Technical Manual

HDAC-Glo™ I/II Assay and Screening System

INSTRUCTIONS FOR USE OF PRODUCTS G6420, G6421, G6422, G6430, G6431, G6550, G6560 AND G6570.

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Part# TM335

HDAC-Glo™ I/II 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 HDAC-Glo™ I/II Assay and Screening System[a–c] are single-reagent-addition, homogeneous, luminescent assays that measure the relative activity of histone deacetylase (HDAC) class I and II enzymes from cells, extracts or purified enzyme sources. This assay is broadly useful for class I and II enzymes, but sensitivity and performance will vary with catalytic efficiency of particular class I and II isoenzymes. The assay uses an acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC 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 HDAC-mediated luminescent signal is persistent and proportional to deacetlyase activity (Figure 2), 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 HDAC-Glo™ I/II 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.

Flexible: May be used with viable cells, extracts or purified HDAC sources.

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.

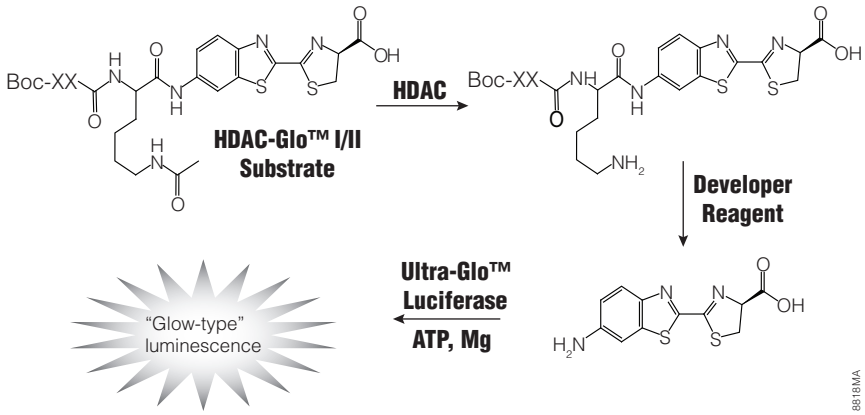

Figure 1. HDAC-Glo™ I/II Assay chemistry. HDAC activity deacetylates the luminogenic HDAC-Glo™ I/II Substrate, making the peptide sensitive to a specific proteolytic cleavage event that is mediated by HDAC-Glo™ I/II Reagent and liberates aminoluciferin. Free aminoluciferin then can be measured using the Ultra-Glo™ firefly luciferase reaction to produce a stable, persistent emission of light. Boc represents an amino-terminal blocking group that protects the substrate from nonspecific cleavage. XXLysine is an HDAC I/II-optimized consensus sequence derived from histone 4 (1).

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

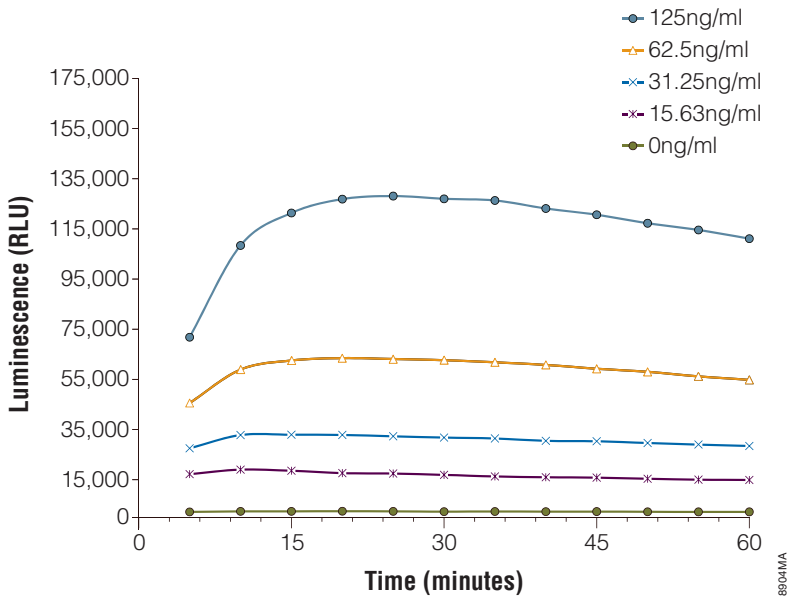

Figure 2. HDAC-mediated luminescent signal is proportional and persistent. Increasing amounts of HeLa Nuclear Extract were assayed using the HDAC-Glo™ I/II Assay as described in Section 3.B.

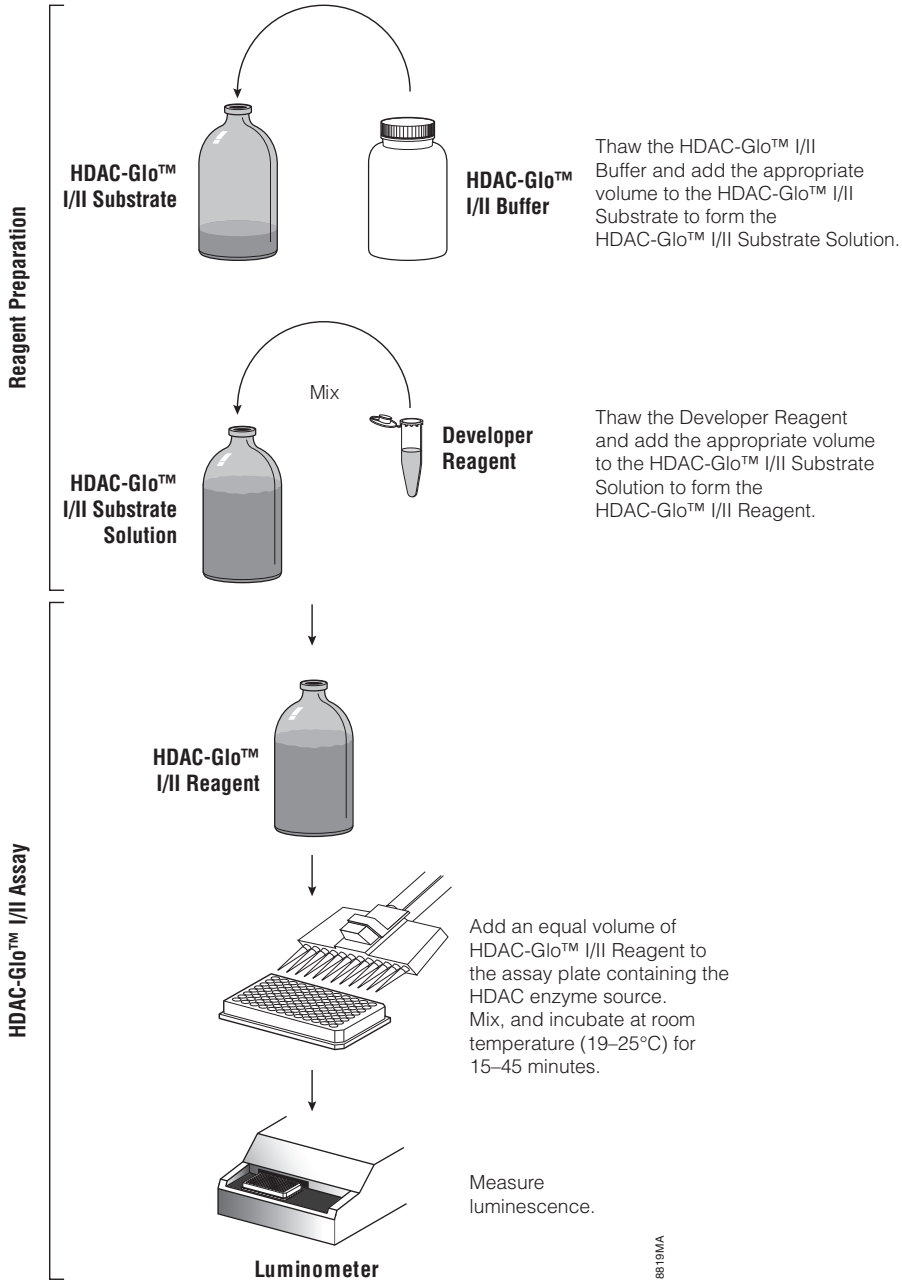

Figure 3. Overview of the HDAC-Glo™ I/II Assay protocol.
Note: Prepare the HDAC-Glo™ I/II Reagent just prior to the assay. HDAC-Glo™ I/II assays using cells as the HDAC enzyme source can be performed in a lytic or nonlytic format as described in Section 3.C.

2. Product Components and Storage Conditions

Product	Size	Cat.#
HDAC-Glo™ I/II Assay	10ml	G6420

G6420 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	HDAC-Glo™ I/II Buffer
1 vial	HDAC-Glo™ I/II Substrate, Lyophilized
10µl	Developer Reagent
10µl	Trichostatin A, 10mM

Product	Size	Cat.#
HDAC-Glo™ I/II Assay	5 × 10ml	G6421

G6421 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	HDAC-Glo™ I/II Buffer
5 vials	HDAC-Glo™ I/II Substrate, Lyophilized
50µl	Developer Reagent
10µl	Trichostatin A, 10mM

Product	Size	Cat.#
HDAC-Glo™ I/II Assay	100ml	G6422

G6422 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	HDAC-Glo™ I/II Buffer
1 vial	HDAC-Glo™ I/II Substrate, Lyophilized
2 × 50µl	Developer Reagent

Product	Size	Cat.#
HDAC-Glo™ I/II Screening System	10ml	G6430

G6430 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	HDAC-Glo™ I/II Buffer
1 vial	HDAC-Glo™ I/II Substrate, Lyophilized
10µl	Developer Reagent
10µl	Trichostatin A, 10mM
10µl	HeLa Nuclear Extract

Product	Size	Cat.#
HDAC-Glo™ I/II Screening System	5 × 10ml	G6431

G6431 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	HDAC-Glo™ I/II Buffer
5 vials	HDAC-Glo™ I/II Substrate, Lyophilized
50µl	Developer Reagent
10µl	Trichostatin A, 10mM
10µl	HeLa Nuclear Extract

2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
HDAC-Glo™ I/II Control Substrate	10µl	G6550

G6550 is supplied at a concentration of 10mM and is sufficient for 480 assays in 96-well plates when combined with the HDAC-Glo™ I/II Reagent.

Product	Size	Cat.#
Trichostatin A	10µl	G6560

G6560 is supplied at a concentration of 10mM in DMSO.

Product	Size	Cat.#
HeLa Nuclear Extract	10µl	G6570

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

Storage Conditions: Store the HDAC-Glo™ I/II Assay components and HDAC-Glo™ I/II Control Substrate at –20°C. Store HeLa Nuclear Extract at –70°C.

Storage Conditions for the HDAC-Glo™ I/II Reagent: For optimal performance, the HDAC-Glo™ I/II 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 HDAC-Glo™ I/II Reagent cannot be used immediately to assay HDAC 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 HDAC-Glo™ I/II Substrate Solution (i.e., the Developer Reagent has not been added) can be stored at 4°C for up to 24 hours or at –20°C for up to 1 month. If the entire volume of HDAC-Glo™ I/II Reagent will not be used in one experiment, combine the HDAC-Glo™ I/II Substrate and HDAC-Glo™ I/II Buffer, then divide the resulting HDAC-Glo™ I/II Substrate Solution into single-use aliquots. To the aliquot to be used immediately, add the appropriate volume of Developer Reagent to create the HDAC-Glo™ I/II Reagent, and freeze the remaining aliquots of HDAC-Glo™ I/II 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.

3. Protocols

Materials to be Supplied by the User

- multichannel pipette or liquid-dispensing robot
- reagent reservoirs
- orbital shaker
- nonacetylated HDAC-Glo™ I/II Control Substrate (Cat.# G6550), optional
- HDAC enzyme source such as intact cells, cell extracts or purified HDAC enzyme
- 20% Triton® X-100, prepared using NANOpure® water or equivalent (for use with the lytic format of the HDAC-Glo™ I/II Assay)
- 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 HDAC-Glo™ I/II Reagent should be used in its entirety on the day it is prepared. If the entire volume of HDAC-Glo™ I/II Reagent will not be used in one day, see Section 2 for recommendations on how to prepare only the amount of HDAC-Glo™ I/II Reagent needed.

3.A. Determining Linear Range Using a Histone Deacetylase Enzyme Source

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.

The HDAC-Glo™ I/II Assay and Screening Systems are provided with sufficient HDAC-Glo™ I/II Buffer to rehydrate the HDAC-Glo™ I/II Substrate and dilute the test compound. For Cat.# G6420 and G6421, be sure to reserve 10ml of buffer for each vial of HDAC-Glo™ I/II Substrate prior to performing the test compound and enzyme dilutions. For Cat.# G6422, be sure to reserve 100ml of buffer for each vial of HDAC-Glo™ I/II Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

1. Prepare an initial dilution of the histone deacetylase enzyme source using the HDAC-Glo™ I/II Buffer as described below:

   HeLa Nuclear Extract: If using the HeLa Nuclear Extract provided with HDAC-Glo™ I/II Screening System, dilute the extract 1:3,000 in HDAC-Glo™ I/II Buffer (i.e., 1µl of HeLa Nuclear Extract and 3.0ml of HDAC-Glo™ I/II Buffer). The diluted HeLa Nuclear Extract also can be used as an HDAC-Glo™ I/II chemistry control as described in Step 2. Do not store diluted HeLa Nuclear Extract.

   Purified HDAC: Dilute the enzyme to 1–5µg/ml in HDAC-Glo™ I/II Buffer.

   Note: There are several commercial sources of purified HDAC 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.

3.A. Determining Linear Range Using a Histone Deacetylase Enzyme Source (continued)

Cells: Dilute cells in serum-free medium or HDAC-Glo™ I/II Buffer to a concentration of 100,000 cells/ml.

Note: See General Considerations (Section 4.A) for more information about the use of serum in the HDAC-Glo™ I/II Assay.

2. Prepare serial twofold dilutions of the HDAC enzyme source in HDAC-Glo™ I/II 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 HDAC-Glo™ I/II Screening System is provided as a source of deacetylase activity to confirm that the assay chemistry is working properly. Add 100µl of diluted HeLa Nuclear Extract described in Step 1 to each well in column 11 instead of the diluted HDAC enzyme source. 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

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

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

   For Cat.# G6420 and G6421, add 10ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 10µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix.

   For Cat.# G6422, add 100ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 100µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix.

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

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

4. Add an equal volume of HDAC-Glo™ I/II 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 HDAC-Glo™ I/II Reagent on ice.
5. Mix the plate at room temperature for 30–60 seconds 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 HDAC-Glo™ I/II Reagent).

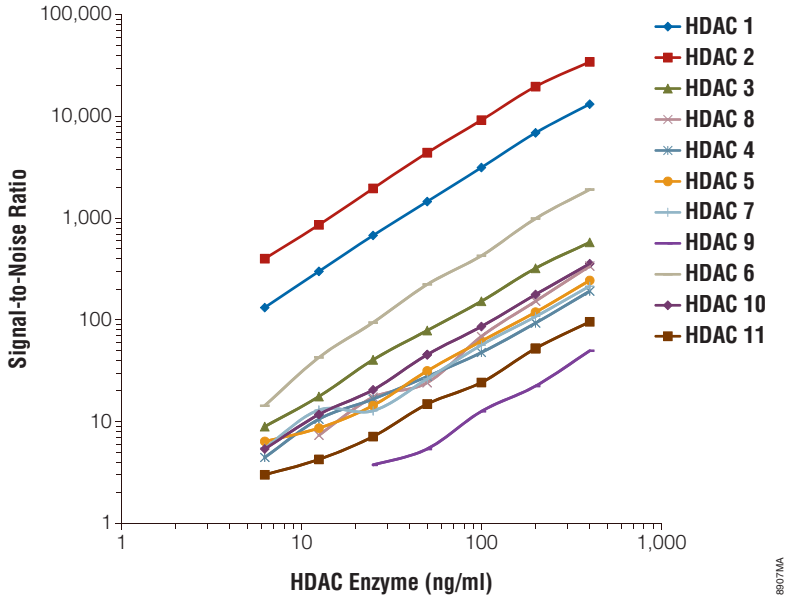

Figure 4. Example of linear range data. Recombinant HDAC enzymes were diluted to 400ng/ml in HDAC-Glo™ I/II Buffer, then serially diluted twofold in 100µl volumes in a 96-well plate. An equal volume of HDAC-Glo™ I/II Reagent was added, and luminescence was measured after 20 minutes at room temperature. The recombinant enzymes were obtained from various vendors (HDAC1, 2, 3, 6 and 8 from Enzo; HDAC4 from Cayman Chemical; HDAC7 from Millipore; and HDAC5, 9, 10 and 11 from SignalChem). Data were plotted using GraphPad Prism® software. Data represent the mean ± standard deviation of four samples.

3.B. Determining HDAC Inhibitor Potency Using Extract or Purified Enzyme

Figure 6 shows representative results for HDAC inhibitor potency determination using cell extracts and intact cells.

This protocol is written for a 96-well plate. Required volumes for 384-well and 1536-well plates are given in parentheses.

The HDAC-Glo™ I/II Assay and Screening Systems are provided with sufficient HDAC-Glo™ I/II Buffer to rehydrate the HDAC-Glo™ I/II Substrate and dilute the test compound. For Cat.# G6420 and G6421, be sure to reserve 10ml of buffer for each vial of HDAC-Glo™ I/II Substrate prior to performing the test compound and enzyme source dilutions. For Cat.# G6422, be sure to reserve 100ml of buffer for each vial of HDAC-Glo™ I/II Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

1. Prepare an initial dilution of unknown compound and the known HDAC inhibitor Trichostatin A in HDAC-Glo™ I/II Buffer. Prepare serial twofold or threefold dilutions 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 HDAC-Glo™ I/II Buffer to wells in columns 11 and 12 to serve as the no-inhibitor and no-HDAC controls.

   Optional: The HeLa Nuclear Extract supplied with the HDAC-Glo™ I/II Screening System (Cat.# G6430, G6431) is provided as a source of deacetylase activity to confirm that the assay chemistry is working properly. Add 100µl of diluted HeLa Nuclear Extract described in Step 2

		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		
Trichostatin A	E	50nM	25nM	12.5nM	6.25nM	3.12nM	1.56nM	0.78nM	0.39nM	0.20nM	0.098nM		
	F	50nM	25nM	12.5nM	6.25nM	3.12nM	1.56nM	0.78nM	0.39nM	0.20nM	0.098nM		
	G	50nM	25nM	12.5nM	6.25nM	3.12nM	1.56nM	0.78nM	0.39nM	0.20nM	0.098nM		
	H	50nM	25nM	12.5nM	6.25nM	3.12nM	1.56nM	0.78nM	0.39nM	0.20nM	0.098nM		

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

to each well in column 10 instead of the diluted test compound or Trichostatin A. Do not store diluted HeLa Nuclear Extract.

Note: Brief centrifugation of Trichostatin A may be required to recover the full volume.

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

   If using HeLa Nuclear Extract provided with HDAC-Glo™ I/II Screening System, dilute the extract 1:3,000. Brief centrifugation may be required to recover HeLa Nuclear Extract from the tube.

3. Dispense 50µl of HDAC enzyme source to each well of inhibitor dilutions prepared in Step 1 and no-inhibitor controls (column 11). Add 50µl of HDAC-Glo™ I/II Buffer to the no-HDAC controls (column 12). (Dispense 10µl for 384-well or 2.5µl for 1536-well plates.)

4. Mix the plate at room temperature for 30–60 seconds using an orbital shaker at 500–700rpm to ensure homogeneity.

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

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

6. Prepare the HDAC-Glo™ I/II Reagent as described below:

   For Cat.# G6420 and G6421, add 10ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 10µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix.

   For Cat.# G6422, add 100ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 100µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix.

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

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

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

7. Add an equal volume of HDAC-Glo™ I/II Reagent to each assay well (100µl for 96-well, 20µl for 384-well or 5µl for 1536-well plates).

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

3.B. Determining HDAC Inhibitor Potency Using Extract or Purified Enzyme (continued)

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

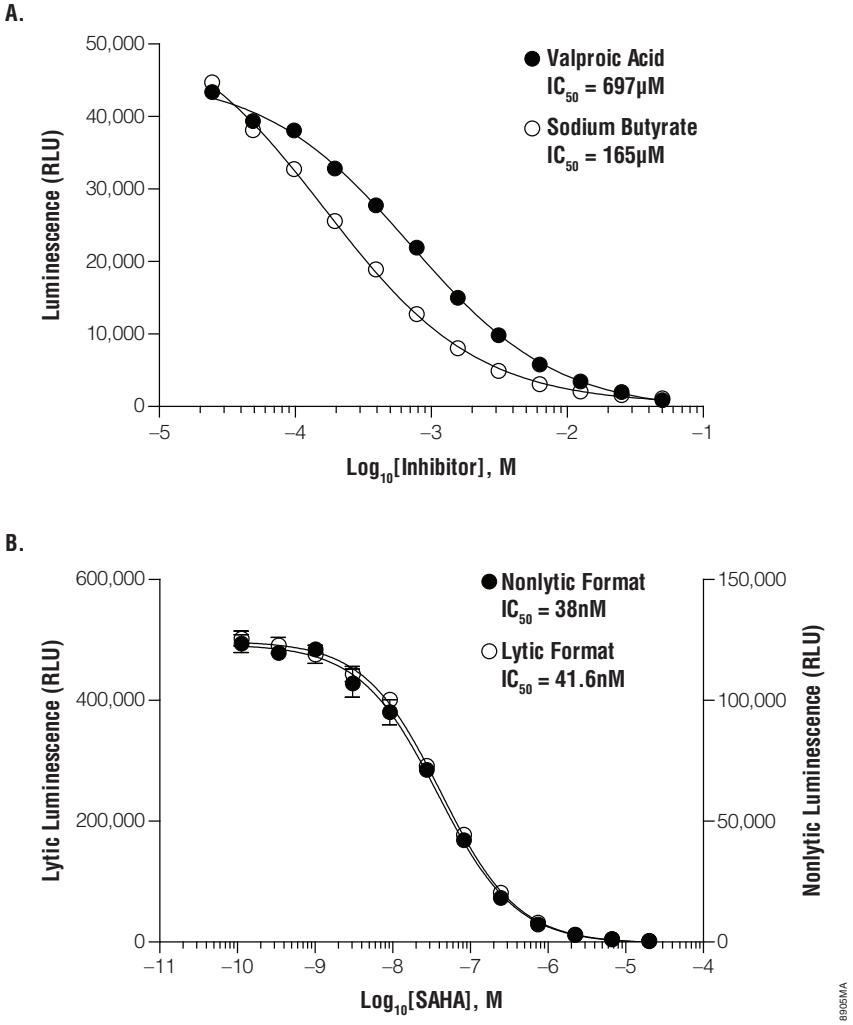

Figure 6. Example of HDAC inhibitor potency data. Panel A. HDAC-Glo™ I/II Assays were performed as described in Section 3.B using valproic acid or sodium butyrate as inhibitors and HeLa Nuclear Extract as the HDAC enzyme source. The final concentration of HeLa Nuclear Extract protein was 1µg/ml. **Panel B.** Assays were performed as described in Section 3.C using suberoylanilide hydroxamic acid (SAHA) as the inhibitor and K562 cells (10,000 cells/well) as the HDAC enzyme source. For lytic assays, Triton® X-100 was added to the HDAC-Glo™ I/II Reagent to a final concentration of 1%. Nonlytic assays did not contain Triton® X-100. Data were plotted and IC_{50} values determined using GraphPad Prism® software. Data represent the mean ± standard deviation of four samples.

3.C. Determining HDAC Inhibitor Potency Using Cells

This protocol is written for a 96-well plate. Required volumes for a 384-well plate are given in parentheses.

The HDAC-Glo™ I/II Assay and Screening Systems are provided with sufficient HDAC-Glo™ I/II Buffer to rehydrate the HDAC-Glo™ I/II Substrate and dilute the test compound. For Cat.# G6420 and G6421, be sure to reserve 10ml of buffer for each vial of HDAC-Glo™ I/II Substrate prior to performing the test compound and enzyme source dilutions. For Cat.# G6422, be sure to reserve 100ml of buffer for each vial of HDAC-Glo™ I/II Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

Typically, a cell lysis component is not necessary in the HDAC-Glo™ I/II Reagent due to cell permeability of HDAC-Glo™ I/II Reagent components. However, a higher signal-to-noise ratio (i.e., a three- to fourfold increase) can be achieved by adding Triton® X-100 to the reagent at a final concentration of 1% in Step 7. The nonlytic format delivers a comparable IC_{50} value when compared to the lytic format (Figure 6). When working with cells that are prone to clumping, you may observe higher variability in the nonlytic format.

1. Seed attachment-dependent cells into a white-walled 96-well plate at a density of 10,000 cells/well or less (or at the cell density empirically determined in Section 3.A) in a volume of 50µl (2,000–5,000 cells/well in 10µl for a 384-well plate). Allow cells to attach by incubating at 37°C for the necessary amount of time.

   Note: For suspension cells, seed cells after diluting the inhibitor (Step 3).

2. In a parallel, white-walled 96-well plate, prepare twofold or threefold serial dilutions of the unknown compound and Trichostatin A in HDAC-Glo™ I/II Buffer; a serial twofold dilution is shown in Figure 7. The final volume in each well should be 75µl (15µl for 384-well plate). Add only HDAC-Glo™ I/II Buffer to wells in columns 11 and 12 to serve as no-inhibitor and no-HDAC controls.

   Note: Brief centrifugation may be required to recover the full volume of Trichostatin A from the tube.

3. Remove culture medium from attachment-dependent cells by gentle aspiration, and replace with 50µl of serum-free medium (10µl for a 384-well plate).

   If using suspension cells, add cells to a white-walled 96-well plate at the cell density empirically determined in Section 3.A in a final volume of 50µl of serum-free medium (10µl for a 384-well plate).

   Note: Optimal performance is achieved in serum-free medium. Assays conducted in serum-supplemented medium may have higher background luminescence, which may or may not adversely affect the assay. See Section 4.A.

3.C. Determining HDAC Inhibitor Potency Using Cells (continued)

4. Transfer 50µl of inhibitor dilutions prepared in Step 2 to cells (10µl for a 384-well plate). The final volume per well of a 96-well plate should be 100µl (i.e., 50µl of inhibitor + 50µl of medium).

5. Mix the plate at room temperature for 30–60 seconds using an orbital shaker at 500–700rpm to ensure homogeneity.

6. Incubate cell/inhibitor mixtures at 37°C for at least 30 minutes (but not longer than approximately 2 hours).

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

7. Prepare the HDAC-Glo™ I/II Reagent as described below.

   For Cat.# G6420 and G6421, add 10ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 10µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix. For lytic assays, add 500µl of 20% Triton® X-100 to the HDAC-Glo™ I/II Reagent for a final concentration of 1%.

   For Cat.# G6422, add 100ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate, then mix. Add 100µl of Developer Reagent to form the HDAC-Glo™ I/II Reagent, then mix. For lytic assays, add 5ml of 20% Triton® X-100 to the HDAC-Glo™ I/II Reagent for a final concentration of 1%.

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

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

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

8. Add 100µl of HDAC-Glo™ I/II Reagent to each well (20µl for 384-well plate).

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

10. Measure luminescence at signal steady-state (15–45 minutes after adding the HDAC-Glo™ I/II Reagent to cells).

    Note: HDAC-Glo™ I/II Assays using cells in the nonlytic format may require more time to reach signal steady state than assays using cells in the lytic format because of reagent permeability constraints.

3.D. Confirming Deacetylase Inhibition

The HDAC-Glo™ I/II Assay and Screening System are robust and resilient to assay interferences. However, a small false-inhibition rate is possible through interference with 1) the Developer Reagent or 2) the luciferase detection component. Promega has developed the nonacetylated HDAC-Glo™ I/II Control Substrate, which has the same amino acid sequence as the HDAC-Glo™ I/II Substrate and can be used with the HDAC-Glo™ I/II Assay and Screening System to confirm true HDAC inhibition in secondary screens. The nonacetylated HDAC-Glo™ I/II Control Substrate (Cat.# G6550) can be purchased separately. Assays with the HDAC-Glo™ I/II Control Substrate do not require deacetylation to produce luminescence and therefore are not inhibited by HDAC inhibitors.

This protocol is written for a 96-well plate.

The HDAC-Glo™ I/II Assay and Screening Systems are provided with sufficient HDAC-Glo™ I/II Buffer to rehydrate the HDAC-Glo™ I/II Substrate and dilute the test compound. For Cat.# G6420 and G6421, be sure to reserve 10ml of buffer for each vial of HDAC-Glo™ I/II Substrate prior to performing the test compound and enzyme source dilutions. For Cat.# G6422, be sure to reserve 100ml of buffer for each vial of HDAC-Glo™ I/II Substrate. Sequential small-volume dilutions may be necessary for compounds that require significant dilution.

3.D. Confirming Deacetylase Inhibition (continued)

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

   The HeLa Nuclear Extract supplied with the HDAC-Glo™ I/II Screening System (Cat.# G6430, G6431) is provided as a source of deacetylase activity to confirm that the assay chemistry is working properly. Dilute the HeLa Nuclear Extract 1:3,000 in HDAC-Glo™ I/II Buffer, then add 100µl of diluted HeLa Nuclear Extract to each well in column 10 instead of the diluted test compound or Trichostatin A. Do not store diluted HeLa Nuclear Extract.

		1	2	3	4	5	6	7	8	9	10	11	12
Assays Using the HDAC-Glo™ I/II 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 HDAC-Glo™ I/II 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		

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

2. Dilute the HDAC enzyme source (cells, HeLa Nuclear Extract or purified enzyme) in HDAC-Glo™ I/II Buffer to a cell number or concentration within the linear range of the assay (Section 3.A).

   Most cells can be used at 2,500 to 10,000 cells per well in a final volume of 50µl. HeLa Nuclear Extract can be used at a final protein concentration of 250–1,000ng/ml and delivered in 50µl.

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

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

5. Prepare the HDAC-Glo™ I/II Reagent and HDAC-Glo™ I/II Control Reagent. The HDAC-Glo™ I/II Control Reagent contains nonacetylated HDAC-Glo™ I/II Control Substrate.

   a. For Cat.# G6420 and G6421, add 10ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate to form the HDAC-Glo™ I/II Substrate Solution. Mix well, then divide into two 5ml aliquots.

      For Cat.# G6422, add 100ml of HDAC-Glo™ I/II Buffer to the HDAC-Glo™ I/II Substrate to form the HDAC-Glo™ I/II Substrate Solution. Mix well, then divide into two 50ml aliquots.

   b. For Cat.# G6420 and G6421, add 5µl of Developer Reagent to one 5ml aliquot of HDAC-Glo™ I/II Substrate Solution to prepare the HDAC-Glo™ I/II Reagent. Mix.

      For Cat.# G6422, add 50µl of Developer Reagent to one 50ml aliquot of HDAC-Glo™ I/II Substrate Solution to prepare the HDAC-Glo™ I/II Reagent. Mix.

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

3.D. Confirming Deacetylase Inhibition (continued)

c. To the other aliquot of HDAC-Glo™ I/II Substrate Solution add the HDAC-Glo™ I/II Control Substrate (10mM) to a final concentration of 500nM.

Dilute the Developer Reagent 1:100 (e.g., 2µl of Developer Reagent and 198µl of HDAC-Glo™ I/II Buffer). For Cat.# G6420 and G6421, add 5µl of this 1:100 dilution of Developer Reagent to prepare the HDAC-Glo™ Control Reagent. For Cat.# G6422, add 50µl of this 1:100 dilution of Developer Reagent to prepare the HDAC-Glo™ Control Reagent.

Note: 100-fold less Developer Reagent is required in HDAC-Glo™ I/II 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 creating the HDAC-Glo™ I/II Control Reagent will compromise the performance of the HDAC-Glo™ I/II Control Reagent (excessive and signal-saturating luminescence with a shortened signal half-life). This can make comparisons to HDAC-Glo™ I/II Reagent reactions difficult to interpret.

6. Add 100µl of HDAC-Glo™ I/II Reagent to all wells in rows A–D. Add 100µl of HDAC-Glo™ I/II 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 HDAC-Glo™ I/II Reagent).

9. Plot luminescence values (RLU) versus inhibitor concentration for data collected using HDAC-Glo™ I/II 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 is HDAC class I/II-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 HDAC-Glo™ I/II Reagent and HDAC-Glo™ I/II Control Reagent. Because of this difference in Developer Reagent, only qualitative determination of inhibition should be made.

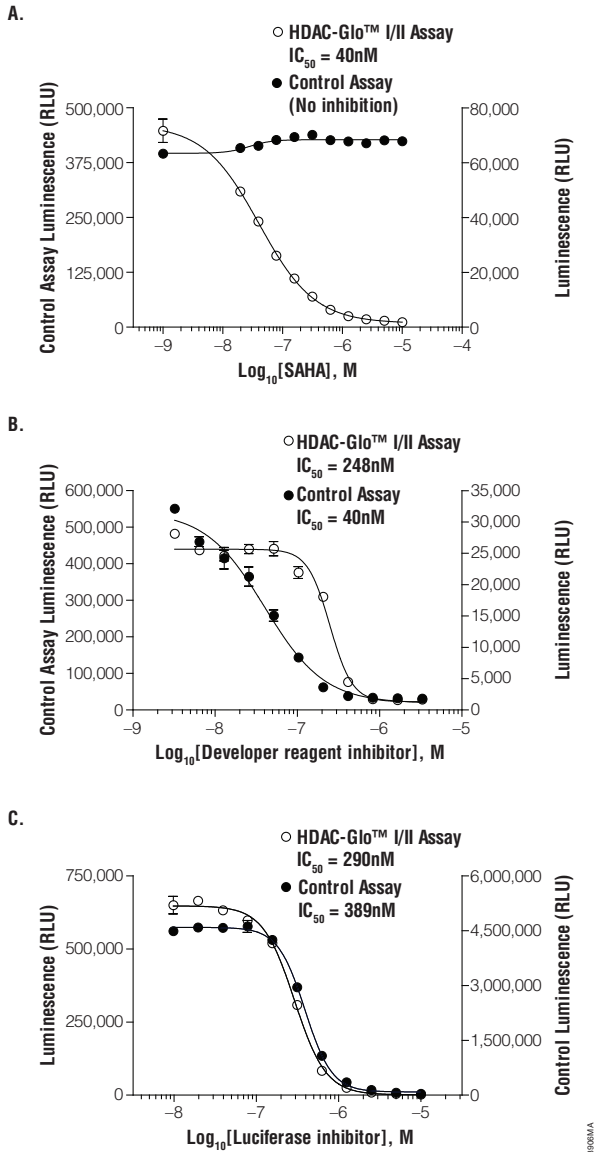

Figure 9. Inhibitor profiles for the HDAC-Glo™ I/II Assay. Panel A. Selective inhibition of HDAC. Assays with the HDAC-Glo™ I/II Control Reagent do not require deacetylation to produce luminescence and, therefore, are not inhibited by HDAC inhibitors. Assays with the HDAC-Glo™ I/II Reagent are inhibited. This profile is consistent with specific inhibition of HDAC activity. **Panel B.** Nonselective inhibition of Developer Reagent. Assays with the HDAC-Glo™ I/II Control Reagent and HDAC-Glo™ I/II Reagent are both inhibited. This profile is consistent with Developer Reagent inhibition, not HDAC 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 HDAC-Glo™ I/II Reagent and HDAC-Glo™ I/II Control Reagent are both inhibited. This profile is consistent with inhibition of luciferase detection component, not HDAC inhibition. Data represent the mean ± standard deviation of four samples.

4. General Considerations

4.A. Background Luminescence and Inherent Serum Activity

Animal serum used to supplement tissue culture medium may contain detectible levels of deacetylase activity. Because assay results can be collected in less than 1 hour, we recommend not using serum-supplemented medium during the assay. If serum is used, a cell-free medium control should be used to determine background luminescence.

4.B. Phenol Red

Phenol red-containing medium may affect overall luminescence by slightly quenching signal. Optimal sensitivity can be achieved in phenol red-free medium.

4.C. 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 HDAC-Glo™ I/II Reagent.

4.D. 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.

HDAC-Glo™ I/II Assays using cells in the nonlytic format may require more time to reach signal steady state than assays using cells in the lytic format because of reagent permeability constraints.

4.E. Assay Controls

No-HDAC (Medium or Buffer Background) Control: Set up at least triplicate wells without cells or HDAC 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 untreated cells or uninhibited HDAC. Add the same solvent used to deliver the test compounds to no-inhibitor control wells.

Known Inhibitor (Trichostatin A) Control (optional): Set up triplicate wells or a dilution series using the provided Trichostatin A as a control for specific inhibition of HDAC activity from cells or the HDAC source.

4.F. High-Throughput Screening

The HDAC-Glo™ I/II Assay and Screening System can be scaled or miniaturized easily 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% Triton® X-100 in the HDAC-Glo™ I/II Assay Reagent and HDAC-Glo™ I/II Control Assay Reagent (Section 3.C, Step 7) 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 I and II enzyme activity and lead to unacceptable assay performance.

5. References

1. Smith, E.R. *et al* (2000) The *Drosophila* MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation. *Mol. Cell. Biol.* **20**, 312–8.
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™ Control Substrate	10µl	G6550
SIRT-Glo™ Assay	10ml	G6450
	5 × 10ml	G6451
	100ml	G6452
SIRT-Glo™ Screening System	10ml	G6470
	5 × 10ml	G6471
SIRT-Glo™ Control Substrate	35µl	G6460
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	100ml	G8092
	10 × 10ml	G8093
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
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
	5 × 10ml	G9291
	2 × 50ml	G9292

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
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

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 Base Instrument with Shaking	each	E8031
GloMax®-Multi+ Detection System Base Instrument with Heating and Shaking	each	E9031
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, Australian Pat. Nos. 754312 and 785294 and other patents and 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.

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