# Selective Bioluminogenic HDAC Activity Assays for Profiling HDAC Inhibitors

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## 1. Abstract

Histone deacetylases (HDACs) play critical roles in the regulation of gene transcription and cell signaling events by deacetylating histones and other important non-histone substrates. Aberrant increases in HDAC enzyme activities are therefore implicated in a number of human infirmities, including cancers, metabolic disease and neurodegeneration. Fortunately, HDAC enzymes represent attractive pharmacological targets because they are readily tractable with small molecule inhibitors. In fact, several HDAC inhibitors (HDACi) have recently proceeded through (or are near) the FDA approval process for the treatment of hematologic malignancies. However, the promise of clinical HDACi therapy has been hampered by significant dose-limiting toxicities. These off-target effects have led to a renewed focus on basic HDAC biology and the development of isoenzyme-specific HDAC inhibitors which could avoid off-target effects. To help facilitate the discovery of compounds with better defined selectivity profiles, we have developed lysine deacetylase assays that selectively measure specific isoenzyme activities in cells, extracts, or purified recombinant preparations. These assays are based on substrates that are selective due to a combination of extended peptide sequence and novel chemical modifications. Deacetylase activity is measured by delivering a single, pro-luminogenic, homogeneous assay reagent to assay wells, resulting in luminescence proportional to HDAC activity. In addition to being isoenzyme selective, these novel substrates are cell permeable allowing for lytic and non-lytic cell-based HDAC assays. Lastly, these assays are also fully compatible with fluorescent viability and/or cytotoxicity assays. This provides additional flexibility for multiplexed formats which examine not only selective HDAC inhibition, but the functional consequences they exert on cell health.

## 2. HDAC-Glo™ Assay Chemistries and Selectivity

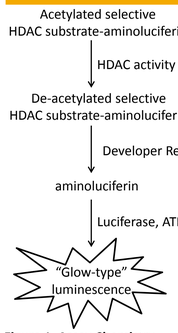


**Figure 1. Assay Chemistry.**
**Note:** All three enzymatic events occur in a coupled, near simultaneous reaction which is proportional to deacetylase activity.

| Assay | Substrate | HDAC selectivity | HDAC isoform | Substrate $K_m$ (µM) |
|---|---|---|---|---|
| HDAC-Glo™ I/II | Boc-GAK(Ac)-aluc | Class I/IIb | HDAC 1 | 45 |
| | | | HDAC 2 | 65 |
| | | | HDAC 3 | 192 |
| | | | HDAC 6 | 207 |
| | | | HDAC 8 | 31 |
| | | | HDAC 10 | 301 |
| HDAC-Glo™ Class IIa | Boc-K(Me2)GGAK(TFA)-aluc | Class IIa | HDAC 4 | 13 |
| | | | HDAC 5 | 61 |
| | | | HDAC 7 | 32 |
| | | | HDAC 9 | 33 |
| HDAC-Glo™ 2 | Boc-K($Me_2$)GGAK(Ac)-aluc | HDAC 2 | HDAC 2 | 10 |

**Table 1. The optimized substrate sequence, selectivity, and $K_m$ for each HDAC isoform for the three HDAC-Glo™ assays.** For substrate $K_m$ determinations, a 2-fold serial titration of each HDAC-Glo™ substrate was performed in the appropriate corresponding assay buffer. Two separate HDAC recombinant enzyme concentrations (n=3 for each) and no enzyme controls (n=2) were added to the diluted substrate to start the coupled enzymatic reaction. Luminescence was measured and the background luminescence for each substrate concentration point (determined from the no enzyme control wells) was subtracted for the two separate HDAC enzyme concentrations. The data was plotted and $K_m$ was determined using a one-site binding, hyperbolic fit.

## 3. Biochemical Isoenzyme Selectivity Panels

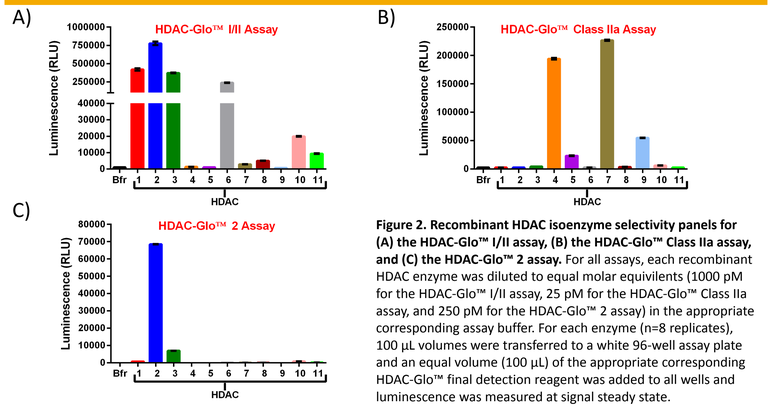


**Figure 2. Recombinant HDAC isoenzyme selectivity panels for (A) the HDAC-Glo™ I/II assay, (B) the HDAC-Glo™ Class IIa assay, and (C) the HDAC-Glo™ 2 assay.** For all assays, each recombinant HDAC enzyme was diluted to equal molar equivilents (1000 pM for the HDAC-Glo™ I/II assay, 25 pM for the HDAC-Glo™ Class IIa assay, and 250 pM for the HDAC-Glo™ 2 assay) in the appropriate corresponding assay buffer. For each enzyme (n=8 replicates), 100 µL volumes were transferred to a white 96-well assay plate and an equal volume (100 µL) of the appropriate corresponding HDAC-Glo™ final detection reagent was added to all wells and luminescence was measured at signal steady state.

## 4. Linear and Dynamic Assay Ranges

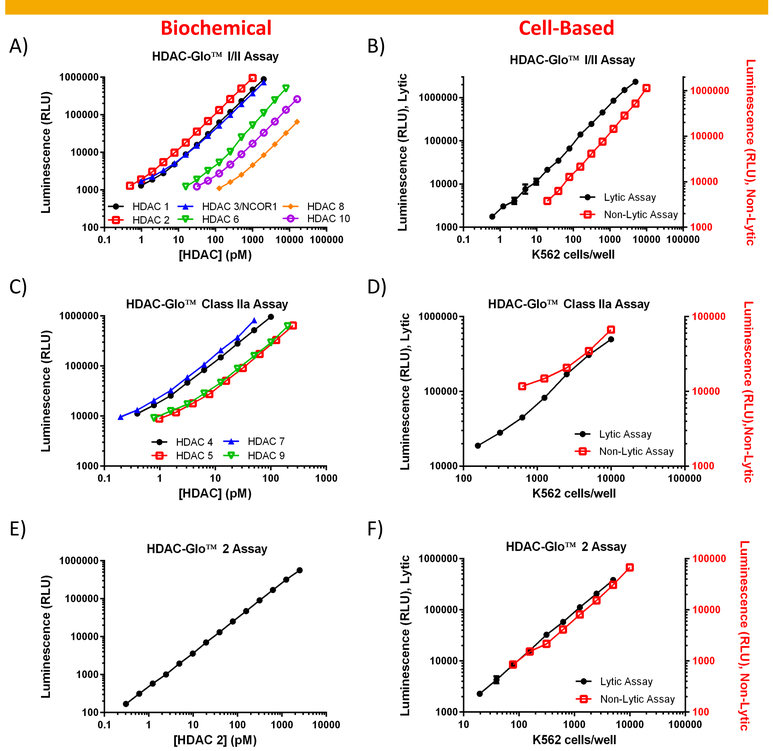


**Figure 3. Recombinant HDAC enzyme titrations (A, C, E) and K562 cell titrations (B, D, F) using the HDAC-Glo™ I/II assay (A, B), HDAC-Glo™ Class IIa assay (C, D), and HDAC-Glo™ 2 Assay (E, F).** For recombinant HDAC enzyme titrations, a 10 µL addition of a 2-fold serial dilution series of recombinant HDAC enzyme in the appropriate HDAC-Glo™ assay buffer was added to a white 384-well assay plate followed by addition of 10 µL of the appropriate corresponding HDAC-Glo™ final detection reagent and luminescence was measured. For K562 cell titrations, a 20 µL addition of 2-fold serial dilution series of K562 cells in serum-free RPMI-1640 media was added to a white 384-well assay plate followed by addition of 20 µL of the appropriate corresponding HDAC-Glo™ final detection reagent and luminescence was measured. For K562 cell titrations, lytic assay luminescence: left y-axis, closed black circles; non-lytic assay luminescence: right y-axis, open red squares.

## 5. Cell-Based Profiling of HDAC Inhibitor Selectivity and Potencies

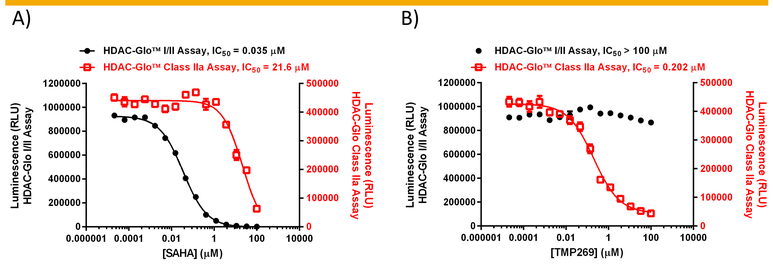


**Figure 4. Example inhibitor potency data demonstrating cell-based isoenzyme selectivity in K562 cells using the HDAC-Glo™ I/II assay or the HDAC-Glo™ Class IIa assay.** SAHA, a selective class I/IIb HDAC inhibitor (Panel A) or TMP269, a selective class IIa HDAC inhibitor (Panel B) were examined. A 10 µL addition of a 15-point 3-fold serial dilution of 2X compound was added to a white 384-well assay plate followed by a 10 µL addition of K562 cells in serum-free RPMI-1640 media (final of 2500 K562 cells/well). Following a 45 minute pre-incubation of cells/inhibitor at room temperature, an equal volume (20 µL) of HDAC-Glo™ I/II final detection reagent or HDAC-Glo™ Class IIa final detection reagent (both with 1% Triton X-100 for lytic assay) was added to the appropriate wells and luminescence was measured. HDAC-Glo™ I/II assay: left x-axis, closed black circles; HDAC-Glo™ Class IIa assay: right x-axis, open red squares.

## 6. Real-Time Assessment of Compound Cell Permeability & Inhibitor Association Rates in a Live-Cell Format

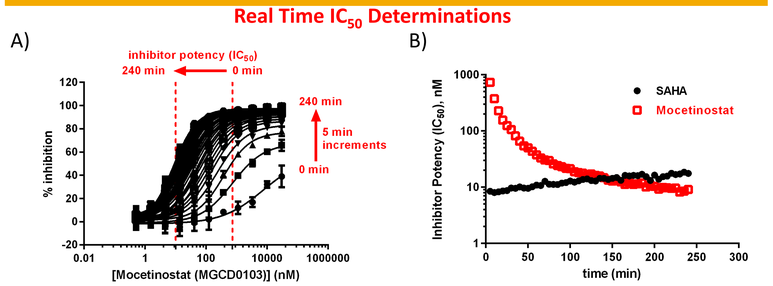


**Figure 5. Example data to demonstrate real time kinetic inhibitor potency determinations in live (non-lytic) K562 cells using the HDAC-Glo™ 2 Assay to assess differences in compound cell permeability and active-site engagement.** In panel (A), a 90 µL addition of K562 cells in serum-free media was added to a white 96-well assay plate to obtain a final of 10,000 cells/well. This was followed by a 100 µL addition of HDAC-Glo™ 2 final detection reagent to initiate the HDAC reaction. Following a pre-incubation for 20 minutes to reach enzyme steady-state, a 10 µL addition of 20X mocetinostat (selective HDAC 1, 2, 3 inhibitor) was added and luminescence was measured every 5 minutes for 4 hours. In panel (B), the same experiment was done for SAHA and the data for both compounds was re-plotted as $IC_{50}$ versus time.

## 7. Mechanistic Cell Cytotoxicity: Multiplexed HDAC Activity and Cytotoxicity Measures

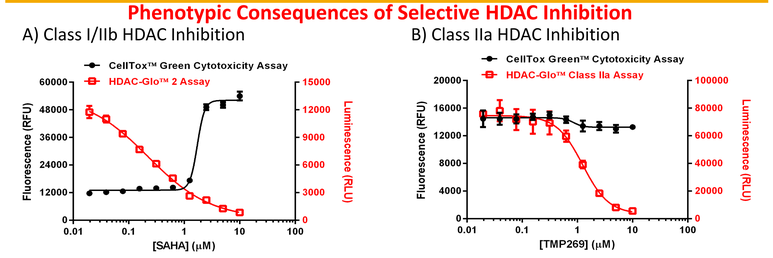


**Figure 6. Example data demonstrating the multiplexed HDAC-Glo™ Assays with the CellTox™ Green Cytotoxicity Assay.** K562 cells were exposed to (A) SAHA, a selective class I/IIb HDAC inhibitor or (B) TMP269, a selective class IIa HDAC inhibitor for 72 hours in the presence of CellTox™ Green, a real-time fluorescent cell cytotoxicity probe. After fluorescence data was gathered, HDAC 2 activity (A) or HDAC Class IIa activity (B) was collected in the luminescence channel by adding an equal volume of HDAC-Glo™ 2 Assay Reagent (A) or HDAC-Glo™ Class IIa Assay Reagent (B) in a multiplexed, same-well format. CellTox™ Green Assay: left x-axis, fluorescence, closed black circles; HDAC-Glo™ 2 Assay (A) and HDAC-Glo™ Class IIa Assay (B): right x-axis, luminescence, open red squares. Note: an increase in RFU with the CellTox™ Green probe is indicative of an increase in cell death.

## 8. Conclusions

The selective HDAC-Glo™ Assays offer significant advantages over existing plate-based methods because they deliver:

- Isofamily (HDAC-Glo™ I/II and Class IIa Assay) and Isoenzyme (HDAC-Glo™ 2 Assay) selectivity for targeting and profiling isoenzyme-selective HDAC inhibitors
- Versatility to use either in a biochemical or cell-based (lytic and non-lytic) format
- An easy-to-use, homogeneous, one-step reagent with a robust, steady, luminescent signal
- Easy scalability into high-density plate formats
- Enhanced sensitivity over current fluorescent-based formats (data not shown)
- Broad linearity responses (2-3 logs) with large dynamic ranges
- Comparable $IC_{50}$ values to published data when using well-characterized HDAC inhibitors
- The ability to assess differences in cell permeability and target association rates in live cells with well-characterized HDAC inhibitors known to have slow or fast on-rates
- The ability to multiplex with fluorescent cell cytotoxicity assays using live cells to not only measure HDAC inhibition, but to measure the phenotypic consequences they exert on cell health (mechanistic cell cytotoxicity)

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