TECHNICAL MANUAL

HDAC-Glo™ 2 Assay

Instructions for Use of Product
G9590

Revised 11/17
TM406

HDAC-Glo™ 2 Assay

All technical literature is available at: www.promega.com/protocols/
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1. Description

The HDAC-Glo™ 2 Assay[a–c] is a single-reagent addition, homogeneous, luminescence assay that selectively measures the relative activity of histone deacetylase (HDAC) 2 enzyme from cells, extracts or recombinant sources. The assay uses an isoenzyme-selective, acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC 2 activity. 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 (Figure 1).

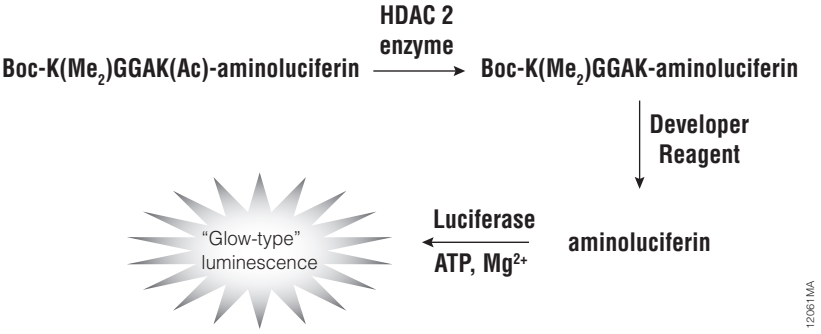

Figure 1. The HDAC-Glo™ 2 Assay chemistry. HDAC 2 enzyme deacetylates the luminogenic substrate, making the peptide sensitive to a specific proteolytic cleavage event that liberates aminoluciferin. Free amino-luciferin can then be measured using the firefly luciferase reaction to produce a stable, persistent emission of light.

Note: The three enzymatic events occur in a coupled, nearly simultaneous reaction that is proportional to deacetylase activity.

Assay Advantages

- **Simple Measurement of Deacetylating Activities:** Single-reagent-addition, homogeneous "add-mix-measure" protocol.
- **Sensitive:** The assay provides 100-fold or better sensitivity than comparable fluorescence methods.
- **Utility:** The assay may be used with recombinant enzyme sources or in either a lytic or nonlytic cell-based format.
- **Fast Data Acquisition:** Collect maximal signal in as little as 20 minutes with persistent, "glow-type" steady state signal half-life.

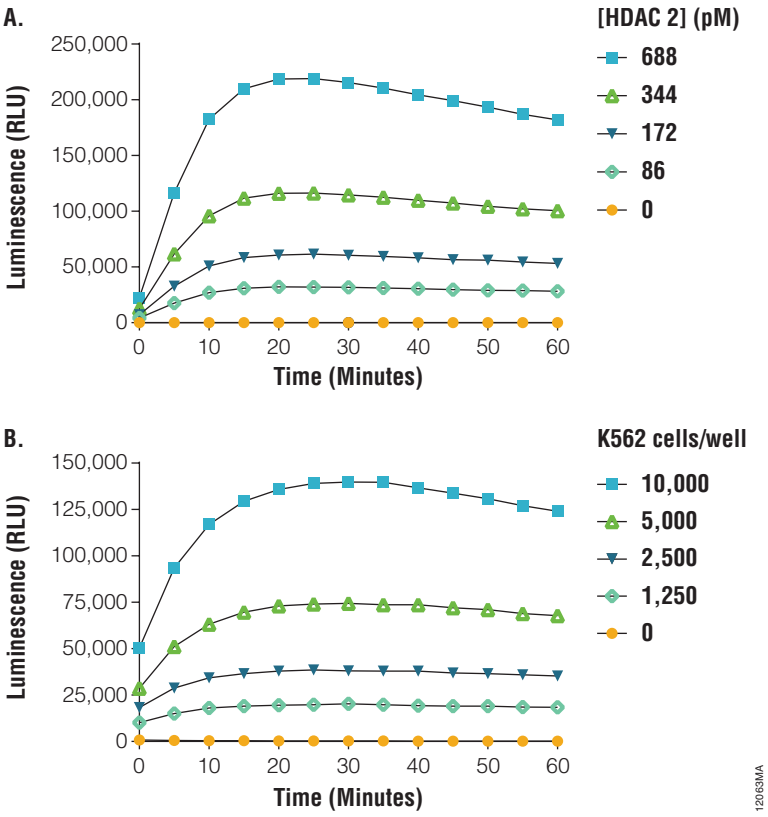

Figure 2. The HDAC 2-mediated luminescent signal is proportional to deacetylase activity.
HDAC 2-mediated signal is persistent in a purified biochemical assay (**Panel A**) and in a cell-based (lytic) assay (**Panel B**). Enzymatic steady state (between deacetylase, developer enzyme and luciferase) is typically achieved within 20 minutes with a signal half-life of 60–90 minutes after steady state is achieved.

2. Product Components and Storage Conditions

Product	Size	Cat.#
HDAC-Glo™ 2 Assay	**10ml**	**G9590**

This system contains sufficient reagents for 100 × 100µl assays in 96-well plates or 1,000 × 10µl biochemical-based assays in 384-well plates (500 × 20µl cell-based assays in 384-well plates). Includes:

- 25ml HDAC-Glo™ 2 Assay Buffer
- 20µl HDAC-Glo™ 2 Substrate, 10mM
- 10µl Developer Reagent
- 1 vial Luciferin Detection Reagent

Storage Conditions: Store the HDAC-Glo™ 2 Activity Assay components at –20°C. For HDAC-Glo™ 2 Assay Buffer, HDAC-Glo™ 2 Substrate and Luciferin Detection Reagent, thaw and equilibrate to room temperature prior to use (see Section 3). For Developer Reagent, thaw and store on ice until addition to the substrate solution just prior to assay. Protect HDAC-Glo™ 2 Substrate from light exposure.

Additional Storage Considerations: Use HDAC-Glo™ 2 Final Detection Reagent on the day it is prepared. Prepare HDAC-Glo™ 2 Final Detection Reagent immediately prior to assaying samples. If HDAC-Glo™ 2 Final Detection Reagent cannot be used immediately for assay of HDAC 2 activity, hold it on ice protected from light until use (with brief equilibration to room temperature before adding to sample). We do not recommend storing beyond 8 hours due to stability issues. In the event that an entire kit will not be used in one experiment, rehydrate Luciferin Detection Reagent with HDAC-Glo™ 2 Assay Buffer, add HDAC-Glo™ 2 Substrate (follow Step 1, Section 3) and divide the Substrate solution into two aliquots. Supplement the aliquot to be used immediately with a proportional volume of Developer Reagent to create HDAC-Glo™ 2 Final Detection Reagent, and freeze the remaining aliquot of HDAC-Glo™ 2 Substrate solution.

3. Preparation of Reagents

Figure 3 provides a schematic diagram of the following detailed instructions.

1. Thaw HDAC-Glo™ 2 Assay Buffer and HDAC-Glo™ 2 Substrate. Add 10ml of HDAC-Glo™ 2 Assay Buffer to Luciferin Detection Reagent (9ml for lytic cell-based assays). Add 20µl of 10mM HDAC-Glo™ 2 Substrate to Luciferin Detection Reagent to make HDAC-Glo™ 2 Substrate solution. Incubate at 37°C for 1 hour.
2. Thaw Developer Reagent on ice. Add 10µl of Developer Reagent to the HDAC-Glo™ 2 Substrate solution prepared in Step 1 to make HDAC-Glo™ 2 Final Detection Reagent. For lytic cell-based assays add 1ml of 10% Triton® X-100 in HDAC-Glo™ 2 Assay Buffer to make HDAC-Glo™ 2 Final Detection Reagent.

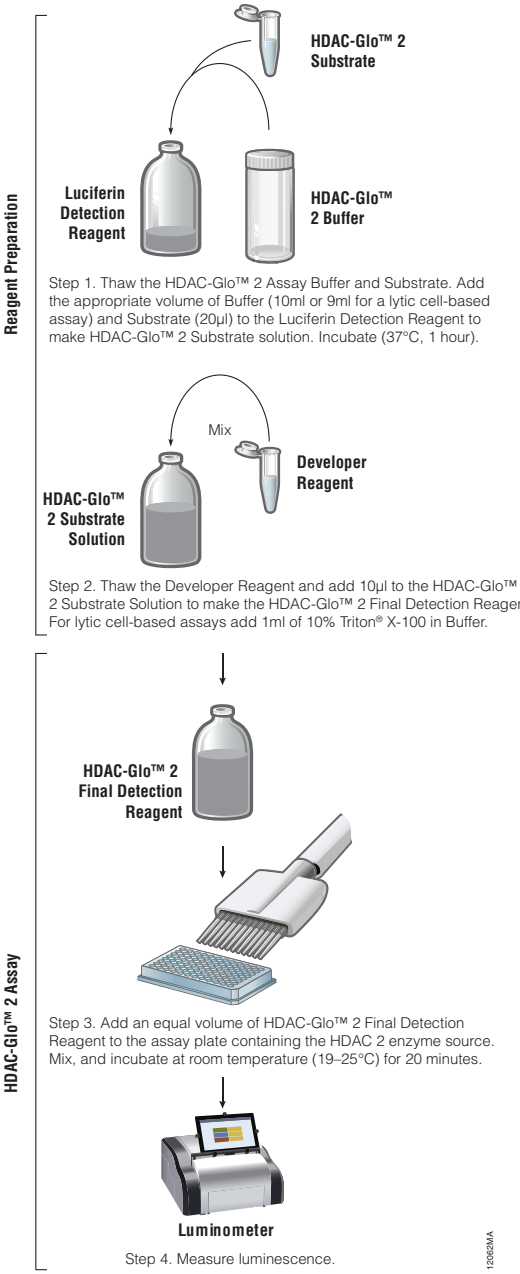

Figure 3. Reagent preparation and assay procedure. See Section 3 for detailed reagent preparation information.

4. Protocols

Materials to Be Supplied by the User

- 96-, 384- or 1536-well, white-walled tissue culture plates compatible with luminometer. Recommended 96-well plates: Corning Costar® Cat.# 3917 (white bottom) or Corning Costar® Cat.# 3903 (clear bottom); for 384-well plates: Corning Cat.# 3673 or 3674 (biochemical), Corning Cat.# 3570 (cell-based)
- multichannel pipettor or liquid-dispensing robot
- reagent reservoirs
- orbital shaker
- recombinant HDAC 2 enzyme source or cells with HDAC 2 activity (such as K562 cells)
- Triton® X-100
- DMSO
- control inhibitor compound (e.g., SAHA)

4.A. HDAC 2 Inhibitor Potency Determination using Recombinant Enzyme

See Figure 5, Panel A, for sample IC_{50} data from a biochemical assay.

1. Follow Step 1 of Figure 3 (see Section 3 for detailed reagent preparation information). During the 1-hour incubation (at 37°C), prepare a compound dilution series in a parallel plate as follows:
    a. Prepare threefold serial dilutions of the unknown compound or SAHA control at 100X of the final assay concentration in 100% DMSO. Be sure to include a no-compound (DMSO-only) control.
    b. Transfer the 100X compound dilution series to HDAC-Glo™ 2 Assay Buffer to obtain a final 2X/2% DMSO concentration (i.e., 5µl of 100X compound + 245µl of HDAC-Glo™ 2 Assay Buffer). This is the master intermediate dilution series of compound.
    c. Transfer 50µl of each dilution from the 2X compound dilution series (Step 1.b) to the white 96-well assay plate (5µl for a 384-well plate). Replicates should be prepared from the same master intermediate dilution series and DMSO percentage should be equal across the compound titration.
2. Dilute HDAC 2 enzyme source (to 2X final desired enzyme concentration) in HDAC-Glo™ 2 Assay Buffer and dispense 50µl into inhibitor dilutions and no-compound controls in the white 96-well assay plate (5µl for a 384-well plate). All assay components should now be at 1X concentration and 1% DMSO. **Note:** An HDAC 2 enzyme titration may be necessary before inhibitor titrations are made to determine the optimal concentration of HDAC 2 to use per well.
3. Mix briefly using an orbital shaker at 500–700rpm to ensure homogeneity.
4. Incubate enzyme/inhibitor mixes for at least 30 minutes at room temperature (19–25°C).
5. Prepare HDAC-Glo™ 2 Final Detection Reagent (see Step 2 of Figure 3 or Section 3).
6. Add 100µl of HDAC-Glo™ 2 Final Detection Reagent to each well (10µl for a 384-well plate).
7. Mix briefly using an orbital shaker at 500–700rpm to ensure homogeneity.
8. Incubate for 20 minutes at room temperature to achieve enzyme steady state, then measure luminescence.

Table 1. Recommended Plate Layout (96-Well Plate Format). Shaded (upper) portion of Table 1 is an example of an unknown compound dilution series (dilution factor is shown). Nonshaded (lower) portion of the table is an example of a control inhibitor (SAHA = 1X nM).

Initial	1:3	1:9	1:27	1:81	1:243	1:729	1:2,187	1:6,561	1:19,683	1:59,049	0
Initial	1:3	1:9	1:27	1:81	1:243	1:729	1:2,187	1:6,561	1:19,683	1:59,049	0
Initial	1:3	1:9	1:27	1:81	1:243	1:729	1:2,187	1:6,561	1:19,683	1:59,049	0
Initial	1:3	1:9	1:27	1:81	1:243	1:729	1:2,187	1:6,561	1:19,683	1:59,049	0
10,000	3,333.3	1,111.1	370.4	123.5	41.2	13.7	4.6	1.5	0.5	0.2	0
10,000	3,333.3	1,111.1	370.4	123.5	41.2	13.7	4.6	1.5	0.5	0.2	0
10,000	3,333.3	1,111.1	370.4	123.5	41.2	13.7	4.6	1.5	0.5	0.2	0
10,000	3,333.3	1,111.1	370.4	123.5	41.2	13.7	4.6	1.5	0.5	0.2	0

4.B. HDAC 2 Inhibitor Potency Determination using Cells

See Figure 5, Panel B, for sample IC_{50} data from a cell-based assay.

1. Seed 50µl of attachment-dependent cells into a white 96-well plate at a density of about 10,000 cells/well (about 5,000 cells/well in 10µl for a 384-well plate). Allow cells to attach by incubation at 37°C.

   Note: Suspension cells can be added directly to inhibitor dilutions in Step 4 at the desired number of cells/well (50µl per well for a 96-well plate; 10µl per well for a 384 well plate). The best assay performance is achieved in serum-free medium.

   Note: A cell titration may be needed before the inhibitor titrations to determine the optimal number of cells to use per well in either a lytic or nonlytic format.

2. Follow Step 1 of Figure 3 (see Section 3 for detailed reagent preparation information). During the 1-hour incubation (at 37°C), prepare a compound dilution series in a parallel plate as follows:

   a. Make threefold serial dilutions of the unknown compound or SAHA control at 100X of the final concentration in 100% DMSO. Be sure to include a no-compound (DMSO-only) control.

   b. Transfer the 100X compound dilution series to serum-free culture medium to obtain a final 2X/2% DMSO concentration (i.e., 5µl of 100X compound + 245µl of serum-free culture medium). This is the compound master intermediate dilution series.

3. Remove culture medium from attachment-dependent cells by gentle aspiration and replace it with 50µl of serum-free medium for a 96-well plate format (10µl for a 384-well format).

4. Transfer 50µl of each dilution from the 2X master intermediate dilution series (prepared in Step 2.b) to the white 96-well assay plate (10µl for a 384-well plate). Replicates should come from the same master intermediate dilution series, and DMSO percentage should be equal across the inhibitor titration.

5. Mix briefly using an orbital shaker at 500–700rpm to ensure homogeneity.

6. Incubate cell/inhibitor mixes for at least 30 minutes at room temperature.

4.B. HDAC 2 Inhibitor Potency Determination using Cells (continued)

7. Prepare HDAC-Glo™ 2 Final Detection Reagent (Step 2 of Figure 3 or Section 3).

   Note: The HDAC-Glo™ 2 Substrate is cell-permeant, making nonlytic cell formats possible. However, a greater signal window (3–4X) can be achieved in a lytic format, by adding Triton® X-100 to the Final Detection Reagent to a final concentration of 1%. If performing the assay in nonlytic mode, it may be necessary to increase the number of cells per assay well to increase the signal window.

8. Add 100µl of HDAC-Glo™ 2 Final Detection Reagent to each well (20µl for a 384-well plate).

9. Mix briefly using an orbital shaker at 500–700rpm to ensure homogeneity.

10. Incubate for 20 minutes at room temperature (19–25°C) to achieve enzyme steady state, then measure luminescence.

5. General Considerations

5.A. Background Luminescence and Inherent Serum Activity

Animal serum used to supplement tissue culture medium may contain detectable levels of the deacetylase activity. Because the assay results can be collected within less than 1 hour, we recommend that you use serum-free medium during the assay. If serum is used, a cell-free medium control should be used as a background control. For longer incubation periods with compounds/cells, when serum-supplemented media is essential, the assay reagent may take a longer period of time to reach a steady state luminescent signal, especially in a nonlytic format.

5.B. Temperature

The enzymes measured in this assay are influenced by temperature. For best results, incubate at a constant controlled temperature to ensure uniformity across the plate. After compound addition and a brief mix, pre-incubate the multiwell plate to equilibrate to room temperature prior to adding the Final Detection Reagent.

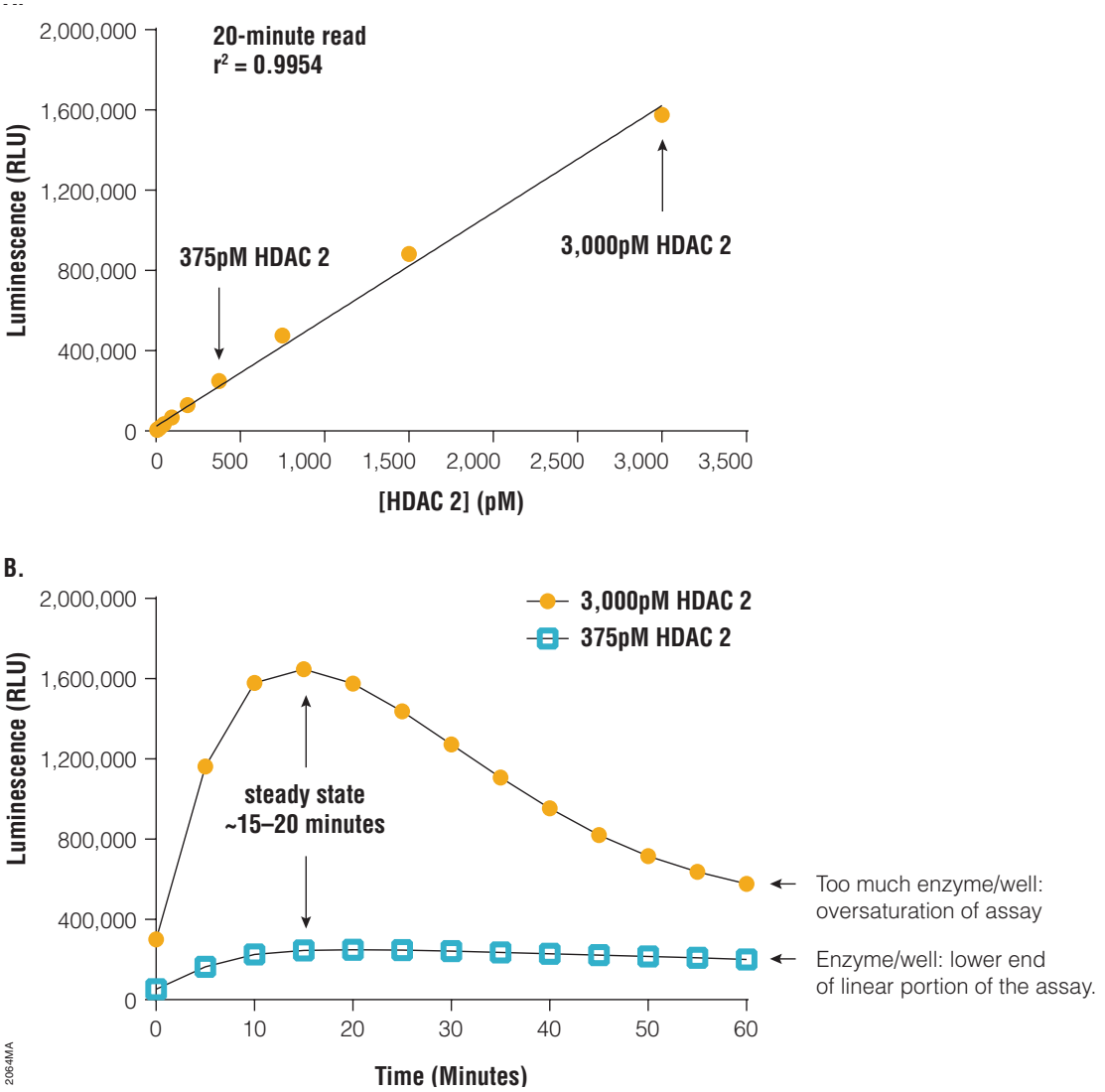

Figure 4. Example of linear range data for the HDAC-Glo™ 2 Assay and signal stability in a biochemical format. Panel A shows the linear range of the HDAC-Glo™ 2 Assay. **Panel B** shows that if the amount of enzyme or number of cells/well is at the lower end of the linear portion of the assay, acceptable signal:background ratios can be achieved once steady state is reached. That signal is stable with a half-life of about 60–90 minutes after steady state is reached.

5.C. Incubation Time and Signal Stability

Enzymatic steady state is typically achieved after approximately 20 minutes at room temperature. We recommend measuring luminescence after this signal plateau is achieved (20 minutes at room temperature or within 30 minutes thereafter). Signal will gradually decay as a function of time with a half-life of about 60–90 minutes after steady-state is achieved. If the assay plate is read before enzymatic steady state, variation may be seen in replicates from one end of the assay plate to the other end, depending on the plate reader pattern. This is especially important in a 384-well plate, where it can take up to 5 minutes to read a single plate. We recommend performing an enzyme or cell titration before running additional experiments in order to determine the optimal concentration of enzyme or number of cells/well to use. After performing an enzyme or cell titration, use a concentration of enzyme or number of cells/well in the lower end of the linear portion of the assay that still gives an acceptable signal:background ratio. If you add too much enzyme or cells/well, you can oversaturate the assay, and once steady state is achieved, the signal will decay faster as a function of time. If the amount of enzyme or cells/well is in the lower end of the linear portion of the assay, once steady state is achieved, the signal is more "stable" and will decay more slowly as a function of time. Figure 4, Panel B, illustrates this principle.

Another factor that can affect the signal stability is the stability of the recombinant HDAC enzyme itself. We have found certain HDAC isoforms to be more stable than other isoforms. We have also found that enzyme stability can vary from vendor to vendor with the same HDAC isoform. If running biochemical assays, it may be useful to evaluate multiple vendors for a given HDAC isoform.

5.D. Assay Controls

Medium or Buffer Background Control: Set up at least triplicate wells without cells or enzyme to serve as the negative control to determine background luminescence.

Untreated Cells or Uninhibited Enzyme Control: The maximal signal control is established by the addition of vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and solvent (DMSO, methanol, etc.) diluted to the same concentration as found in the treatment. Set up at least triplicate wells with untreated cells or uninhibited enzyme. Add the same solvent used to deliver the test compounds to the vehicle control wells.

Optional Inhibitor Control: Set up a dilution series using a known HDAC 2 inhibitor (such as SAHA) as a control for known inhibition of HDAC 2 activity from cells or recombinant enzyme source.

5.E. High-Throughput Screening (HTS) Considerations

When conducting a compound screen in a high-throughput format, it is often beneficial to add a small amount of detergent (such as Triton® X-100) to the final detection reagent whether the assay is run in a biochemical or cell-based format. Although statistically rare, the detergent can reduce the incidence of "false hits"—or compounds that may inhibit luciferase activity. The HDAC-Glo™ 2 Assay is robust and resilient to assay interferences. A small false-inhibition rate is possible through interference with 1) the Developer Reagent; or 2) the luciferase detection component.

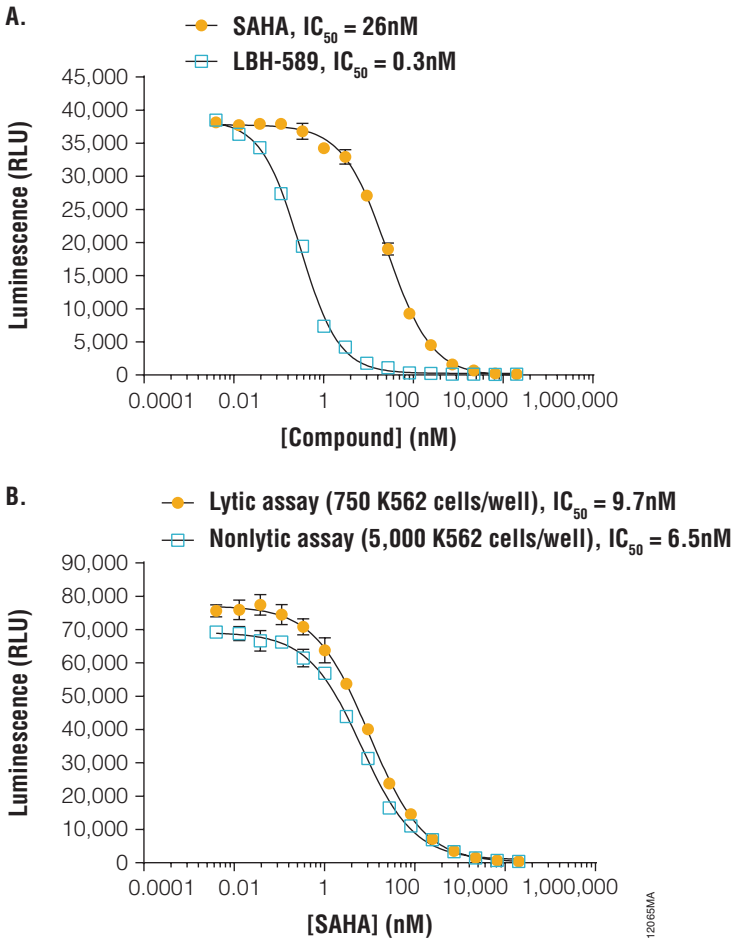

Figure 5. Example of potency data for the HDAC-Glo™ 2 Assay. Panel A, biochemical format. Assay was performed using recombinant HDAC 2 as the enzyme source (1X = 200pM) in a 384-well plate format (Corning Cat.# 3673) at a final assay volume of 20µl. **Panel B, cell-based format**. Assay was performed using K562 cells as the HDAC 2 enzyme source (750 cells/well in lytic format; 5,000 cells/well in a nonlytic format) in a 384-well plate (Corning Cat.# 3570) at a final assay volume of 40µl. For both panels, the final concentration of HDAC-Glo™ 2 Substrate was 10µM. Data was plotted and IC_{50} values were determined using GraphPad Prism® software.

5.F. IC_{50} Value Considerations in Cell-Based Assay Format

Sometimes differences in IC_{50} values can be observed when performing a cell-based assay in a lytic format versus a non-lytic format. There are many possible explanations for this including: 1) lack of compound cell permeability; or 2) the lytic assay conditions adversely affecting certain compound/enzyme interactions. Therefore, when performing IC_{50} value determinations in a cell-based assay, we recommend performing the assay in both lytic and nonlytic formats. Figure 6 illustrates this principle.

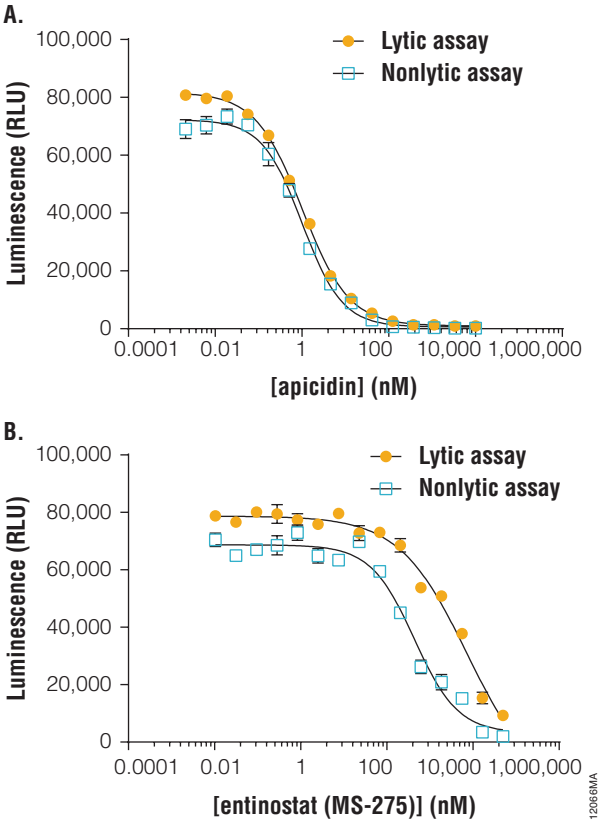

Figure 6. Example of IC_{50} data for the HDAC-Glo™ 2 Assay in a cell-based format (lytic and nonlytic format). These graphs illustrate that differences in IC_{50} values can be observed when performing a cell-based assay in a lytic format versus a nonlytic format. This observation is not assay-specific but is compound-specific. For certain compounds such as apicidin (**Panel A**) or SAHA (Figure 5, Panel B), the lytic and nonlytic IC_{50} values correlate well. However, with compounds such as entinostat (**Panel B**) the lytic and nonlytic IC_{50} values do not correlate well. In the above example, entinostat is a more potent inhibitor of HDAC 2 activity in a nonlytic versus a lytic format. Data was plotted and IC_{50} values determined using GraphPad Prism® software.

6. Supplemental Data

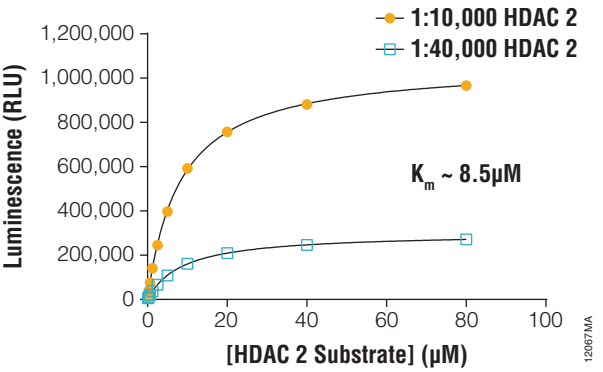

Figure 7. HDAC-Glo™ 2 Substrate K_m determination. An 11-point, twofold serial titration of HDAC-Glo™ 2 Substrate was performed in HDAC-Glo™ 2 Assay Buffer (point 12 was a no-substrate control). After the titration series samples were incubated at 37°C for 1 hour, a proportional volume of Developer Reagent was added to each titration series sample. For each HDAC-Glo™ 2 Substrate titration series sample, 100µl was added to wells of a white 96-well plate (Corning Costar® Cat.# 3917). To this, 100µl of the following samples were added to start the reaction: 1:10,000 HDAC 2 (n = 3 replicates), 1:40,000 HDAC 2 (n = 3 replicates), and no-enzyme controls (n = 2 replicates). Luminescence was measured after 20 minutes at room temperature. The background luminescence for each substrate concentration (determined from the no-enzyme controls) was subtracted from each substrate concentration for the 1:10,000 and 1:40,000 HDAC 2 tests. The data was plotted and K_m was determined using GraphPad Prism® software.

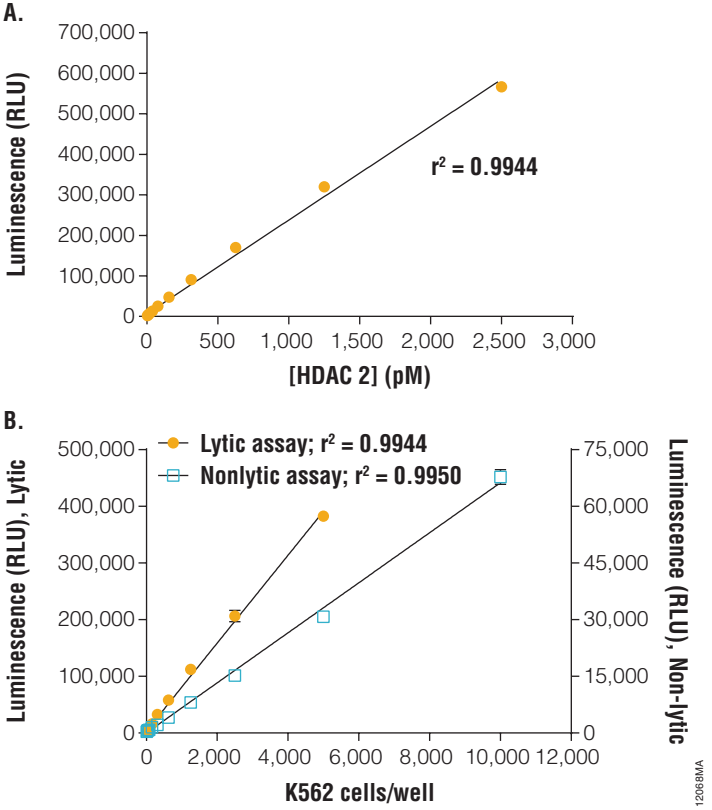

Figure 8. Examples of linear range data for the HDAC-Glo™ 2 Assay. Panel A, biochemical format. Recombinant HDAC 2 was diluted to 2,500pM in HDAC-Glo™ 2 Buffer, then serially diluted twofold (15 points, plus no-enzyme control) in 100µl volumes in a 96-well plate. From this enzyme titration series, 10µl replicates (n = 4) were transferred to a 384-well white assay plate (Corning Cat.# 3673). An equal volume (10µl) of HDAC-Glo™ 2 Final Detection Reagent was added to all wells and luminescence was measured after 20 minutes at room temperature. **Panel B, cell-based assay format (lytic and nonlytic)**. K562 cells were diluted to the appropriate number of cells/ml in serum-free media, then serially diluted twofold (15 points, plus no-cell control) in 100µl volumes in a 96-well plate. From this master cell titration series (lytic assay titration starts at 5,000 cells/well, nonlytic assay titration starts at 10,000 cells/well) 20µl replicates (n = 4) were transferred to a 384-well white assay plate (Corning Cat.# 3570). An equal volume (20µl) of HDAC-Glo™ 2 Final Detection Reagent (with 1% Triton® X-100 for the lytic assay) was added to all wells. Luminescence was measured after 20 minutes at room temperature. For both assays, the final concentration of HDAC-Glo™ 2 Substrate was 10µM. The data for both figures was plotted and linearity was determined using GraphPad Prism® software.

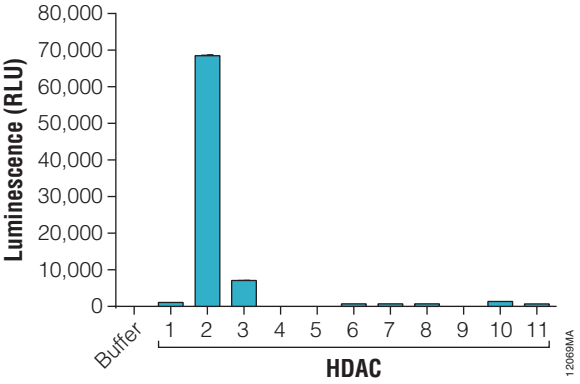

Figure 9. Recombinant HDAC isoenzyme selectivity panel using the HDAC-Glo™ 2 Assay. Each recombinant HDAC enzyme was diluted to equal molar equivalents (250pM) based on concentration, molecular weight and % purity in HDAC-Glo™ 2 Buffer. For each enzyme (n = 8 replicates), 100µl volumes were transferred to a white 96-well assay plate (Corning Costar® Cat.# 3917). An equal volume (100µl) of HDAC-Glo™ 2 Final Detection Reagent was added to all wells and luminescence was measured after 20 minutes at room temperature. For all test conditions, the final concentration of HDAC-Glo™ 2 Substrate was 10µM.

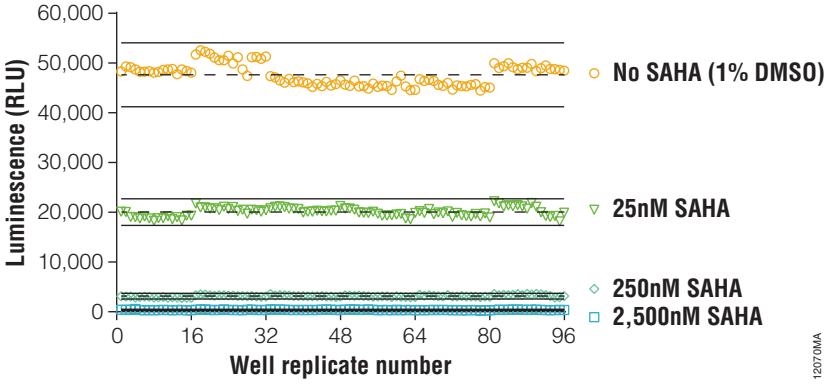

Figure 10. Z´ analysis in 384-well plate format using the HDAC-Glo™ 2 Assay. Four separate pools of 2X SAHA/2% DMSO were prepared in HDAC-Glo™ 2 Assay Buffer, and 5µl for each test condition was added to 96 replicates in a white 384-well assay plate (Corning Cat.# 3673). 2X HDAC 2 (5µl) in HDAC-Glo™ 2 Assay Buffer was added to all wells. The inhibitor/enzyme mix was incubated at room temperature for 30 minutes. An equal volume (10µl) of HDAC-Glo™ 2 Final Detection Reagent was added to all wells (20µl final assay volume), and luminescence was measured after 20 minutes at room temperature. For all test conditions, the final concentration of HDAC-Glo™ 2 Substrate was 10µM. The final concentration of HDAC 2/well was 200pM. The dotted lines for each set of 96 replicates represent the mean, while the solid lines indicate ± 3 standard deviations. The data was plotted using GraphPad Prism® software.

Table 2. Z´ Analysis Data for Figure 10.

Compound Test Condition (n = 96)	Signal:Background	% CV	Z´
no SAHA (1% DMSO)	N.A.	4.5	NA
25nM SAHA	2.4	4.4	0.67
250nM SAHA	15.1	6.1	0.84
2,500nM SAHA	125.0	11.1	0.86

7. 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
SIRT-Glo™ Assay	10ml	G6450
CellTox™ Green Cytotoxicity Assay	10ml	G8741
	50ml	G8742
	100ml	G8743
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
	5 × 10ml	G6081
	2 × 50ml	G6082

Available Separately

Product	Size	Cat.#
HeLa Nuclear Extract	10µl	G6570
Trichostatin A	10µl	G6560
Nicotinamide	30µl	G6540

8. Summary of Change

The following change was made to the 11/17 revision of this document:

Removed any mention of discontinued products.

[a] U.S. Pat. Nos. 6,602,677, 7,241,584, 8,030,017 and 8,822,170, 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.

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