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Product Information

***In Situ* HDAC Activity Fluorometric Assay Kit**

Catalog Number EPI003
Storage Temperature – 20 °C

TECHNICAL BULLETIN

Product Description
Histone deacetylases (HDACs) are a large family of enzymes that remove acetyl groups from histone proteins. Site specific histone acetylation and deacetylation have been shown to activate or repress eukaryotic gene transcription, respectively, and as a consequence, HDACs play a crucial role in mammalian development and disease. HDAC's are localized in both the cytosol and nucleus and some shuttle between the two locations. Increased HDAC expression has been observed in specific developmental stages (1) and various cancers (2).

Sigma's *InSitu* HDAC Activity Fluorometric Assay Kit provides a direct, fast, fluorescence-based method to measure HDAC activity in cultured cells. The procedure requires just two steps, both performed in the original 96-well cell culture plate. First, the cell culture medium is replaced with a cell permeable HDAC Substrate, containing an acetylated lysine side chain. During the subsequent incubation, HDAC Substrate enters the cells and is deacetylated by intracellular HDAC. In the second step, Developer is added to lyse the cells and cleave the deacetylated HDAC Substrate to release a fluorophore. The fluorescence generated can be quantified at Ex/Em = 368/442 nm. The assay is well suited for either individual or high throughput screening.

Summary
Step 1
Cells grown in 96-well plate + HDAC Substrate in medium
Incubate for 1-3 hours
Obtain Deacetylated Substrate (within the cells)

Step 2
Deacetylated Substrate + Developer
Incubate at 37°C for 30 min
Measure Fluorophore (Ex/Em = 368/442 nm)

Components
The kit is sufficient for 100 assays in 96 well plates.

HDAC Assay Buffer (WM cap) 25 mL
Catalog Number EPI003A

HDAC Substrate (Amber cap) 120 µL
Catalog Number EPI003B

Developer (Orange cap) 1.5 ml
Catalog Number EPI003C

HDAC Inhibitor (Blue cap)
(Trichostatin A, 1 mM (TSA)) 10 µL
Catalog Number EPI003D

Positive Control (Jurkat Cell Lysate) (Red cap)
Lyophilized 1 vl
Catalog Number EPI003E

Standard 100 µL
(Deacetylated Substrate, 4 mM) (Yellow cap)
Catalog Number EPI003F

Reagents and equipment required but not provided.
96 well flat-bottom plate – It is recommended to use a black plate with flat clear bottom.
Fluorometric plate reader
Phosphate Buffered Saline for use as control (PBS) Catalog Numbers P4417, P5368

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
The kit is shipped on wet ice. The HDAC Assay Buffer (EPI003A) can be stored at 2-8 °C. All other components should be stored at –20 °C, protected from light.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles. Read the entire protocol before performing the assay.

HDAC Assay Buffer (EPI003A)
Store at –20 °C or 2-8 °C. Allow HDAC Assay Buffer to warm to room temperature before use.

HDAC Substrate (EPI003B)
Aliquot & store at –20 °C. Avoid repeated freeze-thaw.
***Note*:** Use a fresh pipette tip each time to pipette out

Developer (EPI003C)
Aliquot & store at –20 °C. Avoid repeated freeze-thaw.
Keep on ice while in use. Use within 2 months

HDAC Inhibitor (Trichostatin A) (EPI003D)
Aliquot & store at –20 °C. Avoid repeated freeze-thaw.

Positive Control(EPI003E)
Store lyophilized Positive Control at –20 °C
Reconstitute with 25 µL deionized water just prior to use. Mix gently by pipetting. Aliquot and store at -80 °C. Avoid repeated freeze-thaw. Use within 2 months of reconstitution.

Standard (Deacetylated Substrate) (EPI003A)
Store at –20 °C.

Procedure
All samples and standards should be run in duplicate.

1. Sample Preparation
   Seed 10^4 to 10^5 cells per well in a 96 well cell culture plate, in a cell specific medium. Treat log phase cells as desired to alter HDAC activity. Reserve some wells as untreated controls and inhibitor control(s). For background and positive controls use empty well(s) without cells.

2. Assay Reaction

a. Mix enough reagents in a master mix for the number of assays to be performed. Ensure thorough mixing prior to adding to the cells. For each well, prepare a 100 µL mix containing:

Table 1. Reaction Mix

Reagent	Reaction Mix	Inhibitor Control Mix
Cell culture media	99 µL	98 µL
HDAC Substrate	1 µL	1 µL
HDAC Inhibitor	-	1 µL

b. Remove the spent media.
   Note: For suspension cells, spin down the cells at 1000x*g* for 5 minutes prior to removal of media.

c. Add 100 µL of the Reaction Mix to each well containing the test samples & each empty well for the background controls.
   Add 100 µL of the Inhibitor Control Mix to each well for the inhibitor control wells

3. Positive Control
   Add 2 µL of reconstituted Positive Control and 1 µL of HDAC Substrate to each empty positive control and adjust the volume to 100 µL with Phosphate Buffered Saline (not provided).

4. Incubation
   Incubate for one, two or three hours under cell culture conditions.
   Note: Incubation time depends on the HDAC activity of the sample.

5. Standard Curve:
   Dilute Standard to 100 µM by adding 2.5 µL of 4 mM Standard (EPI003F) to 97.5 µL of HDAC Assay Buffer (1:40 dilution) and mix well.

   Add 0, 2, 4, 6, 8 and 10 µL of the diluted Standard into a series of wells to generate 0, 200,400, 600, 800 and 1000 pmol/well of Deacetylated Substrate Standard in HDAC Assay Buffer to give a final volume of 100 µL.
   The Standard Curve is linear up to 5000 pmol/well of Deacetylated Substrate Standard.

6. Developer
   Mix enough reagents for the number of assays to be performed.

a. For each well, prepare 100 µL Developer Mix containing:

	Developer Mix
Developer	10 µL
HDAC Assay Buffer	90 µL

b. Once 1-3 hour incubation is complete, add 100 µL Developer Mix to each well containing Standard, test samples & controls.
c. Incubate for 30 minutes at 37°C to bring cleavage to completion

7. Measurement
   Read fluorescence at Ex/Em = 368/442 nm.in a black plate with clear bottom

Results

Calculations

Subtract the 0 Standard (background) reading from all Standard readings. Plot the Standard Curve. (Standard vs background adjusted reading). Correct sample background by subtracting the value derived from the background control from all sample readings. Apply the corrected sample reading to the Standard curve to get B pmol of Deacetylated Substrate in the sample wells.

Sample HDAC activity = B/T = pmol/min = mU

Where: B is the deacetylated substrate amount (pmol) from Standard Curve

T is the reaction time (min)

Unit Definition: One unit of HDAC is the amount of enzyme that generates one nanomole (1000 pmol) of deacetylated substrate/min at 37°C.

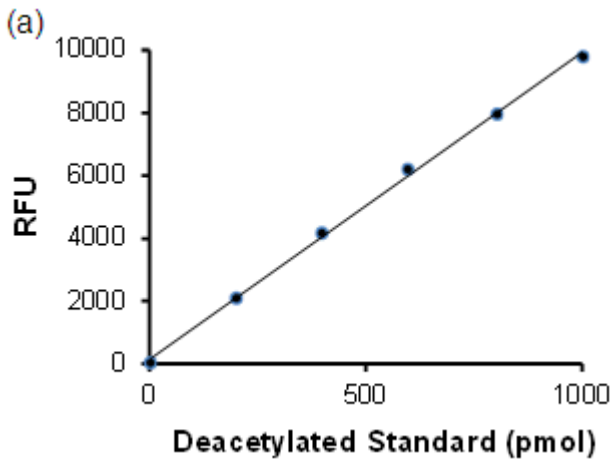

Figure 1 : Deacetylated Substrate Standard Curve

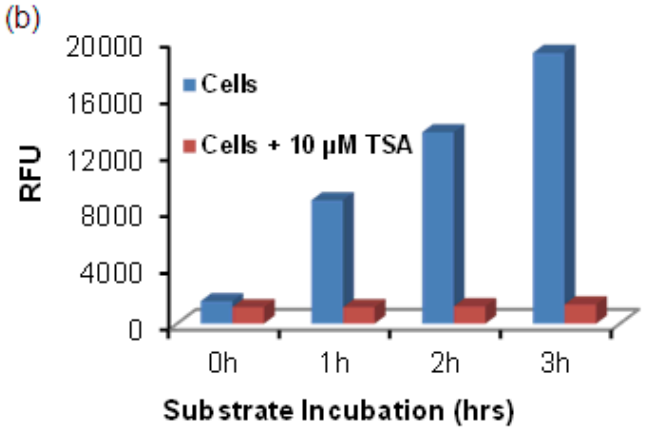

Figure 2 : Deacetylated Substrate Standard Curve Assays were performed following kit protocol

References

1. N. Reynolds, A. O'Shaughnessy and B. Hendrich (2013) Transcriptional repressors: multifaceted regulators of gene expression; Development;140(3):505-12.
2. K. Ververis, A. Hiong, T.C. Karagiannis, and P.V. Licciardi (2013), Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents; Biologics 7: 47–60

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