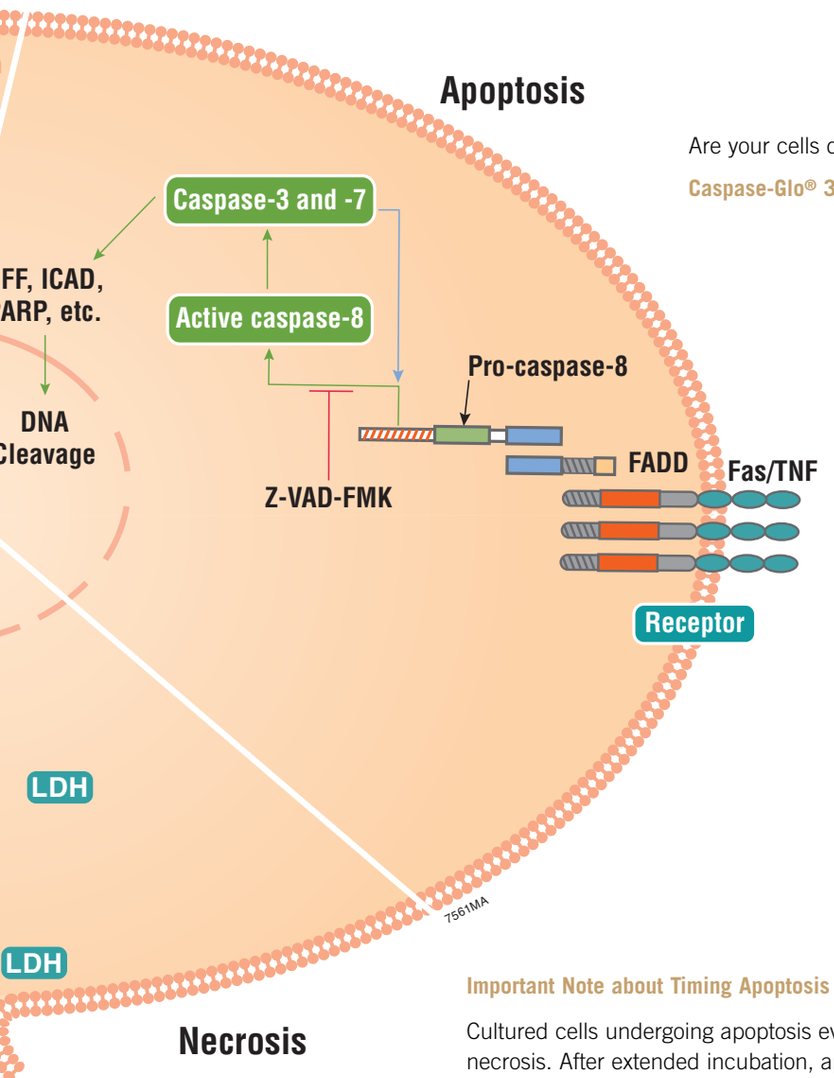
Are your cells dying by necrosis?

CytoTox-ONE™ Homogeneous Membrane Inte

Do you need to distinguish between cytotoxicity and cellular senescence? Perform a proliferation and a cyotoxicity assay.

CellTiter-Glo® Luminescent Cell Viability Assay

MultiTox-Fluor Multiplex Cytotoxicity Assay



Are your cells dying by apoptosis?

Caspase-Glo® 3/7 Assay

Does your treatment induce apoptosis and by what mechanism?

Caspase-Glo® 8 Assay or Caspase-Glo® 9 Assay

ecrosis?

eous Membrane Integrity Assay

Important Note about Timing Apoptosis Assays

Cultured cells undergoing apoptosis eventually undergo secondary necrosis. After extended incubation, apoptotic cells ultimately shut down metabolism and lose membrane integrity. Markers of apoptosis such as caspase activity may be expressed only transiently. Therefore, if apoptosis is the primary mechanism of cell death, understanding the kinetics of the cell death process in your system is critical (7).

Cell Signaling, Cell Viability, Apoptosis, and Cytotoxicity Assays.

Assay	Parameter or Biomarker Measured	Time to Results	Assay Characteristics (*384-well format)	Throughput	Instrumentation Required
CellTiter-Glo® Luminescent Cell Viability Assay	ATP	10 minutes	Sensitive to 10 viable cells*	96-, 384- or 1536-well plates	Luminometer or CCD
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	LDH release	10 minutes	Sensitive to 200 cells*	96- and 384-well plates	Fluorometer, Resorufin $560mn_{Ex}/590nm_{Em}$
MultiTox-Glo Multiplex Cytotoxicity Assay	Live-Cell and Dead-Cell Proteases	0.5 hour	Sensitive to 40 viable cells, 10 dead cells	96-, 384- or 1536-well plates	Fluorometer (AFC $400nm_{Ex}$, $590nm_{Em}$) Luminometer
MultiTox-Fluor Multiplex Cytotoxicity Assay	Live-Cell and Dead-Cell Proteases	0.5–3 hours	Sensitive to 40 viable cells, 10 dead cells	96-, 384- or 1536-well plates	Fluorometer (AFC $400nm_{Ex}$,/$505nm_{Em}$; R110 $485nm_{Ex}$/$520nm_{Em}$)
Caspase-Glo® 3/7 Assay	Caspase-3/7 Activity	0.5 hour	Sensitive to 20 cells*	96-, 384- or 1536-well plates	Luminometer
Caspase-Glo® 8 Assay	Caspase-8 Activity	0.5 hour	Sensitive to ~1,000 cells	96-well plates	Luminometer
Caspase-Glo® 9 Assay	Caspase-9 Activity	0.5 hour	Sensitive to ~1,500 cells	96-well plates	Luminometer
cAMP-Glo® Assay	cAMP (through PKA activation)	~45 minutes	Signal: Background >15 on cells	96-, 384- or 1536-well plates	Luminometer
Kinase-Glo® Luminescent Kinase Assay Platform	ATP remaining after kinase reaction	10 minutes	Linear up from 10–500 µM ATP in kinase reaction depending on system used	96-, 384- or 1536-well plates	Luminometer
ProFluor® PKA or Src-Family Kinase Assay	Phosphorylation of a bisrhodamine peptide substrate	~1 hour	Z′-factor values greater than 0.7 in 96- and 384-well plates	96- or 384-well plates	Fluorometer (R110 $485nm_{Ex}$/$520nm_{Em}$)
SignaTECT® Protein Kinase Assay Systems	Incorporation of radio-labeled phosphate into a biotinylated substrate	Varies	Can be used with crude extracts	Single experiment or 96-well plates	Scintillation Counter, Phosphorimaging system, Autogradiograph

reporters to allow you to observe constellations of regulatory events that result from a treatment strategy or test compound.

Assays for second messengers provide improved correlation with receptor activation because these messengers are spatially closer to receptors in the signaling pathway. Several assays exist for assaying cellular cAMP levels in a homogeneous format (9). While fluorescent, calcium-sensitive dyes can also be used to measure receptor activity, receptor agonists often generate their own fluorescence. Moreover agonists can sometimes affect cellular calcium levels through mechanisms that do not involve receptor binding events. Both of these factors increase the probability of false positive results.

Protein kinases primarily enable the transduction of signals through the cell to bring about a response. Promega offers a variety of systems to measure protein kinase activity, allowing you to assay for virtually any kinase/kinase substrate combination in high-throughput methods (10).

Summary

Determining the molecular response of cells to treatments often requires adopting two lines of questioning. First, looking at the downstream events: Is the regulation of a specific gene modulated by a treatment? Second, looking at the events close to the receptor activation: Are there changes in second messenger activation? Does your treatment affect a specific kinase? Promega provides tools for both of these approaches, allowing you to ask questions of your signal transduction pathway from the bottom up and the top down.

Resources Cited in this Article

1. Choosing the Right Cell-Based Assay for Your Research (www.promega.com/cnotes/cn006/cn006_06.htm)
2. Selecting Cell-Based Assays for Drug Discovery Screening (www.promega.com/cnotes/cn013/cn013_16.htm)
3. Using Protease Biomarkers to Measure Viability and Cytotoxicity (www.promega.com/cnotes/cn019/cn019_16.htm)
4. Multiplexing Homogeneous Cell-Based Assays (www.promega.com/cnotes/cn010/cn010_15.htm)
5. *Protocols and Applications Guide:* Cell Viability (www.promega.com/paguide/chap4.htm)
6. High-Throughput Automation of Multiplexed Cell-Based Assays for Viability and Cytotoxicity (www.promega.com/cnotes/cn020/cn020_26.htm)
7. Timing Your Apoptosis Assays (www.promega.com/cnotes/cn016/cn016_18.htm)
8. Luciferase Reporter Assays: Powerful Adaptable Tools for Cell Biology Research (www.promega.com/cnotes/cn021/cn021_23.htm)
9. Montior GPCR Modulation of Cellular cAMP with an HTS, Bioluminescence Assay (www.promega.com/pnotes/97/15377_24/15377_24.html)
10. Screen for Kinase Modulators in a High-Througput Format (www.promega.com/cnotes/cn020/cn020_21.htm)

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