TECHNICAL MANUAL

NAD(P)H-Glo™ Assay

Instructions for use of Products
G9061 and G9062

Revised 6/13
TM398

NAD(P)H-Glo™ Detection System

All technical literature is available on the Internet at: www.promega.com/protocols/ Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail techserv@promega.com.

1. Description

The NAD(P)H-Glo™ Detection System[a–c] is a homogeneous bioluminescent assay that generates a light signal from biochemical reactions that contain reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). NAD^+, $NADP^+$, NADH and NADPH are important cofactors for many enzymes involved in key cellular pathways, and quantitation of these dinucleotides is a standard approach to determine the activity of many enzymes, either directly or by coupling to other NADH- or NADPH-producing reactions. To that end, Lowry writes "In fact, with the aid of auxiliary enzymes nearly every substance of biological interest could be measured with a pyridine nucleotide system" (1). Metabolic enzymes that use these cofactors include the dehydrogenase family of enzymes. There are over 300 different dehydrogenases, many of which have clinically significant roles in diseases including cancer (2,3).

The NAD(P)H-Glo™ Detection System quantitatively monitors the concentration of the reduced forms, NADH and NADPH, and does not discriminate between them. NADH and NADPH are collectively referred to as NAD(P)H throughout this manual. The oxidized forms, NAD^+ and $NADP^+$, are not detected and do not interfere with quantitation. The assay can be used to monitor the activity of enzymes that produce or use NAD(P)H and to screen for inhibitors.

1. Description (continued)

In the presence of NAD(P)H, the enzyme Reductase reduces a proluciferin reductase substrate to form luciferin. Luciferin then is quantified using Ultra-Glo™ Recombinant Luciferase (rLuciferase), and the light signal produced is proportional to the amount of NAD(P)H in the sample (Figure 1). Both reactions occur with the addition of a single reagent.

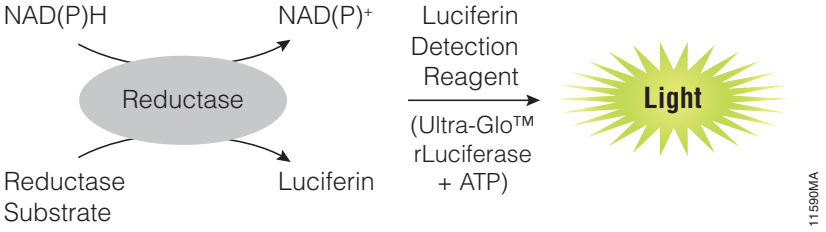

Figure 1. Schematic diagram of the NAD(P)H-Glo™ Detection System technology. In the presence of NAD(P)H, Reductase enzymatically reduces a proluciferin reductase substrate to luciferin. Luciferin is detected by Ultra-Glo™ rLuciferase, and the amount of light produced is proportional to the amount of NAD(P)H in a sample.

The reductase and luciferase reactions are initiated by adding an equal volume of NAD(P)H-Glo™ Detection Reagent, which contains Reductase, Reductase Substrate and Ultra-Glo™ rLuciferase, to a NAD(P)H-containing sample (Figure 2). The assay is rapid, requiring only a 40- to 60-minute incubation, has a broad linear range of 0.1µM to 25µM NADH and has a high signal-to-background ratio (Figure 3). Luminescence is proportional to NAD(P)H concentrations and is specific for the reduced forms. The assay is compatible with 96-, 384-, low-volume 384- and 1536-well plates and is well suited to measure NAD(P)H production or consumption in high-throughput formats.

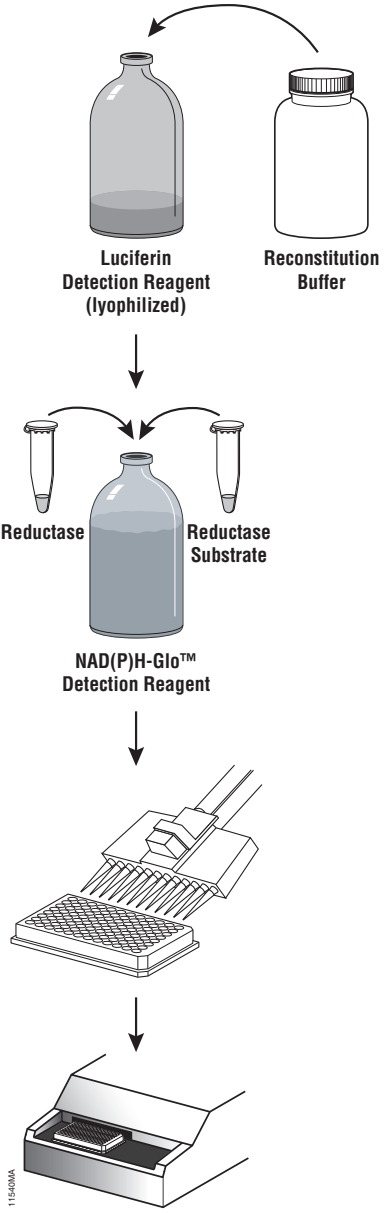

Figure 2. Schematic diagram of the NAD(P)H-Glo™ Detection System protocol.

1. Description (continued)

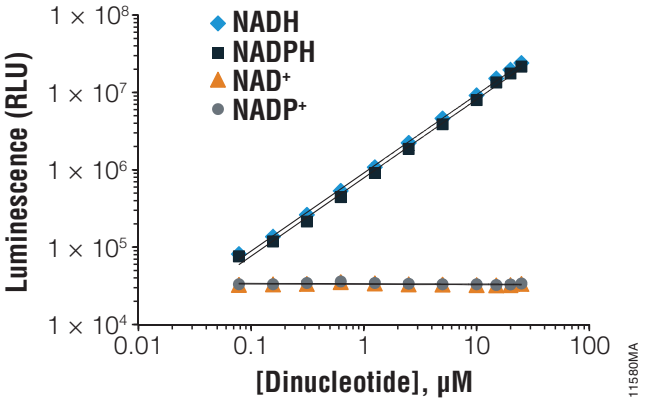

Figure 3. Linear range and specificity of the NAD(P)H-Glo™ Assay. Individual purified nicotinamide adenine dinucleotides were assayed following the protocol described in Section 3.C. NADH, NADPH, NAD^+ and $NADP^+$ stocks were prepared fresh from powder (Sigma Cat.# N6660, N9910, N8285 and N8035, respectively) and diluted to the indicated concentrations in 50mM Tris HCl (pH 7.5). Fifty-microliter samples at each dinucleotide concentration were incubated with 50µl of NAD(P)H-Glo™ Detection Reagent in white 96-well plates. After a 60-minute incubation, luminescence was measured with a GloMax® 96 Microplate Luminometer. Each point represents average luminescence of quadruplicate reactions measured in relative light units (RLU). Error bars are ± 1 standard deviation. The %CV values were ≤5%. The limit of detection was approximately 5nM. The data used to generate this figure are shown in Table 1.

Table 1. Titration of Purified Dinucleotides.

	NADH		NADPH	
Dinucleotide Concentration (µM)	Luminescence (RLU)	Signal-to-Background Ratio[1]	Luminescence (RLU)	Signal-to-Background Ratio[1]
25	23,941,449	787.9	21,568,991	582.1
20	19,569,713	644.0	17,499,318	472.3
15	15,186,715	499.8	13,396,919	361.6
10	9,144,367	300.9	7,924,035	213.9
5	4,609,034	151.7	3,869,484	104.4
2.5	2,226,197	73.3	1,866,620	50.4
1.25	1,079,462	35.5	904,480	24.4
0.625	531,733	17.5	441,820	11.9
0.313	260,486	8.6	214,382	5.8
0.156	136,652	4.5	118,279	3.2
0.078	81,119	2.7	75,989	2.1
0	30,388	1.0	37,054	1.0

[1]Signal of a sample divided by signal of the 0µM control.

Advantages of the NAD(P)H-Glo™ Detection System include:

Broad linear range: The NAD(P)H-Glo™ Detection System detects 0.1µM to 25µM NAD(P)H.

High sensitivity: The limit of detection is ≤0.1µM NADH, with a maximum assay window (i.e., signal-to-background ratio) of 250. The system detects 1µM with a signal higher than fivefold over background.

Compatibility with automation: The add-and-read format is compatible with automated and high-throughput workflow, and reactions are scalable for use in 96-, 384- and 1536-well plates.

Reliability and reproducibility: The NAD(P)H-Glo™ Detection System routinely yields Z´ factors >0.7.

Stable signal: The glow-type signal is stable, with a half-life greater than two hours, allowing batch plate processing.

Luminescence-based NAD(P)H detection: The luminescent format avoids fluorescent interference due to reagents and test compounds sometimes seen in fluorescent assays.

2. Product Components and Storage Conditions

Product	Size	Cat.#
NAD(P)H-Glo™ Detection System	10ml	G9061

The system contains sufficient reagents to perform 200 reactions in 96-well plates (50µl of sample + 50µl of NAD(P)H-Glo™ Detection Reagent), 400 assays in 384-well plates (25µl of sample + 25µl of NAD(P)H-Glo™ Detection Reagent) or 2,000 assays in low-volume 384-well plates (5µl of sample + 5µl of NAD(P)H-Glo™ Detection Reagent). Assay volumes can be varied depending on plate format as long as you maintain a 1:1 ratio of sample to NAD(P)H-Glo™ Detection Reagent. Includes:

- 55µl Reductase
- 55µl Reductase Substrate
- 1 vial Luciferin Detection Reagent (lyophilized)
- 10ml Reconstitution Buffer

Product	Size	Cat.#
NAD(P)H-Glo™ Detection System	50ml	G9062

The system contains sufficient reagents to perform 1,000 reactions in 96-well plates (50µl of sample + 50µl of NAD(P)H-Glo™ Detection Reagent), 2,000 assays in 384-well plates (25µl of sample + 25µl of NAD(P)H-Glo™ Detection Reagent) or 10,000 assays in low-volume 384-well plates (5µl of sample + 5µl of NAD(P)H-Glo™ Detection Reagent). Assay volumes can be varied depending on plate format as long as you maintain a 1:1 ratio of sample to NAD(P)H-Glo™ Detection Reagent. Includes:

- 275µl Reductase
- 275µl Reductase Substrate
- 1 vial Luciferin Detection Reagent (lyophilized)
- 50ml Reconstitution Buffer

Storage Conditions: Store all components at –20°C (–30°C to –10°C). Minimize freeze-thaw cycles of all reagents.

3. NAD(P)H-Glo™ Detection System Protocol

3.A. Preparing the Luciferin Detection Reagent

1. Thaw the Reconstitution Buffer, and equilibrate the Reconstitution Buffer and lyophilized Luciferin Detection Reagent to room temperature.
2. Transfer the entire contents of the Reconstitution Buffer bottle to the amber bottle of lyophilized Luciferin Detection Reagent.

3. Mix by swirling or inversion to obtain a uniform solution. Do not vortex. The Luciferin Detection Reagent should go into solution easily in less than 1 minute.

   Note: Store the reconstituted Luciferin Detection Reagent at room temperature while preparing the NAD(P)H-Glo™ Detection Reagent. If the reconstituted Luciferin Detection Reagent is not used immediately, the reagent can be stored at room temperature (approximately 22°C) for up to 24 hours or dispensed into single-use aliquots and stored at 4°C for up to 1 week or –20°C for up to 3 months with no change in activity.

3.B. Preparing the NAD(P)H-Glo™ Detection Reagent

Determine the number of NAD(P)H-Glo™ assays being performed, and calculate the volume of NAD(P)H-Glo™ Detection Reagent needed. An equal volume of NAD(P)H-Glo™ Detection Reagent will be added to each sample containing NAD(P)H. We recommend preparing extra reagent to compensate for pipetting error. Do not store unused NAD(P)H-Glo™ Detection Reagent.

1. Equilibrate the reconstituted Luciferin Detection Reagent to room temperature.
2. Thaw the Reductase and Reductase Substrate just prior to use, and store on ice.
3. Prepare the required amount of NAD(P)H-Glo™ Detection Reagent by adding the volumes of Reductase and Reductase Substrate indicated in Table 2 per 1ml of reconstituted Luciferin Detection Reagent.

For best results, we recommend preparing the NAD/NADH-Glo™ Detection Reagent immediately before use. If necessary, the prepared NAD/NADH-Glo™ Detection Reagent can be kept at room temperature and used within 6 hours.

Table 2. Preparing the NAD(P)H-Glo™ Detection Reagent.

Component	Volume
Reconstituted Luciferin Detection Reagent	1ml
Reductase	5µl
Reductase Substrate	5µl

4. Mix by gently inverting five times.
5. Return unused Reductase and Reductase Substrate to –20°C storage. Do not store unused NAD(P)H-Glo™ Detection Reagent. Minimize the number of freeze-thaw cycles for all reagents.

3.C. Protocol

This protocol is for a standard reaction of 50µl of sample and 50µl of NAD(P)H-Glo™ Detection Reagent in a 96-well plate. The final reaction volume can be varied as long as you maintain a 1:1 ratio of sample and NAD(P)H-Glo™ Detection Reagent. Throughout this manual, sample refers to the starting biochemical reaction that contains NAD(P)H.

Note: Avoid the presence of DTT and other reducing agents in the samples. Reducing agents will react with the Reductase Substrate to increase background. Also avoid the presence of chelating compounds such as EDTA.

1. Add 50µl of sample to wells of a 96-well white luminometer-compatible plate.
2. Add 50µl of NAD(P)H-Glo™ Detection Reagent to each well.
3. Gently shake the plate to mix.
4. Incubate for 40–60 minutes at room temperature.
5. Record luminescence using a luminometer.

   Note: Luminescence should stabilize during the 40- to 60-minute incubation. If luminescence does not stabilize, you may want to take additional steps as described in Section 5.C.

4. General Considerations

Plates and Luminometers

Use opaque, white multiwell plates that are compatible with your luminometer. Light signal is diminished in black plates, and well-to-well cross-talk is increased in clear plates. All standard instruments capable of measuring luminescence are suitable for this assay. Instrument settings depend on the luminometer manufacturer. Use an integration time of 0.25–1 second per well as a guide. Although relative light output will vary with different instruments, variation should not affect assay performance.

Temperature

The intensity and stability of the luminescent signal from the NAD(P)H-Glo™ Detection System depend on the Reductase and luciferase reaction rates. Environmental factors such as temperature affect reaction rates and consequently the intensity and stability of the luminescent signal. For consistent results, equilibrate assay plates and reagents to room temperature (approximately 22°C) before adding the NAD(P)H-Glo™ Detection Reagent. Insufficient equilibration may result in a temperature gradient and variability across the plate.

Chemical Environment

The chemical environment of the sample containing NAD(P)H can affect the Reductase and luciferase enzymatic rates and light signal intensity. We recommend a pH of ~7–8 for the NAD(P)H-containing samples. Avoid the presence of chelating compounds such as EDTA in the samples. The luciferase reaction requires the divalent magnesium cation, which is included in the Luciferin Detection Reagent. Also avoid the presence of DTT and other reducing agents in samples. Reducing agents will react with the Reductase Substrate and increase background.

The NAD(P)H-Glo™ Detection System is compatible with samples containing up to 10% DMSO.

5. Appendix

5.A. Generating a Standard Curve

A standard curve allows conversion of luminescence, measured in RLUs, to NAD(P)H concentration by directly comparing the luminescent measurements from samples to the light signals generated from purified NAD(P)H. We recommend using purified NADH (Sigma Cat.# N6660) or NADPH (Sigma Cat.# N9910) to prepare a 200µM stock solution by directly adding phosphate-buffered saline (PBS) or 50mM Tris (pH 8.0) to the vial. Immediately before the assay, dilute the 200µM stock in the same buffer used to prepare the experimental samples, as pH and some buffer components can affect the light signal (see Section 4). We recommend assaying each standard concentration in triplicate or quadruplicate on the same plate as the experimental samples.

For each point on the standard curve, calculate the average luminescence of the replicate reactions, and subtract the average luminescence of the blank reactions [reactions at 0µM NAD(P)H]. Use these net luminescence values to generate the standard curve and perform linear regression analysis. Interpolate the amount of NAD(P)H present in the sample by comparing net luminescence values to the values in the standard curve.

Representative data are shown in Table 3, and an example of a standard curve is shown in Figure 4.

5.B. Extending the Linear Range

The linear range of the assay is 0.1µM to 25µM of NAD(P)H. The linear range is limited by Reductase Substrate concentration. To measure higher concentrations of NAD(P)H, more Reductase Substrate can be added, but this will increase background signal and reduce sensitivity.

Table 3. Data Used to Generate the NADH Standard Curve Shown in Figure 4.

Dinucleotide Concentration (µM)	Luminescence (RLU)	Net Luminescence (RLU)
25	18,083,128	18,051,000
12.5	7,934,108	7,901,980
6.25	3,943,710	3,911,582
3.125	1,922,829	1,890,701
1.56	958,328	926,200
0.78	489,796	457,668
0.39	256,005	223,877
0.195	140,531	108,403
0.098	85,185	53,057
0.049	57,351	25,223
0.024	45,498	13,370
0	32,128	0

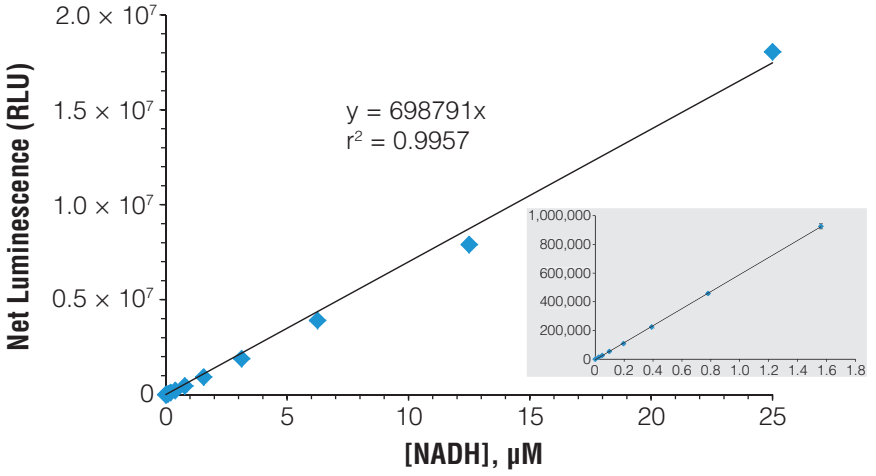

Figure 4. A representative NADH standard curve. An NADH standard curve was generated by serially diluting an NADH stock (Sigma Cat.# N6660) in 1X PBS buffer (pH 6.8). Each dilution was assayed in triplicate in 100µl reactions in a white 96-well plate. Luminescence was recorded after a 40-minute incubation using a GloMax® 96 Microplate Luminometer. To generate the standard curve, net luminescence was calculated as luminescence at each standard concentration minus luminescence at 0µM NADH. Error bars are ± (1 standard deviation + 1 standard deviation of the blank). The %CV values were ≤5%.

5.C. Light Signal Stability

Upon addition of NAD(P)H-Glo™ Detection Reagent to the sample, the signal will continue to increase until all NAD(P)H is consumed. The maximal light signal occurs after 40 to 60 minutes, resulting in a glow-type luminescence signal with a half-life greater than 2 hours (Figure 5).

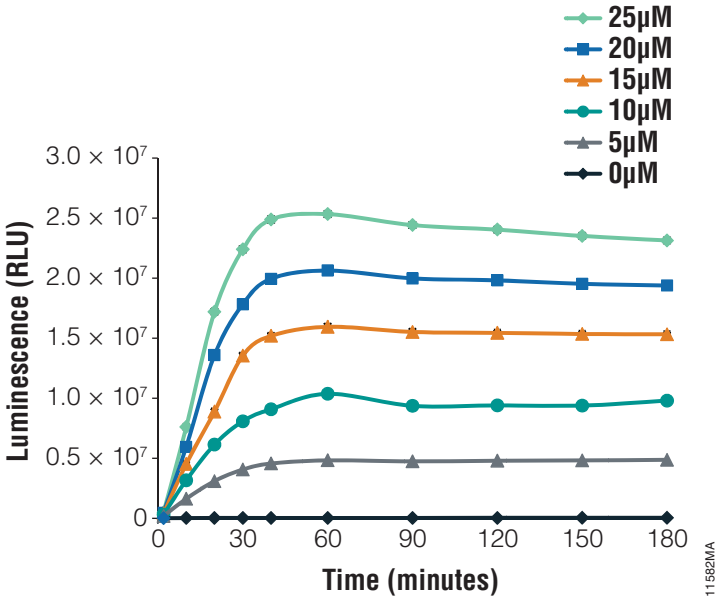

Figure 5. Light signal kinetics. Fifty microliters of purified NADH in 50mM Tris HCl (pH 7.5) at the indicated concentrations was assayed in quadruplicate wells of a white 96-well plate. Luminescence was measured at the indicated time points after addition of 50µl of NAD(P)H-Glo™ Detection Reagent. Each point represents the average luminescence from quadruplicate reactions. Error bars are ± 1 standard deviation. The %CV values were ≤5%.

Some enzymes continue to be active even after addition of the detergent-containing NAD(P)H-Glo™ Detection Reagent. If a stable light signal is desired, stop the production or consumption of NAD(P)H by adding an enzyme-specific inhibitor to the NAD(P)H-Glo™ Detection Reagent.

If production of NAD(P)H is not stopped by adding the NAD(P)H-Glo™ Detection Reagent or enzyme-specific inhibitor, the signal will continue to increase, and the changes in light output can be monitored over time or at a single time point. Light output will remain proportional to the amount of NAD(P)H in the sample until all of the Reductase Substrate is converted to luciferin.

The increase in signal after adding the NAD(P)H-Glo™ Detection Reagent can be stopped at any time by adding the reductase inhibitor menadione at 1/10 the reaction volume to a final concentration of 0.25mM as described below. This protocol is for a reaction with a final volume of 100µl.

5.C. Light Signal Stability (continued)

Materials to Be Supplied by the User

- menadione (e.g., Sigma Cat. #M5625)
- 100% ethanol
- 20% DMSO prepared with water

1. Prepare a 27.5mM menadione solution in 100% ethanol. For 200 reactions of 100µl each, prepare 250µl.
2. Combine 200µl of the 27.5mM menadione solution prepared in Step 1 and 1,800µl of 20% DMSO for a final concentration of 2.75mM menadione.
3. At the desired time point, add 10µl of 2.75mM menadione to each 100µl reaction.
4. Mix the plate by gently shaking.
5. Incubate at room temperature for 10 minutes.
6. Record luminescence using a luminometer.

6. References

1. Lowry, O.H. *et al.* (1961) The measurement of pyridine nucleotides by enzymatic cycling. *J. Biol. Chem.* **236**, 2746–55.
2. Ward, P.S. and Thompson, C.B. (2012) Metabolic reprogramming: A cancer hallmark even Warburg did not anticipate. *Cancer Cell* **21**, 297–308.
3. Vander Heiden, M.G. (2011) Targeting cancer metabolism: A therapeutic window opens. *Nature Reviews Drug Discovery* **10**, 671–84.

7. Related Products

Product	Size	Cat.#
NAD/NADH-Glo™ Assay	10ml	G9071
	50ml	G9072
NADP/NADPH-Glo™ Assay	10ml	G9081
	50ml	G9082

Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Cytotoxicity Assays

Product	Size	Cat.#
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml	G6410

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 2 Assay	10ml	G0940
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 6 Assay	10ml	G0970
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790

Mitochondrial Toxicity Assay

Product	Size	Cat.#
Mitochondrial ToxGlo™ Assay	10ml	G8000

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611

Detection Instrumentation

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct® Software: Base Instrument with Shaking*	1 each	E8032
GloMax®-Multi+ Luminescence Module	1 each	E8041
GloMax®-Multi+ Fluorescence Module	1 each	E8051
GloMax®-Multi+ Visible Absorbance Module	1 each	E8061
GloMax®-Multi+ UV-Visible Absorbance Module	1 each	E9061

*Cat.# E8032 cannot be sold separately and must be purchased with at least one detection module (Cat.# E8041, E8051, E8061 or E9061).

[a]Patent Pending.

[b]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.

[c]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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