SensoLyte® 520 HDAC Activity Assay Kit *Fluorimetric*

Revision number: 1.2	Last updated: October 2014
Catalog #	**AS-72084**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect HDAC activity.
- ***Enhanced Value:*** It provides enough reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	HDAC 520 substrate, Ex/Em=490 nm/520 nm	25 mM in DMSO, 40 µL
Component B	Deacetylated 520 standard, Ex/Em=490 nm/520 nm	25 mM in DMSO, 10 µL
Component C	HeLa Nuclear Extract	~ 4 mg protein/mL, 15 µL
Component D	Assay Buffer	20 mL
Component E	Trichostatin A (Inhibitor of HDAC)	300 µM in DMSO, 100 µL
Component F	HDAC Developer (10X)	0.5 mL
Component G	Cell Lysis Buffer	20 mL

Other Materials Required (but not provided)

- Additional HDAC source: Purified enzymes, extracts or immunoprecipitated complex.
- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components, except Component C, at -20°C.
- Store Component C at –80°C. Aliquot as needed to avoid freeze-thaw cycles.
- Protect Components A and B from light and from moisture.
- Components D and G can be stored at room temperature for convenience.

Introduction

Histones are the chief protein components of chromatin. They act as spools around which DNA winds. Covalent modification of histone proteins through acetylation and deacetylation affects chromatin structure and regulates gene expression. Histone hyperacetylation is well correlated with increased transcription, whereas hypoacetylation correlates with transcriptional repression.[1] Histone deacetylases (HDACs), which catalyze the removal of acetyl groups from a ε-N-acetyl lysine amino acid on a histone, act as transcriptional repressors of genes. Histone deacetylases have been grouped into three classes. Class I (HDAC 1, 2, 3, 8) and Class II (HDAC 4, 5, 6, 7, and 9) are zinc-containing hydrolase's enzymes.[2,3] The third class of deacetylases consists of the members of the sirtuin family of enzymes (Sir 1 to 7).[4] Inhibitors of HDAC classes I and II are being studied as a treatment for cancer and neurodegenerative diseases such as Huntington's and Alzheimer's diseases.[5-7] The Sirtuin 1 (class III) enzyme represents a target for treatment of age-related diseases and type II diabetes.[8,9]

The SensoLyte® 520 HDAC Activity Assay Kit provides a convenient, two-step homogeneous procedure for measuring HDAC activity from various sources of enzyme. It provides quantification of HDAC activity using a fluorogenic substrate described in the literature.[10] In the first step, an acetylated substrate is incubated with HDAC-containing samples. Deacetylation of substrate sensitizes it to the HDAC developer, which, in the second step, generates the green fluorophore. It can be detected with excitation at 490 nm and emission at 520 nm. The substrate included in the kit is cell-permeable, and the assay can measure HDAC activity directly in cell culture in a 96-well plate without a time-consuming cell extraction step. The kit also can be used for high throughput screening of HDAC inhibitors with extracts or purified enzymes. The long wavelength spectra and higher extinction coefficient of the HDAC 520 substrate provide less interference from compounds and cell components. The kit provides all the reagents required for HDAC assay, including HeLa nuclear extract as a source of HDAC activity for positive control and Trichostatin A as an inhibitor for the class I and class II HDACs. The assay can detect 0.78 μg/mL active HDAC of HeLa nuclear extract.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screening HDAC inhibitors using HeLa nuclear extract.

1. Prepare working solutions.

Note: Allow all kit components to thaw before starting the experiment. Component C should be kept on ice after thawing.

1.1 HDAC substrate solution: Dilute HDAC substrate (Component A) 1:500 in assay buffer (Component D) giving a substrate solution of 50 μM. 50 μL of this diluted HDAC substrate is enough for one reaction (96-well plate). For each experiment prepare fresh substrate solution.

Note: The K_m value for the HDAC substrate with HeLa nuclear extract is 16.3 μM, and we recommend a final substrate concentration of 25 μM for inhibitor screening. For screening with a different source of HDAC activity, the optimal substrate concentration should be determined in additional experiments.

Table 1. Diluted HDAC substrate solution for one 96-well plate (96 assays).

Components	Volume
HDAC substrate, 25 mM (Component A)	10 μL
Assay buffer (Component D)	4.99 mL
Total volume	5 mL

1.2 HeLa nuclear extract diluent: Dilute nuclear extract (Component C) 1:40 in assay buffer (Component D). The suggested volume of this diluted HDAC solution for one well of a 96-well plate is 40 μL.

Note 1: The amount of HeLa nuclear extract (Component C) provided in this kit is enough to serve only as a positive control. More HeLa nuclear extract may be ordered from AnaSpec (Cat#72082).
Note 2: Dilute extract immediately before use. Store the solution on ice.

1.3 HDAC inhibitor: Dilute an aliquot of the 300 μM Trichostatin A solution (Component E) 1:30 in assay buffer (Component D). Add 10 μL of this 10 μM Trichostatin solution into each inhibitor control well.

1.4 HDAC Developer: Dilute the developer (Component F) 1:10 in assay buffer (Component D). Dilute the 300 μM Trichostatin A (Component E) 100 fold in the 1X developer (the final concentration of Trichostatin in developer is 3 μM). Each assay requires 50 μL of HDAC developer.

Note 1: The developer, containing Trichostatin, is a bi-functional buffer, which works as a stop solution for HDACs and initiates fluorescent signal releasing fluorophore.
Note 2: Prepare developer before use. Otherwise keep prepared solution on ice until use.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted nuclear extract or enzyme solution to the microplate wells. For one well of a 96-well plate, the suggested volume of extract or enzyme solution is 40 μL and 10 μL of test compound.

2.2 Establish the following control wells at the same time, as deemed necessary:

- Positive control contains HeLa nuclear extract without test compound.
- Inhibitor control contains HeLa nuclear extract and Trichostatin A.
- Vehicle control contains HeLa nuclear extract and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component D) and test compound. Some test compounds may themselves be fluorescent or interfere with the Ex/Em wavelengths and thereby give false results.
- Substrate control contains assay buffer (Component D).

2.3 Using the assay buffer (Component D), bring the total volume of all controls to 50 μL.

2.4 Pre-incubate the plate for 10 min at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

3. Run the enzymatic reaction.

3.1 Add 50 μL of HDAC substrate solution into each well, except the test compound control wells. For best accuracy, it is advisable to have the HDAC substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for no more than 30 sec.

3.2 Incubate the plate for 30-60 minutes at assay temperature.

3.3 Add 50 µL of the prepared HDAC developer and mix thoroughly.

3.4 Incubate the plate for additional 15 min at room temperature.

3.5 Measure fluorescence signal at Ex/Em=490 nm/520 nm.

3.6 For methods of data analysis: Refer to Appendix I.

3.7 An example of the determination of the inhibition of HDAC by trichostatin is shown in Figure 1.

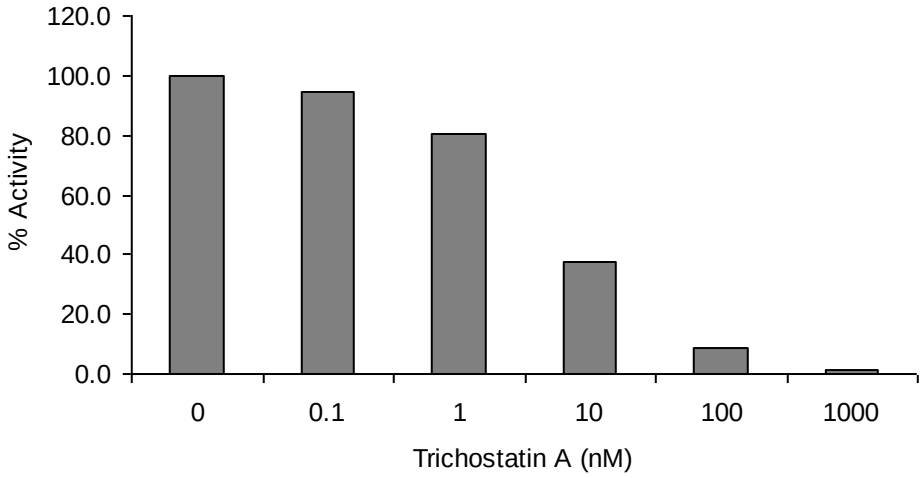

Figure 1. Trichostatin A inhibition of HDACs activity in HeLa nuclear extract measured with SensoLyte® 520 HDAC Activity Assay Kit.

Protocol B. Measuring HDAC activity using cell culture

Note 1: The protocol was tested on HeLa cells. For other cell lines, the optimal cell densities have to be determined.

1. Prepare cells.

- Seed 6 x 10^4 cells in 100 µL media per well in a 96-well plate and grow overnight to reach confluency.

2. Prepare working solutions.

Note: Allow all necessary kit components to thaw before starting the experiment. If using Component C, keep it on ice after thawing.

2.1 HDAC substrate solution: Dilute HDAC substrate (Component A) 1:100 in media giving a substrate solution of 250 µM. 40 µL of diluted HDAC substrate is enough for one reaction (96-well plate). For each experiment prepare fresh substrate solution.

Table 1. HDAC substrate solution for one 96-well plate (100 assays)

Components	Volume
HDAC 520 substrate, 25 mM (Component A)	40 µL
Media for HeLa cells	3.96 mL
Total volume	4 mL

2.2 HDAC inhibitor: Dilute the 300 µM Trichostatin A solution (Component E) 1:60 in media. 10 µL of this 5 µM Trichostatin stock solution are enough for the inhibitor control well.

2.3 HDAC Developer: Dilute the developer (Component F) 1:10 in cold cell lysis buffer (Component G). Dilute the 300 μM Trichostatin A (Component E) 1:150 fold in this 1X developer (the final concentration of Trichostatin in developer is 2 μM). Each assay requires 50 μL of this HDAC developer.

Note 1: The prepared developer is a multi-functional buffer, which works as a stop solution for HDACs, lyses cells and initiates fluorescent signal releasing fluorophore.
Note 2: Prepare developer before use. Otherwise keep prepared solution on ice until use.

3. Set up enzymatic reaction.

3.1 Retrieve cells from the 37°C incubator. Replace media with 40 μL/well of HDAC substrate solution (media containing 250 μM of HDAC substrate). Add test compounds to the microplate wells. For one well of a 96-well plate, the suggested volumes are 40 μL of substrate solution and 10 μL of test compound.

Note: Some test compounds may require preincubation with the cells before addition of substrate.

3.2 Establish the following control wells, as deemed necessary:

- Positive control contains substrate solution without test compound.
- Inhibitor control contains Trichostatin A and substrate solution.
- Vehicle control contains the vehicle used to deliver test compounds and substrate solution.
- Baseline control contains media without substrate. This media should be removed at the end of incubation and replaced with media containing developer and substrate.

Note: The baseline control or "zero time point" serves as background in cellular deacetylation assay. Baseline control cells are incubated with media without substrate. The substrate is added simultaneously with HDAC developer only at the end of incubation.

- Test compound control contains media without substrate. This media should be removed at the end of incubation and replaced with media containing test compound, developer and substrate.

Note: Some test compounds may themselves be fluorescent or interfere with the Ex/Em wavelengths and thereby give false results. Test compound is added to the baseline control to evaluate how it affects background fluorescence in cellular deacetylation assay.

3.3 Bring the total volume of all controls to 50 μL using media.

3.4 Incubate cells in a 37^{o}C incubator for the desired exposure period (signal can be detected by incubating cells with substrates for one to several hours).

3.5 Stop the deacetylation reaction by adding 50 μL/well of the prepared HDAC inhibitor/developer solution and mix thoroughly.

Note: Add 50 μL/well of media containing substrate into baseline control wells, and add 50 μL/well of media containing substrate and test compound in test compound control wells.

3.6 Incubate the plate an additional 15 min at room temperature.

3.7 Measure the fluorescence signal at Ex/Em=490 nm/520 nm.

3.8 For data analysis, refer to Appendix I.

Appendix I. Data Analysis

- For Protocol A: The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).
- For Protocol B: The fluorescence reading from the baseline control well is the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).

  Note: Slight modifications of Protocol B allow measurement and analysis of the amount of deacetylation in the cells and cell supernatant separately, if desired.
- For kinetics analysis:
  - Plot data as RFU versus time for each sample. If you want to convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50,}$ IC_{50}, K_m, K_i, etc.
- For endpoint analysis:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- The standard for this assay is the deacetylated substrate. It is not fluorescent until acted upon by the developer. Production of fluorescence is complete within the 15 min incubation used in the protocol below, and will be directly proportional to the concentration of the deacetylated standard.
- Deacetylated 520 standard: Dilute the 25 mM deacetylated standard (Component B) 1:1000 in assay buffer (Component D) to give 25 μM stock. Perform 2-fold serial dilutions with this diluted standard to obtain 12.5, 6.25, 3.1, 1.56, 0.78, 0.39 μM deacetylated solutions, including an assay buffer blank. Add 100 μL/well of these serially diluted standard solutions into the plate.

  Note: To prepare the reference standard for Protocol B, use the cell culture media for serial dilutions instead of the assay buffer. Add only 50 μL/well of these serially diluted standard solutions into each well.
- Add 50 μL of HDAC developer (prepare as in Step 1 from Protocol you use) to each well. Mix the reagents by shaking the plate gently for 3 to 5 sec.
- Incubate the plate for an additional 15 min at room temperature.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.

- Plot the deacetylated 520 standard curves as RFU (relative fluorescent units) versus concentration as shown in Figure 2.

  Note: The concentration of deacetylated 520 reference standard solutions are 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of enzymatic reaction final product.

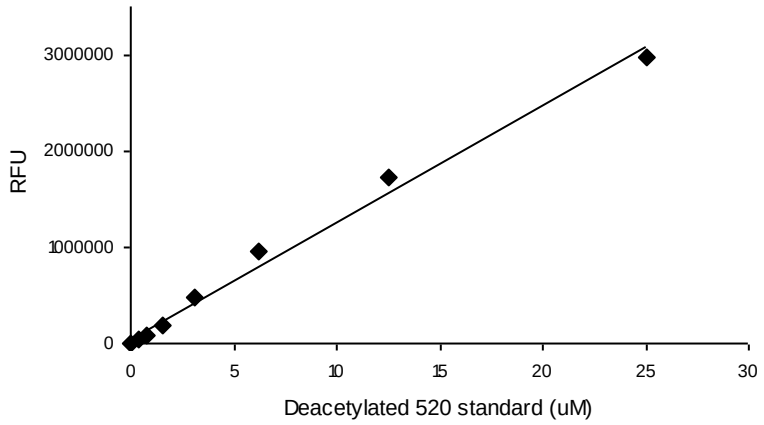

Figure 2. Example of deacetylated 520 reference standard curve. Deacetylated 520 standard was serially diluted with assay buffer, and after 15 min incubation with HDAC developer, fluorescence was recorded at Ex/Em=490 nm/520 nm (Flexstation 384II, Molecular Devices).

References

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