

# Instruction Manual for HDAC Assay Kit (Colorimetric Detection)

Catalog # 17-374

Sufficient reagents for 96 assays per kit.

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DO NOT USE IN HUMANS.**

## I. STORAGE AND STABILITY

**Storage:** Upon receipt, store individual components at recommended temperatures. Store the half volume 96 well plate at room temperature. Store all other components at -20°C.

**Stability:** Components stable for 6 months from date of shipment if stored and handled correctly.

## II. ASSAY OVERVIEW

### Introduction

In the eukaryotic nucleus, the genome is organized into chromatin. Two molecules each of the core histones (H2A, H2B, H3 and H4) comprise a single histone octamer, around which 146 base pairs of DNA are wrapped, forming the nucleosome, the basic subunit of chromatin (1). Nucleosomes are packaged into successively higher order structures, allowing for the compaction and storage of genetic material. The post-translational modifications of histones are of great importance to their function as governors of genome access. They can have a direct impact by altering local chromatin architecture, or work indirectly through the recruitment of trans-acting factors that recognize specific histone modifications (referred to as the “histone code” hypothesis, see ref. 2)

### Histone Acetylation / Deacetylation and Gene Expression

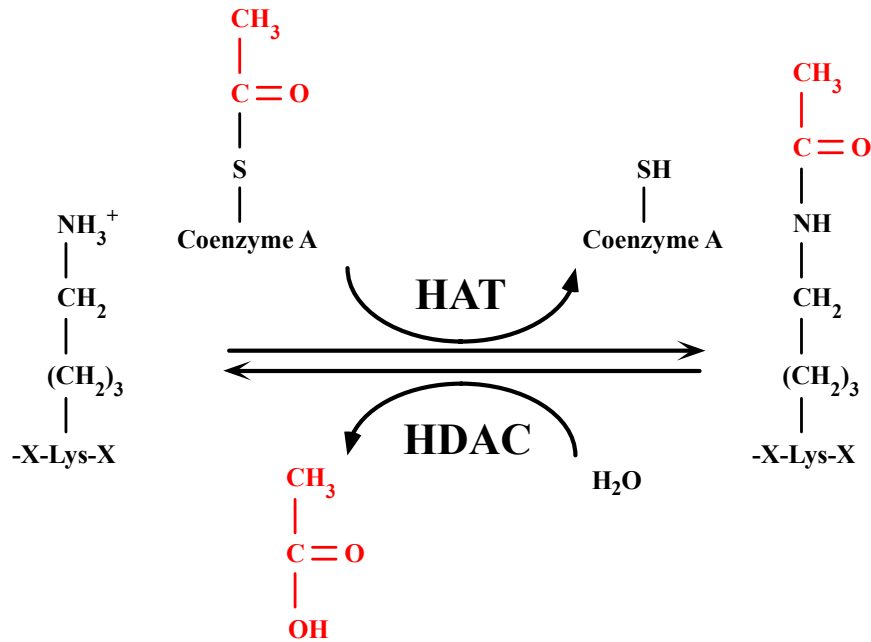
One of the best understood modifications of histones is acetylation. The epsilon amino group of lysine residues in histones is modified by the addition of an acetyl group (Figure 1), a reaction catalyzed by a class of enzymes known as histone acetyltransferases. The reverse reaction, the removal of the acetyl group, is carried out by histone deacetylases (HDACs). The long standing correlation between acetylation and transcription (3) has been nearly solidified by the identification of HAT enzymes that function in concert with protein complexes to activate transcription (reviewed in refs. 4 and 5). Conversely, the repression of transcription involves the recruitment of HDAC enzymes to specific genes (reviewed in ref. 6). Acetylation is thought to exert its influence on transcription by loosening histone-DNA contacts (7, reviewed in ref. 8). Acetylated histones also serve to recruit transcriptional activators containing an acetyl-lysine binding module, the bromodomain (9).

### HDAC Function in the Cell

Since the cloning of the first HDAC gene (10), three distinct families of HDACs have been described, comprising a group of at least 20 proteins in humans (table 1, reviewed in ref 11). The Class I HDACs are most similar in amino acid sequence to the yeast Rpd3 protein. The Class II HDACs, despite sharing sequence similarities to the Class I proteins, are grouped together based on their homology to the yeast Hda1 protein. The Class III HDACs are distinctly different in sequence and function to the HDACs in classes I and II, as they require the cofactor nicotinamide-adenine dinucleotide (NAD) for enzymatic activity. The founding member of Class III is the yeast Sir2 protein. Class III HDACs are not at all sensitive to the chemical inhibitors (e.g. butyrate, trichostatin, trapoxin) that have been used to study the function of the Class I and Class II proteins. The human Class III enzymes are also collectively referred to as “sirtuins”. HDAC proteins are not restricted to the nuclear compartment, and indeed several non-histone substrates have been identified for both NAD-dependent and independent enzymes (12, 13).

Of profound importance is the involvement of HDACs in aging and a variety of human cancers. Many tumor suppressor genes are silenced by a mechanism involving DNA methylation and histone deacetylation, contributing to many types of cancer (reviewed in ref. 14). HDAC inhibitors currently show tremendous early promise as agents in the treatment of these diseases (11).

**Figure 1:** Schematic overview of histone acetylation and deacetylation. The class III HDACs require the cofactor NAD to achieve deacetylation.



**Table 1:** List of identified human HDAC proteins.

| <b>CLASS I</b> | <b>CLASS II</b> | <b>CLASS III</b> |
|----------------|-----------------|------------------|
| (Rpd3 related) | (Hda1 related)  | (Sir2 related)   |
| HDAC1          | HDAC4           | SIRT1            |
| HDAC2          | HDAC5           | SIRT2            |
| HDAC3          | HDAC6           | SIRT3            |
| HDAC8          | HDAC7           | SIRT4            |
| HDAC11         | HDAC9a & 9b     | SIRT5            |
|                | HDRP / MITR     | SIRT6            |
|                | HDAC10          | SIRT7            |

This assay is a simple two-step homogeneous procedure performed in a microtiter plate which can be completed in as little as 90 min. In the first step, samples are incubated with the HDAC Assay substrate, allowing deacetylation of the colorimetric substrate. Subsequent addition of the Activator Solution selectively releases the colorimetric molecule from the deacetylated substrate.

Included in this kit are all reagents needed for this procedure. In addition, Trichostatin A, an inhibitor of HDAC classes I and II, and an unacetylated HDAC assay standard are also included.

### III. SYSTEM COMPONENTS

#### A. Provided Kit Components

**Half volume clear plate**

One half volume 96 well clear plate.

**HDAC Assay Buffer, 10X**

Catalog # 20-311

One vial containing **1ml** of 250mM Tris pH 8.0, 1.37M NaCl, 27mM KCl, 10mM MgCl<sub>2</sub>.

**Activator Solution**

Catalog # 20-268

One vial containing **200µl** of activator solution, **20X stock**. Aliquot upon receipt as needed to avoid future freeze-thaw cycles.

**HDAC Assay Substrate, Colorimetric Detection**

Catalog # 12-561

One vial containing **100µl** of 40mM substrate in DMSO.

**HDAC Assay Standard, Colorimetric Detection**

Catalog # 12-562

One vial containing **50µl** of 20mM unacetylated standard in DMSO.

**Trichostatin A**

Catalog # 20-269

One vial containing **100µl** of 200µM Trichostatin A in DMSO.

**HeLa Nuclear Extract**

Catalog # 12-309

Two vials, each vial containing **50µg** in 25µl of nuclear extract prepared from human HeLa cells in RIPA buffer. Aliquot upon receipt as needed to avoid future freeze-thaw cycles.

#### B. Required Materials Not Provided

- Enzyme preparation or cell extract containing active HDAC(s)
- Ice bucket
- Timer
- Variable volume (5-200µl) pipet + tips
- Variable volume (5-200µl) multichannel pipet + tips
- Reagent troughs for multichannel pipettes
- Microplate incubator
- 96-well plate reader capable of measuring p-nitroanilide absorbance at 405nm

## IV. HDAC ASSAY (Colorimetric Detection) PROCEDURE

**Safety Warnings and Precautions:** The HDAC Assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

### A. General Notes

1. The source of HDAC activity may be an extract, purified enzyme or immunoprecipitated complex.
2. We recommend performing the following experimental controls:
  - A 'no enzyme' negative control.
  - A positive control, using HeLa Nuclear Extract (Catalog # 12-309) provided as a source of HDAC activity.
  - A control or test sample treated with an HDAC inhibitor. Trichostatin A (Catalog # 20-269) is provided as an inhibitor for the Class I and Class II HDACs. The Class III HDACs are not sensitive to Trichostatin A, but the Class III inhibitor nicotinamide is available separately (Catalog # 19-171). The Class III HDACs require NAD<sup>+</sup> as a cofactor, which is also available separately (Catalog # 20-221).
3. The HDAC activity in the HeLa Nuclear Extract is stopped with 1 $\mu$ M Trichostatin.
4. Compounds identified as potential inhibitors of HDAC activity should be confirmed by incubating the potential inhibitor with the unacetylated HDAC Assay Standard, Colorimetric Detection (Catalog # 12-562) and then proceeding on to the substrate activation step. This will allow discrimination between HDAC inhibition versus interference with the activator step.
5. Thaw the HeLa Nuclear Extract (Catalog # 12-309) immediately before use to maintain HDAC activity. To avoid future freeze-thaw cycles, aliquot the extract upon initial thawing. The end user should determine the optimum incubation time and assay temperature for their source of HDAC activity.
6. The undiluted Activator Solution (Catalog # 20-268) is stable on ice for up to one hour. Dilution of the Activator Solution should be done immediately prior to the activation step. Storing or refreezing the diluted Activator Solution is not recommended.
7. It is strongly recommended that a standard curve be generated prior to performing the assay, using the HDAC Assay Standard (Catalog # 12-562). This will help ensure that the assay is within the linear range and that conditions are optimized for the plate reader being used. See Section E (page 8) for a detailed Standard Curve procedure.

## B. Preparation of Assay Solutions

Prepare sufficient volume of each solution based on the number of assays to be performed, plus a slight overage to account for pipetting inaccuracies (either 10% extra or one extra assay point is generally sufficient).

### Reaction Component Preparation

1. Rapidly thaw the **HDAC Assay Substrate, Colorimetric Detection** (Catalog # 12-561), **HDAC Assay Standard, Colorimetric Detection** (Catalog # 12-562), **HDAC Assay Buffer, 10X** (Catalog # 20-311) and **Trichostatin A** (Catalog # 20-269) to room temperature.
2. **2X HDAC Assay Buffer:** Prepare the 2X HDAC Assay Buffer by diluting the HDAC Assay Buffer, 10X (Catalog # 20-311) 5-fold with water (e.g., for 2ml of 2X HDAC Assay Buffer combine 1600 $\mu$ l water and 400 $\mu$ l HDAC Assay Buffer, 10X). Store on ice.
3. **4mM HDAC Assay Substrate:** Prepare the 4mM HDAC Assay Substrate by diluting the 40mM HDAC Assay Substrate, Colorimetric Detection (Catalog # 12-561) 10-fold with 2X HDAC Assay Buffer (e.g., for 1ml of 4mM HDAC Assay Substrate combine 900 $\mu$ l of 2X HDAC Assay Buffer and 100 $\mu$ l 40mM HDAC Assay Substrate, Colorimetric Detection). Each assay point requires 10 $\mu$ l. Store on ice.
4. **2X HDAC Assay Buffer plus 4 $\mu$ M Trichostatin A (used for assay points requiring an inhibitor and used to prepare Activation Solution):** Prepare the 4 $\mu$ M Trichostatin A by diluting the 200 $\mu$ M Trichostatin A (Catalog # 20-269) 50-fold using 2X HDAC Assay Buffer. Prepare a sufficient volume of 2X HDAC Assay Buffer plus 4 $\mu$ M Trichostatin A for each assay point requiring an inhibitor; prepare an additional volume for diluting the Activator Solution (Step 5). Prepare 10 $\mu$ l for each assay point requiring an inhibitor and 20 $\mu$ l for each assay point requiring activation solution. Store on ice.
5. **HeLa Nuclear Extract (positive control):** Immediately prior to use, add 25 $\mu$ l of water to one vial of HeLa Nuclear extract (Catalog # 20-269). Each positive control assay point requires 20 $\mu$ l. Store on ice.
6. **Test Sample:** Prepare a sufficient volume of test sample for the number of assay points. Each assay point requires 20 $\mu$ l. If the test sample is less than 20 $\mu$ l, add 1X HDAC Assay Buffer or water to bring the volume to 20 $\mu$ l. Store on ice.
7. **Diluted Activator Solution:** During Step 6 of the assay protocol (Section C), prepare the Diluted Activator Solution by diluting the Activator Solution (Catalog # 20-268) 20-fold **immediately prior to use** in ice cold 2X HDAC Assay Buffer containing 4 $\mu$ M Trichostatin A (e.g., for 2ml of 1X Activator Solution combine 1900 $\mu$ l 2X HDAC Assay Buffer plus 4 $\mu$ M Trichostatin A and 100 $\mu$ l Activator Solution). Each assay (including the standard curve and the negative controls) will require 20 $\mu$ l of Diluted Activator Solution. Store on ice.

## C. Protocol

This assay is a simple two-step procedure performed in a microtiter plate. In the first step, samples are incubated with the HDAC assay substrate, allowing deacetylation of the substrate. Next, the Activator Solution releases p-nitroanilide from the deacetylated substrate or standard.

1. Prepare the Assay Solutions (see Section IV-B).
2. Pipet 10 $\mu$ l of 2X HDAC Assay Buffer, or 2X HDAC Assay Buffer containing 4 $\mu$ M Trichostatin A into each well.
3. Add 20 $\mu$ l of test sample, or 20 $\mu$ l of HeLa Nuclear Extract (positive control), or 20 $\mu$ l of water (negative control).
4. Allow the wells to equilibrate to assay temperature (37°C).
5. Add 10 $\mu$ l of the 4mM HDAC Assay Substrate and mix thoroughly.
6. Incubate the microtiter plate at 37°C for 60 minutes. The optimum incubation time (30-90 minutes) and temperature (30°C or 37°C) should be determined by the end user.
7. Add 20 $\mu$ l of the **Diluted** Activator Solution (see step 7 of “Preparation of Assay solutions”, Section IV-B) to each well; mix thoroughly.
8. Incubate the microtiter plate at room temperature for 10-20 minutes.
9. Read the absorbance in a plate reader at 405nm.

**Table 2: Assay Incubation Setup**

|   | <b>Negative Control</b> | <b>Positive Control or Test Sample</b> | <b>Positive Control containing Trichostatin A</b> |
|---|-------------------------|--|---|
| <b>2X HDAC Assay Buffer</b>                                     | 10 $\mu$ l              | 10 $\mu$ l                             | 0 $\mu$ l   |
| <b>HDAC Assay Buffer Plus 4<math>\mu</math>M Trichostatin A</b> | 0 $\mu$ l               | 0 $\mu$ l                              | 10 $\mu$ l  |
| <b>HeLa Nuclear Extract or test sample(s)</b>                   | 0 $\mu$ l               | 20 $\mu$ l                             | 20 $\mu$ l  |
| <b>4mM HDAC Assay Substrate</b>                                 | 10 $\mu$ l              | 10 $\mu$ l                             | 10 $\mu$ l  |
| <b>Water</b>  | 20 $\mu$ l              | 0 $\mu$ l                              | 0 $\mu$ l   |
| <b>Total Assay Volume</b>                                       | 40 $\mu$ l              | 40 $\mu$ l                             | 40 $\mu$ l  |

## D. Standard Curve

A standard curve should be generated for each plate reader used to ensure that the assay is within the linear range. Typically, a 1mM-18.75 $\mu$ M standard curve (using Catalog # 12-562) has been used in testing of this kit.

*Generate a standard curve (0-1mM) using the following procedure. We recommend performing the standard curve in duplicate.*

1. Prepare 1X HDAC Assay Buffer by diluting HDAC Assay Buffer, 10X 10 fold (e.g., for 1ml of 1X HDAC Assay Buffer combine 900 $\mu$ l water and 100 $\mu$ l HDAC Assay Buffer, 10X).
2. Pipet 40 $\mu$ l of 1X HDAC Assay Buffer in all wells required for the standard curve except the first wells.
3. Dilute the HDAC Assay Standard, Colorimetric Detection (Catalog # 12-562) to 1mM by adding 10 $\mu$ l of HDAC Assay Standard, Colorimetric Detection to 190 $\mu$ l of 1X HDAC Assay Buffer. Pipet 80 $\mu$ l of the diluted HDAC Assay Standard to each of two empty wells.
4. Transfer 40 $\mu$ l of the 1mM HDAC Assay Standard to the next well containing 40 $\mu$ l of 1X HDAC Assay Buffer, mixing thoroughly before the next transfer. Repeat this process to make successive 2-fold dilutions. Discard 40 $\mu$ l from the final dilution wells to keep a consistent volume of 40 $\mu$ l in each well. Use 40 $\mu$ l of 1X HDAC Assay Buffer in the last wells for the zero standard.
5. The standards may be incubated with the test samples.
6. Add 20 $\mu$ l of the **diluted** Activator Solution (see Section IV-B., Step 7) to each well. Mix thoroughly.
7. Incubate the microtiter plate at room temperature for 10-20 minutes.
8. Read the absorbance in a plate reader at 405nm.

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