CREATIVE BIOMART®
Assay Kit

HDAC colorimetric activity assay Kit

Product Information

Common Name

HDAC

Cat.No.

Kit-0413

Product Overview

The HDAC Colorimetric Assay/Drug Discovery Kit is a complete assay system designed to measure histone deacetylase (HDAC) activity in cell or nuclear extracts, immunoprecipitates or purified enzymes. It comes in a convenient 96-well format, with all reagents necessary for Colorimetric HDAC activity measurements and calibration of the assay. In addition, a HeLa nuclear extract, rich in HDAC activity, is included with the kit. The extract is useful as either a positive control or as the source of HDAC activity for inhibitor/drug screening. Also included is the potent HDAC inhibitor, Trichostatin A, which may be used as model inhibitor.
The HDAC Colorimetric Assay/Drug Discovery Kit is based on the unique Substrate and Developer combination. The system (Colorimetric Histone deAcetylase Lysyl Substrate/Developer) is a highly sensitive and convenient alternative to radiolabeled, acetylated histones or peptide/HPLC methods for the assay of histone deacetylases. The assay procedure has two steps. First, the Substrate, which comprises an acetylated lysine side chain, is incubated with a sample containing HDAC activity (HeLa nuclear or other extract, purified enzyme, bead-bound immunocomplex, etc.). Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the Developer causes an increase in yellow intensity and absorption at 405nm. There is a linear correlation between the absorption and the amount of dye released within instrument limitations.

Description

Histones form the protein core of nucleosomes, the DNA/protein complexes that are the subunits of eukaryotic chromatin. The histones' N-terminal "tails" are subject to a variety of posttranslational modifications, including phosphorylation, methylation, ubiquitination, ADP-ribosylation and acetylation. These modifications have been proposed to constitute a 'histone code' with profound regulatory functions in gene transcription1. The best studied of these modifications, acetylation of

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the ε-aminos of specific histone lysine residues, are catalyzed by histone acetyltransferases (HATs). Histone deacetylases (HDACs) are responsible for hydrolytic removal of these acetyl groups.

Applications

Colorimetric detection, HTS

Stability

Store all components except the microtiter plate at -70°C for the highest stability. The HeLa Nuclear Extract must be handled with particular care in order to retain maximum enzymatic activity. Defrost it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused extract should be refrozen quickly, by placing at -70°C. If possible, snap freeze in liquid nitrogen or a dry ice/ethanol bath. To minimize the number of freeze/thaw cycles, aliquot the extract into separate tubes and store at -70°C. The Substrate, when diluted in Assay Buffer, may precipitate after freezing and thawing. It is best, therefore, to dilute only the amount needed to perform the assays of that day.

Storage

-80°C

Size

96 wells

Kit Components

1. Nuclear Extract from HeLa Cells (human cervical cancer cell line)
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 500µl
2. Substrate
FORM: 50mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 50µl
3. Developer Concentrate (20x)
FORM: 20x Stock Solution; Dilute in HDAC Assay Buffer before use.
STORAGE: -70°C

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QUANTITY: 300µl
4. Trichostatin A (HDAC Inhibitor)
FORM: 0.2mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 100µl
5. Deacetylated Standard
FORM: 10mM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
QUANTITY: 30µl
6. HDAC ASSAY BUFFER (50mM Tris/Cl, pH 8.0, 137mM NaCl, 2.7mM KCl, 1mM MgCl2)
STORAGE: -70°C
QUANTITY: 20ml
7. 1/2 VOLUME MICROPLATE
1 clear, 96-well
STORAGE: Room temperature

Materials Required but Not Supplied

1. Microtiter-plate reader capable of measuring A405 to >3- decimal accuracy
2. Pipetman or multi-channel pipetman capable of pipetting 2- 100µl accurately
3. Ice bucket to keep reagents cold until use.
4. Microtiter plate warmer or other temperature control device (optional)

Preparation

1. Defrost all kit components and keep these, and all dilutions described below, on ice until use. All undiluted kit components are stable for several hours on ice.
2. Prepare a sufficient amount of HeLa Nuclear Extract or other HDAC source to provide for the assays to be performed (# of wells x 5µl). 5µl of undiluted Extract is needed for the standard assay. If the undiluted HDAC source generates excessive signal, dilute it with HDAC Assay Buffer and reassay.
3. Prepare dilution(s) of Trichostatin A and/or Test Inhibitors in Assay Buffer. Since 10µl will be used per well, and since the final volume of the HDAC reaction is 50µl, these inhibitor dilutions will be 5x their

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final concentration.
4. Prepare dilution(s) of the Substrate (50mM) in Assay Buffer that will be 2x the desired final concentration(s). For inhibitor screening, substrate concentrations at or below the Km are recommended. Twentyfiveμl will be used per well. Initial dilutions of the 50mM stock to 2.5mM or less in Assay Buffer yield stable solutions. Again, note that freezing/thawing of Substrate solutions in Assay Buffer may cause precipitation of the Substrate.
5. Shortly before use (<30 min.), prepare sufficient Developer for the assays to be performed (50μl per well). First, dilute the Developer Concentrate 20-fold (e.g. 50μl plus 950μl Assay Buffer) in cold Assay Buffer. Second, dilute the 0.2mM Trichostatin A 100-fold in the 1x Developer just prepared (e.g. 10μl in 1ml; final Trichostatin A concentration in the 1x Developer = 2μM; final concentration after addition to HDAC/Substrate reaction = 1μM). Addition of Trichostatin A to the Developer insures that HDAC activity stops when the Developer is added. Keep Developer on ice until use.
Sample Assay Buffer HeLa Extract Inhibitor(5x) Substrate(2x)
Blank 25μl 0 0 25μl
Control 20μl 5μl 0 25μl
Trichostatin A* 10μl 5μl 10μl* 25μl
Test Sample** 10μl 5μl 10μl** 25μl
6. HDAC reaction mixtures, prior to addition of Developer.
* Refers to dilution of trichostatin A in Assay Buffer, which will be 5x the final concentration. Examples: 1) As a measure of non-HDAC background, 5μM would produce final 1μM concentration and essentially complete HDAC inhibition; 2) As a model inhibitor "hit", 250nM would produce final 50nM and ~50% inhibition.
** Refers to dilution of potential inhibitor in Assay Buffer, which will be 5x its final concentration.

Assay Protocol

1. Add Assay buffer, diluted trichostatin A or test inhibitor to appropriate wells of the microtiter plate. Table 1 lists examples of various assay types and the additions required for each.
2. Add HeLa extract or other HDAC sample to all wells except those that are to be "No Enzyme Controls".
3. Allow diluted Substrate and the samples in the microtiter plate to equilibrate to assay temperature (e.g. 37°C).

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4. Initiate HDAC reactions by adding diluted substrate (25µl) to each well and mixing thoroughly.
5. Allow HDAC reactions to proceed for desired length of time and then stop them by addition of Developer (50µl). Incubate plate at 37°C for 10-15 min. Signal is stable for at least 30 min. beyond this time.
6. Read plate in a microtiter-plate reader at 405nm.

Uses Of The Deacetylated Standard

Preparation of a Standard Curve:

1. Standard curves prepared with the Deacetylated Standard can be used to correlate changes in OD405nm with molar amounts of deacetylation. Concentration ranges of 0-250µM or 0-500µM will both produce good results. A single standard curve will suffice; there is no need to do a standard curve each time assays are done. However, it can be useful to include a single well of Deacetylated Standard (e.g. at a concentration of 50µM) as a positive control, each time an assay is performed. (See also the next section on testing for interference with the Developer.)
2. After choosing a concentration range, prepare, in Assay Buffer, a series of Deacetylated Standard dilutions that span this range. Pipet 50µl of each of these dilutions, and 50µl of Assay Buffer as a 'zero', to a set of wells on the microtiter plate.
3. Prepare, as described in "Preparing Reagents For Assay", sufficient Developer for the standard wells (50µl per well).
4. Mix 50µl of the Developer with the 50µl in each standard well and incubate 5-10 min. at 37°C.
5. Read samples in a microtiter-plate reader at 405nm.
6. Plot absorbance (y-axis) versus concentration of the Deacetylated Standard (x-axis). Determine slope as OD/µM.

Testing of Potential HDAC Inhibitors for Interference with the Developer:

1. The Developer is formulated so that, under normal circumstances, the reaction goes to completion in less than 1 min. at 37°C. That, together with the recommended 10-15 min. reaction time, should help insure that in most cases, even when some retardation of the development reaction occurs, the signal will fully develop prior to the reading of the plate.
2. It should be possible to identify many cases in which there is interference with the development reaction by taking a series of absorbance readings immediately following addition of the Developer (e.g. readings at 1 or 2 min. intervals for 30 min.). The absorbance of control samples (no inhibitor)

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will change very little after the first or second reading. Samples containing compounds which inhibit HDACs, but which do not interfere with the Developer, will display similarly rapid kinetics, although a lower final absorbance. Trichostatin A (5nM) provides a good model of this behavior. Any sample in which the approach to the final absorbance is substantially slower than in the above examples should be suspected of interference with the development reaction. For samples in which little or no color development has occurred, it may be impossible to assess the development kinetics.

3. Absolute certainty regarding interference with the Developer can only be obtained through an assay in which the compound in question is tested for its effect on the reaction of Deacetylated Standard with the Developer. Using a standard curve such as that described in the previous section, determine the concentration of Deacetylated Standard that will yield a signal similar to that produced after development of a control (no inhibitor) HDAC reaction. Mix 40µl of the diluted Standard with 10µl inhibitor or 10µl Assay Buffer. Initiate development by adding 50µl of 1x Developer to each well. Follow color development by reading at 1 or 2 min. intervals for 30 min. If a test inhibitor sample reaches its final yellow color intensity more slowly than the control or if the final value is significantly below that of the control, then there is interference with the Developer reaction.

4. Once it is determined that a particular substance does interfere with the Developer reaction, it may be possible to adjust reaction conditions to eliminate this effect. In cases where the same final yellow color intensity is achieved, but more slowly than the control (e.g. 25 min. rather than 1 min.), simply extending the incubation time after addition of the Developer would be sufficient. Other possible adjustments include increasing the volume of Developer used per well (e.g. to 100µl) and diluting the Developer Concentrate 10-fold, rather 20-fold. All three of these approaches may be used separately or in combination.

TABLE 2. ASSAY MIXTURES FOR INHIBITOR RETESTING WITH DEACETYLATED STANDARD

Sample Assay Buffer Inhibitor (5x) Diluted* Standard (1.25x) DEVELOPER (1x).

Control 10µl 0 40µl 50µl

Tricho-statin A** 0 10µl 40µl** 50µl

Test Inhibitor& 0 10µl 40µl& 50µl

* The appropriate dilution of the Deacetylated Standard, may be determined from the standard curve and should be the concentration producing a Colorimetric signal equal to that produced by

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control (no inhibitor) samples in the HDAC assay. The dilution in Assay Buffer is prepared at 1.25x this concentration to compensate for the 4/5 dilution due to addition of 10µl of Assay Buffer or inhibitor.
** Refers to dilution of trichostatin A in Assay Buffer, which will be 5x its final concentration in the 50µl volume, prior to addition of Developer. Example: As a model inhibitor that does not interfere with the Developer, 25nm trichostatin A would produce a final 5nm concentration.
& Refers to dilution of potential inhibitor in Assay Buffer, which will be 5x its final concentration in the 50µl volume, prior to addition of Developer.

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