Technical Manual

SIRT-Glo™ Assay and Screening System

INSTRUCTIONS FOR USE OF PRODUCTS G6450, G6451, G6452, G6470, G6471, G6460, G6540 AND G6570.

PRINTED IN USA.
Revised 9/11

Part# TM336

SIRT-Glo™ Assay and Screening System

All technical literature is available on the Internet at: www.promega.com/tbs/ 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 SIRT-Glo™ Assay and Screening System[a–c] are single-reagent-addition, homogeneous, luminescent assays that measure 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.

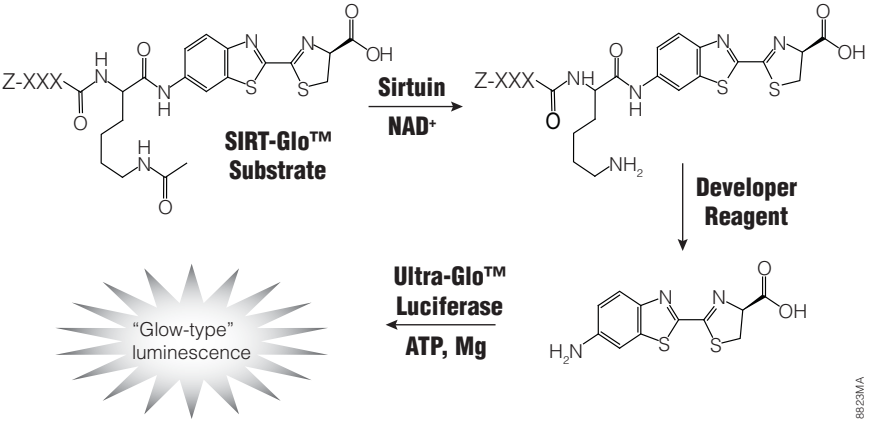

Figure 1. SIRT-Glo™ Assay chemistry. SIRT activity deacetylates the luminogenic SIRT-Glo™ Substrate, making the peptide sensitive to a specific proteolytic cleavage event that is mediated by SIRT-Glo™ Reagent and liberates aminoluciferin. Free aminoluciferin then can be measured using the Ultra-Glo™ 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.

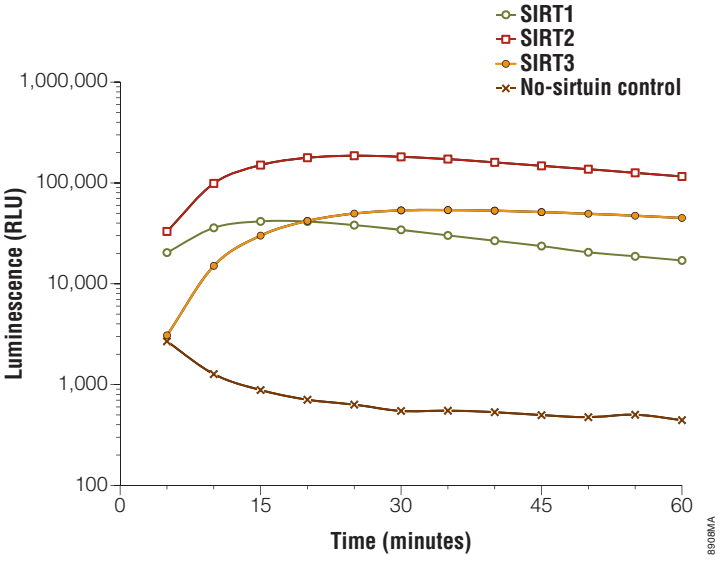

Figure 2. SIRT-mediated luminescent signal is persistent. Recombinant SIRT1 (1,000mU/ml), SIRT2 (1,250mU/ml) and SIRT3 (350mU/ml) proteins were assayed using the SIRT-Glo™ Assay as described in Section 3.B.

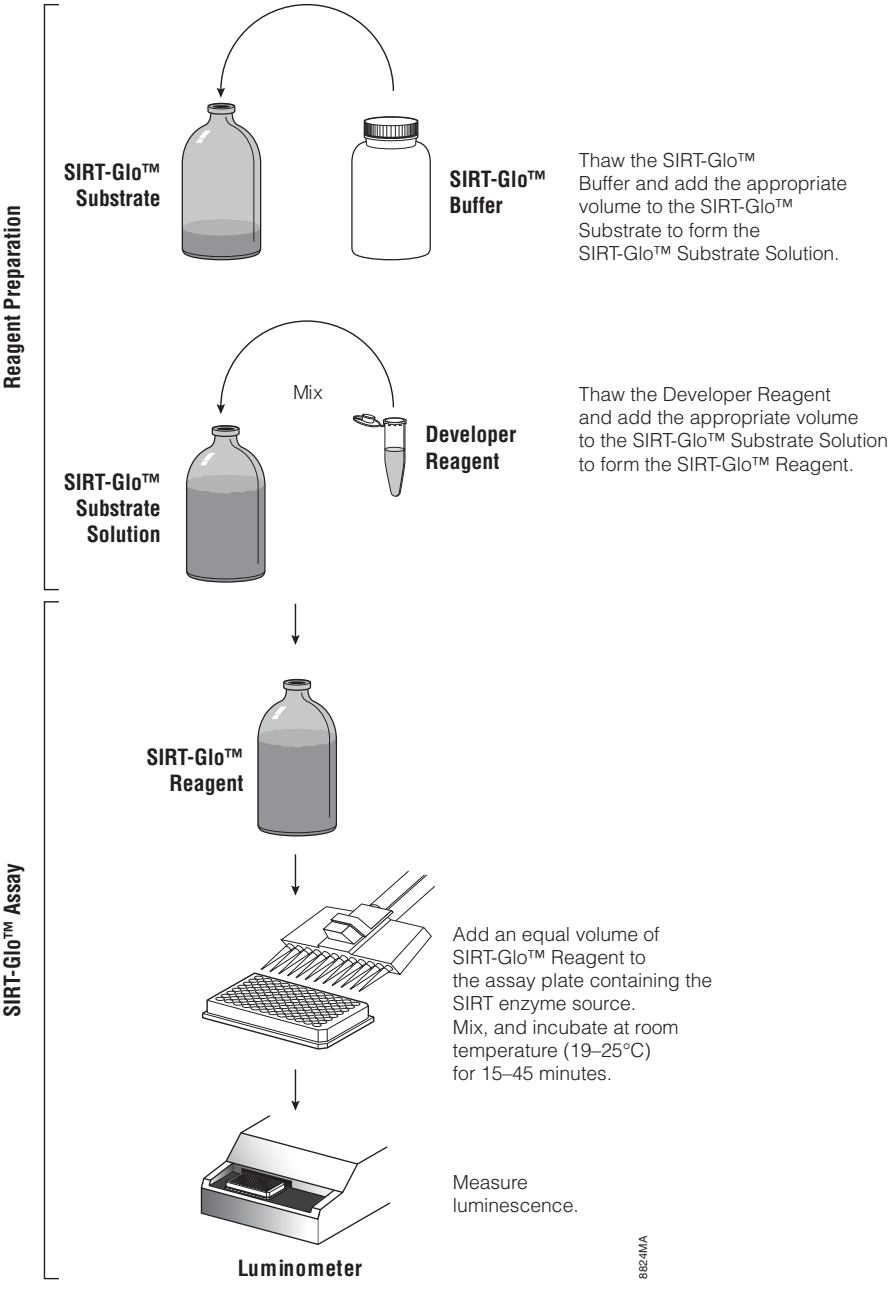

Figure 3. Overview of the SIRT-Glo™ Assay protocol.
Note: Prepare the SIRT-Glo™ Reagent just prior to the assay.

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.#
SIRT-Glo™ Assay	5 × 10ml	G6451

G6451 is sufficient for 500 assays at 100µl/assay in a 96-well plate or 2,000 assays at 25µl/assay in a 384-well plate. Includes:

125ml	SIRT-Glo™ Buffer
5 vials	SIRT-Glo™ Substrate, Lyophilized
50µl	Developer Reagent
30µl	Nicotinamide, 1M

Product	Size	Cat.#
SIRT-Glo™ Assay	100ml	G6452

G6452 is sufficient for 1,000 assays at 100µl/assay in a 96-well plate or 4,000 assays at 25µl/assay in a 384-well plate. Includes:

2 × 125ml	SIRT-Glo™ Buffer
1 vial	SIRT-Glo™ Substrate, Lyophilized
2 × 50µl	Developer Reagent

Product	Size	Cat.#
SIRT-Glo™ Screening System	10ml	G6470

G6470 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
10µl	HeLa Nuclear Extract

Product	Size	Cat.#
SIRT-Glo™ Screening System	5 × 10ml	G6471

G6471 is sufficient for 500 assays at 100µl/assay in a 96-well plate or 2,000 assays at 25µl/assay in a 384-well plate. Includes:

125ml	SIRT-Glo™ Buffer
5 vials	SIRT-Glo™ Substrate, Lyophilized
50µl	Developer Reagent
30µl	Nicotinamide, 1M
10µl	HeLa Nuclear Extract

2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
SIRT-Glo™ Control Substrate	35µl	G6460

G6460 is supplied at a concentration of 10mM and is sufficient for 480 assays in 96-well plates when combined with the SIRT-Glo™ Reagent.

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 and SIRT-Glo™ Control Substrate at –20°C. Store HeLa Nuclear Extract at –70°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. Purified sirtuins should be used in screening assays.

3. Protocols

Materials to be Supplied by the User

- multichannel pipette or liquid-dispensing robot
- reagent reservoirs
- orbital shaker
- purified SIRT enzyme
- nonacetylated SIRT-Glo™ Control Substrate (Cat.# G6460), optional
- 96-well, 384-well or 1536-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-well and 1536-well plates are given in parentheses. Representative data are shown in Figure 4.

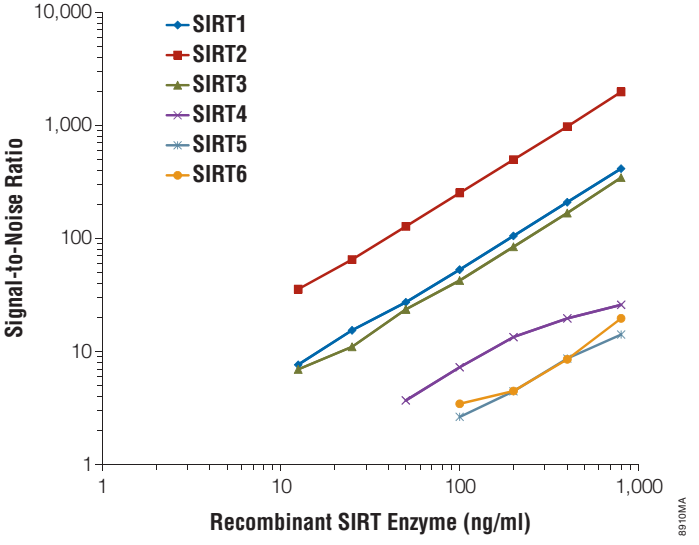

Figure 4. Example of linear range data. Recombinant sirtuins were diluted to 800ng/ml in SIRT-Glo™ Buffer, then serially diluted twofold in 100µl volumes in a 96-well plate. An equal volume of SIRT-Glo™ Reagent was added, and luminescence was measured after 20 minutes at room temperature. The recombinant 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 and Screening Systems are provided with sufficient SIRT-Glo™ Buffer to rehydrate the SIRT-Glo™ Substrate and dilute the test compound. For Cat.# G6450 and G6451, be sure to reserve 10ml of buffer for each vial of SIRT-Glo™ Substrate prior to performing the test compound and enzyme dilutions. For Cat.# G6452, be sure to reserve 100ml of buffer for each vial of SIRT-Glo™ Substrate. 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 1536-well plates).

   Optional: The HeLa Nuclear Extract supplied with the SIRT-Glo™ Screening System is provided 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 and G6451, 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.

   For Cat.# G6452, add 100ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate, then mix. Add 100µl 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.

4. Add an equal volume of SIRT-Glo™ Reagent to each assay well (100µl for 96-well, 20µl for 384-well or 5µl for 1536-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 1536-well plates are given in parentheses.

The SIRT-Glo™ Assay and Screening Systems are provided with sufficient SIRT-Glo™ Buffer to rehydrate the SIRT-Glo™ Substrate and dilute the test compound. For Cat.# G6450 and G6451, be sure to reserve 10ml of buffer for each vial of SIRT-Glo™ Substrate prior to performing the dilutions. For Cat.# G6452, be sure to reserve 100ml of buffer for each vial of SIRT-Glo™ Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

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

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 1536-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 supplied with the SIRT-Glo™ Screening System is provided 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.

		1	2	3	4	5	6	7	8	9	10	11	12
Unknown Test Compound	A	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	no-inhibitor (maximum-signal) controls	no-enzyme, no-inhibitor (background) controls
	B	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	C	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	D	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
Nicotinamide	E	5000µM	2500µM	1250µM	625µM	312.5µM	156µM	78µM	39µM	19µM	9.5µM		
	F	5000µM	2500µM	1250µM	625µM	312.5µM	156µM	78µM	39µM	19µM	9.5µM		
	G	5000µM	2500µM	1250µM	625µM	312.5µM	156µM	78µM	39µM	19µM	9.5µM		
	H	5000µM	2500µM	1250µM	625µM	312.5µM	156µM	78µM	39µM	19µM	9.5µM		

8825MA

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 1536-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.

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

   For Cat.# G6450 and G6451, 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.

   For Cat.# G6452, add 100ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate, then mix. Add 100µl 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 1536-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).

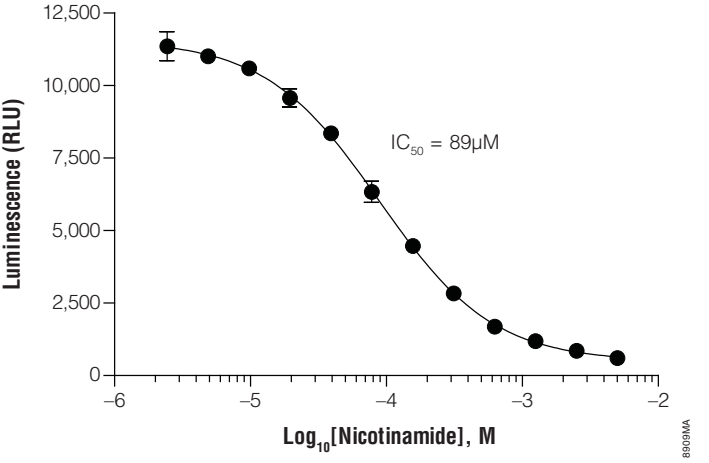

Figure 6. Example of potency data generated using recombinant SIRT1 and Nicotinamide. SIRT-Glo™ Assays were performed as described in Section 3.B. The final concentration of recombinant SIRT1 protein (Biomol Cat.# BML-SE239) was 400ng/ml. Data were plotted and IC_{50} value determined using GraphPad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

3.C. Confirming Sirtuin Inhibition or Activity Augmentation

The SIRT-Glo™ Assay and Screening System are robust and resilient to assay interferences. However, a small false inhibition or augmentation rate is possible through interference with 1) the Developer Reagent or 2) the luciferase detection component. Promega has developed the nonacetylated SIRT-Glo™ Control Substrate, which has the same sequence as the SIRT-Glo™ Substrate and can be used with the SIRT-Glo™ Assay and Screening System to confirm true SIRT inhibition or augmentation in secondary screens. The nonacetylated SIRT-Glo™ Control Substrate (Cat.# G6460) is available separately. Assays with the SIRT-Glo™ Control Substrate do not require deacetylation to produce luminescence and therefore are not inhibited by SIRT inhibitors.

This protocol is written for a 96-well plate.

The SIRT-Glo™ Assay and Screening Systems are provided with sufficient SIRT-Glo™ Buffer to rehydrate the SIRT-Glo™ Substrate and dilute the test compound. For Cat.# G6450 and G6451, be sure to reserve 10ml of buffer for each vial of SIRT-Glo™ Substrate prior to performing the test compound and enzyme source dilutions. For Cat.# G6452, be sure to reserve 100ml of buffer for each vial of SIRT-Glo™ Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

1. Prepare serial twofold dilutions of the putative sirtuin inhibitor or activator identified in the primary screen in SIRT-Glo™ Buffer in wells in columns 1 through 10 as shown in Figure 8. The final volume should be 50µl per well. Add only SIRT-Glo™ Buffer to wells in columns 11 and 12. These wells will serve as no-inhibitor (maximum-signal) and no-sirtuin (background) controls.

   Optional: The HeLa Nuclear Extract supplied with the SIRT-Glo™ Screening System is provided 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 adding 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
Assays Using the SIRT-Glo™ Reagent	A	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	no-inhibitor (maximum-signal) controls	no-enzyme, no-inhibitor (background) controls
	B	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	C	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	D	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
Assays Using the SIRT-Glo™ Control Reagent	E	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	F	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	G	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
	H	initial dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		

8826MA

Figure 8. Example of a plate layout showing dilution ratios to confirm selective inhibition.

2. Dilute the SIRT enzyme in SIRT-Glo™ Buffer to a concentration within the linear range of the assay (Section 3.A).

   Note: Appropriate purified enzyme concentrations will vary greatly between isoenzymes and vendors and should be determined empirically as described in Section 3.A.

3. Add 50µl of SIRT enzyme to all wells in rows A–D, except wells in column 12. Add 50µl of SIRT-Glo™ Buffer to all wells of rows E–H except wells in column 12. Add 50µl of SIRT-Glo™ Buffer to all wells in column 12. Column 12 contains the no-enzyme, no-inhibitor controls (see Figure 8).

3.C. Confirming Sirtuin Inhibition or Activity Augmentation (continued)

4. Mix the plate at room temperature for 30–60 seconds using an orbital shaker at 500–700rpm, then incubate at room temperature for at least 30 minutes (but not longer than approximately 2 hours).

5. Prepare the SIRT-Glo™ Reagent and SIRT-Glo™ Control Reagent. The SIRT-Glo™ Control Reagent contains nonacetylated SIRT-Glo™ Control Substrate.

   a. For Cat.# G6450 and G6451, add 10ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate to form the SIRT-Glo™ Substrate Solution. Mix well, then divide into two 5ml aliquots.

      For Cat.# G6452, add 100ml of SIRT-Glo™ Buffer to the SIRT-Glo™ Substrate to form the SIRT-Glo™ Substrate Solution. Mix well, then divide into two 50ml aliquots.

   b. For Cat.# G6450 and G6451, add 5µl of Developer Reagent to one 5ml aliquot of SIRT-Glo™ Substrate Solution to prepare the SIRT-Glo™ Reagent. Mix.

      For Cat.# G6452, add 50µl of Developer Reagent to one 50ml aliquot of SIRT-Glo™ Substrate Solution to prepare the SIRT-Glo™ Reagent. Mix.

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

   c. To the other aliquot of SIRT-Glo™ Substrate Solution, add the SIRT-Glo™ Control Substrate (10mM) to a final concentration of 5µM.

      Dilute the Developer Reagent 1:100 (e.g., 2µl of Developer Reagent and 198µl of SIRT-Glo™ Buffer). For Cat.# G6450 and G6451, add 25µl of this 1:100 dilution of Developer Reagent to prepare the SIRT-Glo™ Control Reagent. For Cat.# G6452, add 250µl of this 1:100 dilution of Developer Reagent to prepare the SIRT-Glo™ Control Reagent.

      Note: 20-fold less Developer Reagent is required in SIRT-Glo™ Control Reagent reactions because the developer enzyme, not deacetylase enzyme, is now the rate-limiting step of the reaction. Failure to dilute the Developer Reagent prior to preparing the SIRT-Glo™ Control Reagent will compromise the performance of the SIRT-Glo™ Control Reagent (excessive and signal-saturating luminescence with a shortened signal half-life). This can make comparisons to SIRT-Glo™ Reagent reactions difficult to interpret.

6. Add 100µl of SIRT-Glo™ Reagent to all wells in rows A–D. Add 100µl of SIRT-Glo™ Control Reagent to all wells in rows E–H.

7. Mix the plate at room temperature for 30–60 seconds using an orbital shaker at 500–700rpm. Incubate at room temperature for 15–45 minutes.

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

9. Plot luminescence values (RLU) versus inhibitor concentration for data collected using the SIRT-Glo™ Assay Reagent and Control Assay Reagent. Use a linear scale for luminescence and a log scale for inhibitor concentration.

10. Determine if the unknown inhibitor or activator is sirtuin-selective by comparing the shapes of inhibition profiles to the examples shown in Figure 9.

    Note: The true IC_{50} values may be slightly different than the IC_{50} values shown in Figure 9 due to the different amounts of Developer Reagent added to SIRT-Glo™ Reagent and SIRT-Glo™ Control Reagent. Because of this difference in Developer Reagent, only qualitative determination of inhibition should be made.

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.

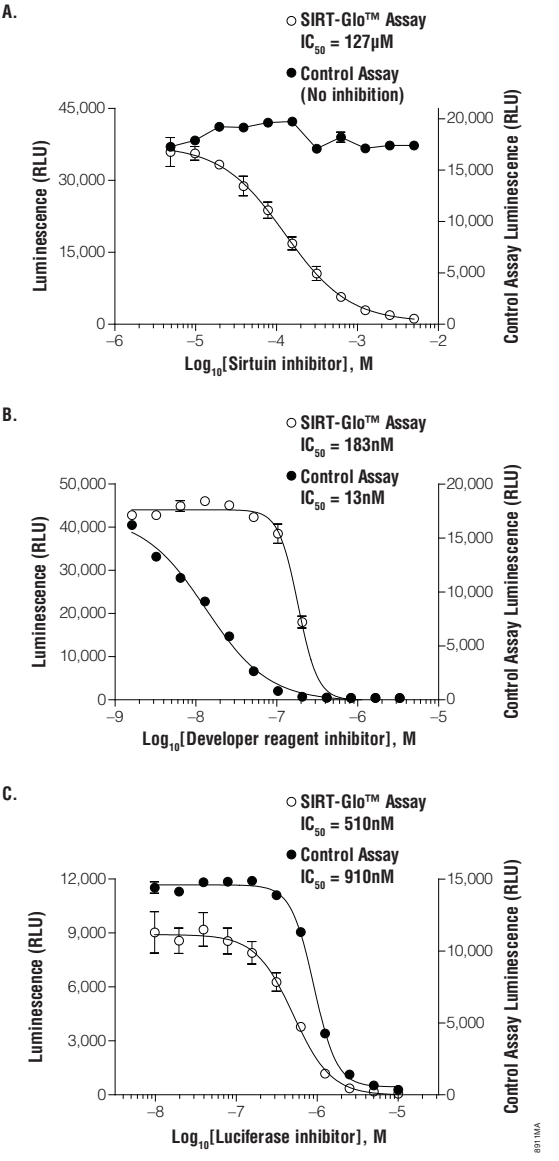

Figure 9. Inhibitor profiles for the SIRT-Glo™ Assay. Panel A. Selective inhibition of sirtuins. Assays with the SIRT-Glo™ Control Reagent do not require deacetylation to produce luminescence and, therefore, are not inhibited by sirtuin inhibitors. Assays with the SIRT-Glo™ Reagent are inhibited. This profile is consistent with specific inhibition of SIRT activity. **Panel B.** Nonselective inhibition of Developer Reagent. Assays with the SIRT-Glo™ Control Reagent and SIRT-Glo™ Reagent are both inhibited. This profile is consistent with Developer Reagent inhibition, not sirtuin inhibition. The difference in IC_{50} values in Panel B is due to the difference in the Developer Reagent concentration. **Panel C.** Nonselective inhibition of the luciferase detection reagent. Assays with the SIRT-Glo™ Reagent and SIRT-Glo™ Control Reagent are both inhibited. This profile is consistent with inhibition of luciferase detection component, not sirtuin inhibition. Data represent the mean ± standard deviation of four samples.

Promega

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: 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 and Screening System 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 and SIRT-Glo™ Control 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.

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.#
SIRT-Glo™ Control Substrate	10µl	G6460
HDAC-Glo™ I/II Assay	10ml	G6420
	5 × 10ml	G6421
	100ml	G6422
HDAC-Glo™ I/II Screening System	10ml	G6430
	5 × 10ml	G6431
HDAC-Glo™ Control Substrate	10µl	G6550
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	10 × 10ml	G8093
	100ml	G8092
Caspase-Glo® 2 Assay	10ml	G0940
	50ml	G0941
Caspase-Glo® 6 Assay	10ml	G0970
	50ml	G0971
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

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
	5 × 10ml	G9291
	2 × 50ml	G9292
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
DUB-Glo™ Protease Assay (DUB/SENP/NEDP)	10ml	G6260
	50ml	G6261
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

6. Related Products (continued)

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®-Multi Base Instrument	each	E7031
GloMax®-Multi Luminescence Module	each	E7041
GloMax®-Multi Fluorescence Module	each	E7051
GloMax®-Multi Absorbance Module	each	E7061
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Shaking	each	E8032
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Heating and Shaking	each	E9032
GloMax®-Multi+ Luminescence Module	each	E8041
GloMax®-Multi+ Fluorescence Module	each	E8051
GloMax®-Multi+ Visible Absorbance Module	each	E8061
GloMax®-Multi+ UV-Visible Absorbance Module	each	E9061

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents 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]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.

Furthermore, the rights above are extended to purchasers of licensed products from Promega to use, possess, distribute, market and sell such licensed products for the same purposes and for the same scope as granted to Promega.

Caspase-Glo, CellTiter-Glo and GloMax are registered trademarks of Promega Corporation. ApoLive-Glo, ApoTox, CellTiter-Fluor, CytoTox-Glo, DUB-Glo, GSH-Glo, GSH/GSSG-Glo, HDAC-Glo, Instinct, Proteasome-Glo, SIRT-Glo and Ultra-Glo are trademarks of Promega Corporation.

GraphPad Prism is a registered trademark of GraphPad Software, Inc. Tergitol is a trademark of The Dow Chemical Company or an affiliated company of Dow.

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

All prices and specifications are subject to change without prior notice.

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.