TECHNICAL MANUAL

SIRT-Glo™ Assay System

Instructions for Use of Product
G6450

Revised 2/18
TM336

SIRT-Glo™ Assay System

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

1. Description

The SIRT-Glo™ Assay System[a,b,c] is a single-reagent-addition, homogeneous, luminescent assay that measures the relative activity of the NAD^+-dependent histone deacetylase (HDAC) class III enzymes (sirtuins; SIRTs) from purified enzyme sources. This assay is broadly useful for class III enzymes, but sensitivity and performance will vary with catalytic efficiency of particular isoenzymes. The assay uses an acetylated, luminogenic peptide substrate that can be deacetylated by SIRT activities (Figure 1). Deacetylation of the peptide substrate is measured using a coupled enzymatic system in which a protease in the Developer Reagent cleaves the peptide from aminoluciferin, which is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase. The SIRT-mediated luminescent signal is persistent (Figure 2) and proportional to deactetylase activity (Figure 4), allowing batch processing of multiwell plates. Enzymatic steady state (between deacetylase, protease and luciferase) is typically achieved within 15–45 minutes, and the signal has a half-life of greater than 3 hours. An overview of the SIRT-Glo™ Assay protocol is shown in Figure 3.

1. Description (continued)

Assay Advantages

Simple Measurement of Deacetylating Activities: Uses a single-reagent-addition, homogeneous, "Add-Mix-Measure" protocol.

Highly Sensitive: Provides 10- to 100-fold higher sensitivity than comparable fluorescence methods.

Fast Data Acquisition: Achieves maximum signal in as little as 15 minutes with persistent, "glow-type" steady-state signal, making the protocol amenable to automation in high-throughput formats and compatible with luminometers without injectors.

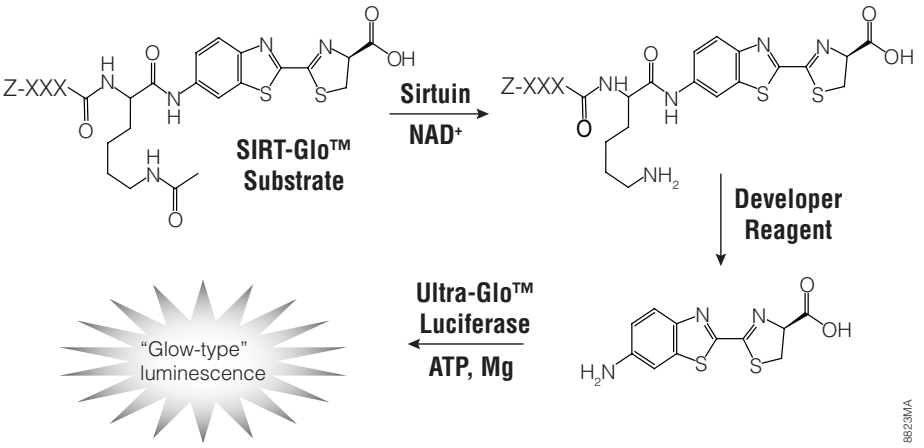

Figure 1. SIRT-Glo™ Assay chemistry. SIRT activity deacetylates the luminogenic SIRT-Glo™ Substrate, making the peptide sensitive to a specific proteolytic cleavage event that is mediated by SIRT-Glo™ Reagent and liberates aminoluciferin. Free aminoluciferin then can be measured using the Ultra-Glo™ firefly luciferase reaction to produce a stable, persistent emission of light. Z represents an amino-terminal blocking group that protects the substrate from nonspecific cleavage. XXXLysine is a SIRT-optimized amino acid sequence based on a consensus sequence derived from p53 (1).

Note: All three enzymatic events occur in coupled, homogeneous, nearly simultaneous reactions that reach steady state in 15–45 minutes.

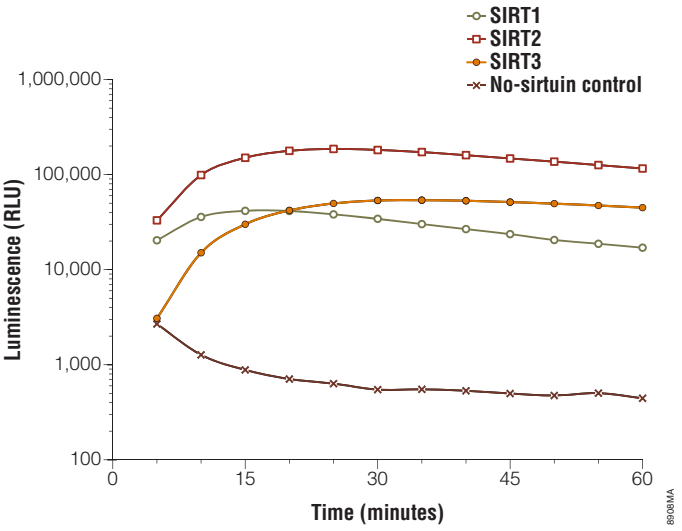

Figure 2. SIRT-mediated luminescent signal is persistent. Recombinant SIRT1 (1,000mU/ml), SIRT2 (1,250mU/ml) and SIRT3 (350mU/ml) proteins were assayed using the SIRT-Glo™ Assay as described in Section 3.B.

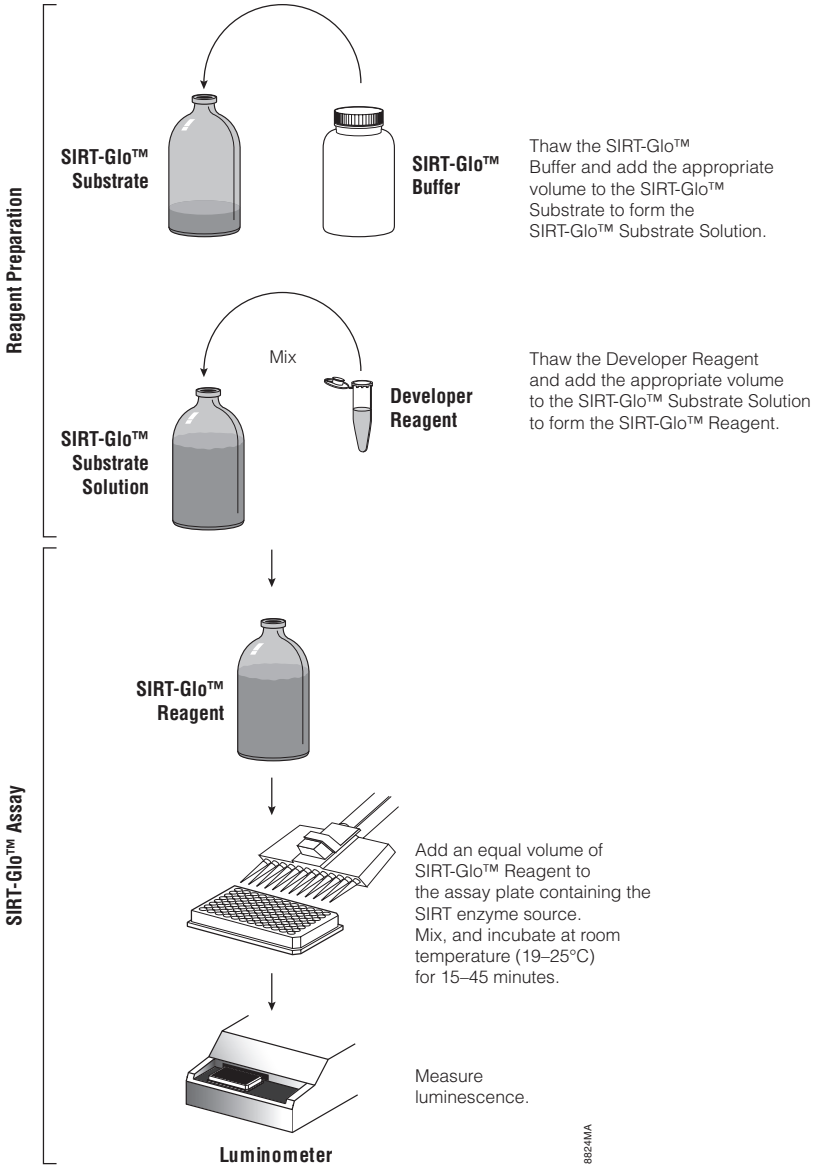

Figure 3. Overview of the SIRT-Glo™ Assay protocol.

Note: Prepare the SIRT-Glo™ Reagent just prior to the assay.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
SIRT-Glo™ Assay	**10ml**	**G6450**

G6450 is sufficient for 100 assays at 100µl/assay in a 96-well plate or 400 assays at 25µl/assay in a 384-well plate. Includes:

- 25ml SIRT-Glo™ Buffer
- 1 vial SIRT-Glo™ Substrate, Lyophilized
- 10µl Developer Reagent
- 30µl Nicotinamide, 1M

PRODUCT	SIZE	CAT.#
Nicotinamide	**30µl**	**G6540**

G6540 is supplied at a concentration of 1M in SIRT-Glo™ Buffer.

PRODUCT	SIZE	CAT.#
HeLa Nuclear Extract	**10µl**	**G6570**

G6570 is supplied at a concentration of 5mg/ml.

Storage Conditions: Store the SIRT-Glo™ Assay components at –20°C.

Storage Conditions for the SIRT-Glo™ Reagent: For optimal performance, the SIRT-Glo™ Reagent should be used in its entirety on the day prepared. However, the reagent can be stored at –20°C for up to 2 weeks with minimal change in performance. Just prior to assaying samples, prepare the reagent as described in Section 3. If the SIRT-Glo™ Reagent cannot be used immediately to assay SIRT activity, it should be stored on ice until use (with brief equilibration to room temperature before use). Storage on ice for more than 8 hours is discouraged due to decreased assay performance. The SIRT-Glo™ Substrate Solution (i.e., the Developer Reagent has not been added) can be stored at 4°C for up to 24 hours or at –70°C for up to 1 month. If the entire volume of SIRT-Glo™ Reagent will not be used in one experiment, combine the SIRT-Glo™ Substrate and SIRT-Glo™ Buffer, then divide the resulting SIRT-Glo™ Substrate Solution into single-use aliquots. To the aliquot to be used immediately, add the appropriate volume of Developer Reagent to create the SIRT-Glo™ Reagent, and freeze the remaining aliquots of SIRT-Glo™ Substrate Solution.

Storage Conditions for the HeLa Nuclear Extract: Store the HeLa Nuclear Extract at –70°C. Minimize freeze-thaw cycles. Store the thawed HeLa Nuclear Extract on ice when in use.

HeLa Nuclear Extract is not intended to be a source of SIRT activity. HeLa Nuclear Extract contains HDAC class I and II enzymes that use and deacetylate the SIRT-Glo™ Assay Substrate.

3. Protocols

Materials to be Supplied by the User

- multichannel pipette or liquid-dispensing robot
- reagent reservoirs
- orbital shaker
- purified SIRT enzyme
- 96-well, 384-well or 1,536-well, white-walled, opaque- or clear-bottom tissue culture plates compatible with luminometer (see note below)

Note: Commercial plate vendors use different proprietary compositions of plastic, which may affect overall luminescence values. Although many different sources of plates can be used, best results are obtained using Costar plates (Cat.# 3917) in 96-well formats.

The SIRT-Glo™ Reagent should be used in its entirety on the day it is prepared. If the entire volume of SIRT-Glo™ Reagent will not be used in one day, see Section 2 for recommendations on how to prepare only the amount of SIRT-Glo™ Reagent needed.

3.A. Determining Linear Range Using Sirtuin Enzymes

This protocol is written for a 96-well plate. Required volumes for 384- and 1,536-well plates are given in parentheses. Representative data are shown in Figure 4.

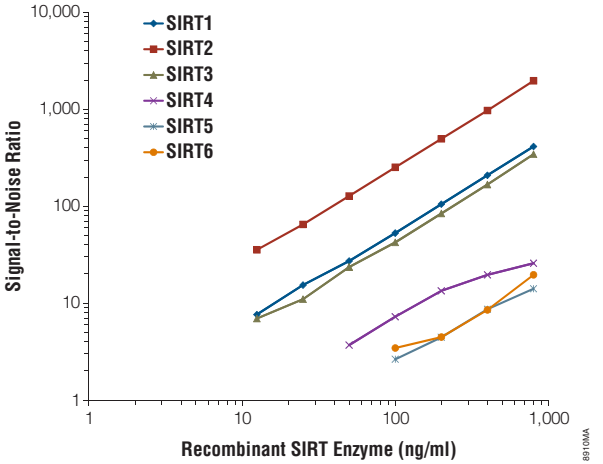

Figure 4. Example of linear range data. Recombinant sirtuins were diluted to 800ng/ml in SIRT-Glo™ Buffer, then serially diluted twofold in 100µl volumes in a 96-well plate. An equal volume of SIRT-Glo™ Reagent was added, and luminescence was measured after 20 minutes at room temperature. The recombinant enzymes (SIRT1, 2, 3, 4, 5 and 6) were obtained from SignalChem. Data were plotted using GraphPad Prism® software. Data represent the mean ± standard deviation of four samples.

3.A. Determining Linear Range Using Sirtuin Enzymes (continued)

The SIRT-Glo™ Assay is provided with sufficient SIRT-Glo™ Buffer to rehydrate the SIRT-Glo™ Substrate and dilute the test compound. For Cat.# G6450, be sure to reserve 10ml of buffer for each vial of SIRT-Glo™ Substrate prior to performing the test compound and enzyme dilutions. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

1. Prepare an initial dilution of the purified sirtuin enzyme at 1–5µg/ml in SIRT-Glo™ Buffer.

   Note: There are several commercial sources of purified sirtuins with varying specific activities. Specific isoenzymes also will display different activities. Therefore, the useful enzyme dilution may vary greatly and should be determined experimentally prior to inhibitor potency determinations.

2. Prepare serial twofold dilutions of the SIRT enzyme in SIRT-Glo™ Buffer in rows A–D of a white-walled 96-well plate as described in Figure 5. The final volume of diluted enzyme in each well should be 100µl for 96-well plates (20µl for 384-well or 5µl for 1,536-well plates).

   Optional: The HeLa Nuclear Extract can be used as a source of deacetylase activity to confirm that the assay chemistry is working properly. Dilute the extract 1:5,000 (i.e., 1µl of HeLa Nuclear Extract and 5.0ml of SIRT-Glo™ Buffer), then add 100µl of diluted HeLa Nuclear Extract to each well in column 11 instead of the diluted sirtuin prepared in Step 1. Do not store diluted HeLa Nuclear Extract.

	1	2	3	4	5	6	7	8	9	10	11	12
A	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	no enzyme
B	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	no enzyme
C	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	no enzyme
D	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	no enzyme

8820MA

Figure 5. Example of a plate layout showing dilution ratios for the SIRT enzyme when determining linear range.

3. Equilibrate the SIRT-Glo™ Buffer and Substrate at room temperature. Thaw the Developer Reagent. Prepare the SIRT-Glo™ Reagent as described below:

   For Cat.# G6450, add 10ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate, then mix. Add 10µl of Developer Reagent to form the SIRT-Glo™ Reagent, then mix.

If the SIRT-Glo™ Reagent cannot be used immediately to assay SIRT activity, it should be stored on ice until use (with brief equilibration to room temperature before use). If the entire volume of SIRT-Glo™ Reagent will not be used in one day, see Section 2 for recommendations on how to prepare only the amount of SIRT-Glo™ Reagent needed.

Note: Brief centrifugation may be required to recover the full volume of Developer Reagent.

3.A. Determining Linear Range Using Sirtuin Enzymes (continued)

4. Add an equal volume of SIRT-Glo™ Reagent to each assay well (100µl for 96-well, 20µl for 384-well or 5µl for 1,536-well plates). The final volume per well of a 96-well plate is 200µl. Place any unused SIRT-Glo™ Reagent on ice.
5. Mix briefly at room temperature using an orbital shaker at 500–700rpm to ensure homogeneity. Incubate at room temperature for 15–45 minutes.
6. Measure luminescence at signal steady-state (15–45 minutes after adding the SIRT-Glo™ Reagent).

3.B. Determining SIRT Inhibitor or Activator Potency

Figure 6 shows representative results for SIRT inhibitor potency determination using purified SIRT1 enzyme.

This protocol is written for a 96-well plate. Required volumes for 384-well and 1,536-well plates are given in parentheses.

The SIRT-Glo™ Assay is provided with sufficient SIRT-Glo™ Buffer to rehydrate the SIRT-Glo™ Substrate and dilute the test compound. For Cat.# G6450, be sure to reserve 10ml of buffer for each vial of SIRT-Glo™ Substrate prior to performing the dilutions. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

1. Prepare serial twofold or threefold dilutions of unknown compound and the known SIRT inhibitor Nicotinamide in SIRT-Glo™ Buffer in a white-walled 96-well plate; a serial twofold dilution is shown in Figure 7. The final volume in each well should be 50µl for 96-well plates (10µl for 384-well or 2.5µl for 1,536-well plates). Add only SIRT-Glo™ Buffer to wells in columns 11 and 12 to serve as the no-inhibitor and no-sirtuin controls.

   Optional: The HeLa Nuclear Extract can be used as a source of deacetylase activity to confirm that the assay chemistry is working properly. Dilute the extract at least 1:5,000, then add 100µl of diluted HeLa Nuclear Extract to each well in column 10 instead of the diluted test compound or Nicotinamide. Do not store diluted HeLa Nuclear Extract.

   Note: Brief centrifugation of Nicotinamide and HeLa Nuclear Extract may be required to recover the full volume.

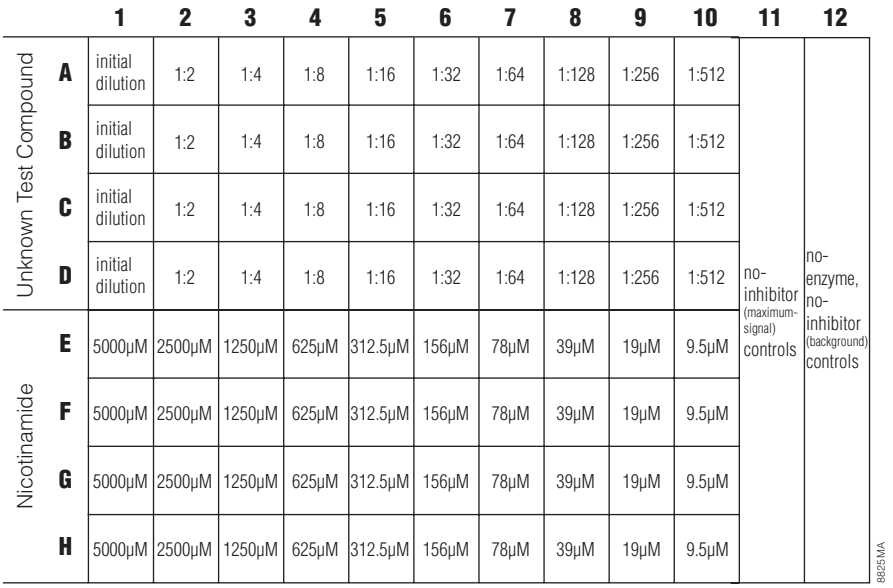

Figure 7. Example of a plate layout showing a twofold serial dilution of unknown test compound and final concentrations of Nicotinamide.

2. Dilute the SIRT enzyme using SIRT-Glo™ Buffer to the desired concentration. Be sure that the enzyme concentration is within the linear range determined in Section 3.A.

3. Dispense 50µl of SIRT enzyme to each well of inhibitor dilutions prepared in Step 1 and no-inhibitor controls (column 11). Add 50µl of SIRT-Glo™ Buffer to the no-sirtuin controls (column 12). (Dispense 10µl for 384-well or 2.5µl for 1,536-well plates.)

4. Mix briefly at room temperature using an orbital shaker at 500–700rpm to ensure homogeneity.

5. Incubate sirtuin/inhibitor mixes at room temperature for at least 30 minutes (but not longer than approximately 2 hours).

   Note: During this incubation, equilibrate the SIRT-Glo™ Buffer and Substrate at room temperature. Thaw the Developer Reagent.

3.B. Determining SIRT Inhibitor or Activator Potency (continued)

6. Prepare the SIRT-Glo™ Reagent as described below:

   For Cat.# G6450, add 10ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate, then mix. Add 10µl of Developer Reagent to form the SIRT-Glo™ Reagent, then mix.

   If you have already prepared the reagent to determine the linear range of the SIRT enzyme (Section 3.A), remove the SIRT-Glo™ Reagent from ice and warm the reagent to room temperature.

   If the SIRT-Glo™ Reagent cannot be used immediately to assay SIRT activity, it should be stored on ice until use (with brief equilibration to room temperature before use). If the entire volume of SIRT-Glo™ Reagent will not be used in one day, see Section 2 for recommendations on how to prepare only the amount of SIRT-Glo™ Reagent needed.

   Note: Brief centrifugation may be required to recover the full volume of Developer Reagent.

7. Add an equal volume of SIRT-Glo™ Reagent to each assay well (100µl for 96-well, 20µl for 384-well or 5µl for 1,536-well plates).

8. Mix briefly at room temperature using an orbital shaker at 500–700rpm to ensure homogeneity. Incubate at room temperature for 15–45 minutes.

9. Measure luminescence at signal steady-state (15–45 minutes after adding the SIRT-Glo™ Reagent).

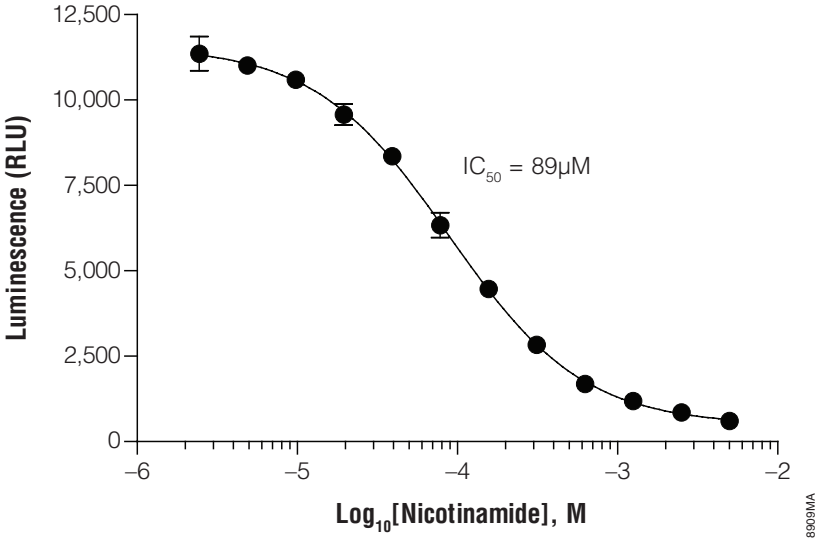

Figure 6. Example of potency data generated using recombinant SIRT1 and Nicotinamide. SIRT-Glo™ Assays were performed as described in Section 3.B. The final concentration of recombinant SIRT1 protein (Biomol Cat.# BML-SE239) was 400ng/ml. Data were plotted and IC_{50} value determined using GraphPad Prism® software. Data represent the mean ± standard deviation of four samples.

4. General Considerations

4.A. Background Luminescence

The no-sirtuin controls should be used to determine background luminescence.

4.B. Temperature

The enzyme activities measured in this assay are influenced by temperature. For best results, incubate at a constant, controlled temperature to ensure uniformity across the plate. Samples should be equilibrated at room temperature (19–25°C) prior to adding the SIRT-Glo™ Reagent.

4.C. Incubation Time

Steady-state of reactions is typically achieved within 15–45 minutes at room temperature. Luminescence can be measured at any time after the signal plateau. Signal will gradually decay as a function of time, and measurement should be completed within 1–2 hours.

4.D. Assay Controls

No-Sirtuin (Buffer Background) Control: Set up at least triplicate wells without SIRT enzyme to serve as the negative control to determine background luminescence.

No-Inhibitor Control: The maximum-signal control is established by adding vehicle (used to deliver the test compound) to wells. In most cases, this consists of a buffer system or medium plus solvent (e.g., DMSO or methanol) at the same concentration as that found in the treated samples. Set up at least triplicate wells with uninhibited SIRT enzyme. Add the same solvent used to deliver the test compounds to no-inhibitor control wells.

Known Inhibitor (Nicotinamide) Control (optional): Set up triplicate wells or a dilution series using the provided Nicotinamide as a control for specific inhibition of SIRT activity. The use of Nicotinamide as an inhibitor control is strongly encouraged when using recombinantly expressed or purified preparations of SIRTs. The sensitive SIRT-Glo™ Assay can measure the activities of nonSIRT deacetylase impurities that may copurify with target enzymes.

Hela Nuclear Extract Control (optional): This enzyme source is a control for the deacetylase chemistry and should not be considered a source of NAD^+-dependent sirtuin activity. HeLa Nuclear Extract contains HDAC class I and II enzymes that use and deacetylate the SIRT-Glo™ Assay Substrate.

4.E. High-Throughput Screening

The SIRT-Glo™ Assay can be easily scaled and miniaturized for high-density formats in high-throughput screening. To minimize variability, we recommend the following:

1. **Thorough mixing of assay reagent and sample:** Assay well geometry and small dispense volumes may affect the effectiveness of mixing and cause some degree of partitioning of reagent or sample. Poor assay homogeneity in individual wells may result in reduced signals, which can complicate hit scoring.
2. **Longer incubation after assay reagent addition:** If vibrational mixing is not possible, extend the incubation time prior to measuring luminescence to at least 30–60 minutes.
3. **Detergent addition:** Every assay chemistry exhibits some degree of susceptibility to false hits. Inclusion of 1–2% Tergitol™ NP-9 in the SIRT-Glo™ Assay Reagent may reduce compound aggregation and inhibition due to weak, nonspecific assay inhibitors observed in single-concentration screens (2,3). Other common detergents may not be compatible with HDAC class III enzyme activity and lead to unacceptable assay performance.

4.F. Potential Assay Interference

No assay measuring sirtuin is perfect and the SIRT-Glo™ Assay System could be negatively affected by compounds that act directly on the Developer Reagent or the luminescent detection SIRT-Glo Substrate Solution. We recommend all assay results should be confirmed by orthogonal methods.

5. References

1. Abraham, J. *et al.* (2000) Post-translational modification of p53 protein in response to ionizing radiation analyzed by mass spectrometry. *J. Mol. Biol.* **295**, 853–64.
2. Thorne, N. *et al.* (2010) Apparent activity in high-throughput screening: Origins of compound-dependent assay interference. *Curr. Opin. Chem. Biol.* **14**, 315–24.
3. Auld, D. S. *et al.* (2008) Characterization of chemical libraries for luciferase inhibitory activity. *J. Med. Chem.* **51**, 2372–86.

6. Related Products

Product	Size	Cat.#
HDAC-Glo™ I/II Assay	10ml	G6420
	5 × 10ml	G6421
	100ml	G6422
HDAC-Glo™ I/II Screening System	10ml	G6430
	5 × 10ml	G6431
MTase-Glo™ Methyltransferase Assay	400 assays	V7601
	2,000 assays	V7602
Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay	1,000 assays	V7990
	10,000 assays	V7991
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	10 × 10ml	G8093
	100ml	G8092
Caspase-Glo® 2 Assay	10ml	G0940
Caspase-Glo® 6 Assay	10ml	G0970
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
	100ml	G8212
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
	5 × 10ml	G6081
	2 × 50ml	G6082
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
	5 × 10ml	G9291
	2 × 50ml	G9292

6. Related Products (continued)

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
	5 × 10ml	G9201
	2 × 50ml	G9202
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
	5 × 10ml	G9271
	2 × 50ml	G9272
ApoTox-Glo™ Triplex Assay	10ml	G6320
	5 × 10ml	G6321
ApoLive-Glo™ Multiplex Assay	10ml	G6410
	5 × 10ml	G6411
Proteasome-Glo™ Chymotrypsin-Like Assay	10ml	G8621
	50ml	G8622
Proteasome-Glo™ Trypsin-Like Assay	10ml	G8631
	50ml	G8632
Proteasome-Glo™ Caspase-Like Assay	10ml	G8641
	50ml	G8642
Proteasome-Glo™ 3-Substrate System	10ml	G8531
	50ml	G8532
Proteasome-Glo™ 3-Substrate Cell-Based Assay System	10ml	G1180
	50ml	G1200
GSH-Glo™ Glutathione Assay	10ml	V6911
	50ml	V6912
GSH/GSSG-Glo™ Assay	10ml	V6611
	50ml	V6612

Detection Instrumentation

Product	Size	Cat.#
GloMax® 96 Microplate Luminometer	each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	each	E6521
GloMax® Discover System	each	GM3000
GloMax® Explorer System, Fully Loaded	each	GM3500
GloMax® Explorer System, Luminescence and Fluorescence	each	GM3510

7. Summary of Changes

The 2/18 version of this technical manual has been modified to remove any mention of discontinued products.

[a]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.

[b]U.S. Pat. No. 8,632,992 and other patents pending.

[c]Promega has a nonexclusive, worldwide license to U.S. Pat. Nos. 7,033,778, 7,256,013, Europe Pat. No. 1243568 and Japan Pat. No. 4267043 and related applications to manufacture, have manufactured, use, possess, distribute, market, sell, offer for sale, and import deacetylase activity assay kits and related products for research and laboratory use (including the use on human derived cell lines, but excluding such use on human subjects for diagnostic or therapeutic purposes), product control, process control, product development and process development.

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