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Product Information

HDAC3 Immunoprecipitation (IP) and Activity Assay Kit

Catalog Number **EPI013**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Histone deacetylases (HDACs) play a central role in controlling cell cycle regulation, cell differentiation, and tissue development. These proteins have crucial roles in development and physiology. They are also deeply involved in cellular proliferation, cell cycle, and apoptosis. HDAC3 is primarily localized in the nucleus, but can also be found in the cytoplasm and at the plasma membrane.

The HDAC3 Immunoprecipitation (IP) and Activity Assay Kit provides an antibody-based method to specifically immunoprecipitate the HDAC3 complex from cells and tissues, and to measure HDAC3 activity fluorometrically. HDAC3 is immunoprecipitated from cell, nuclear, or tissue extract(s) using a HDAC3 specific antibody followed by capturing the complex on protein-A/G beads. The immunoprecipitated complex reacts with the HDAC substrate. Only the deacetylated substrate is cleaved by the Developer to produce a fluorophore, which can be easily analyzed using a fluorescence plate reader. The kit is suitable for measurement of HDAC3 activity of immunoprecipitated complex or purified enzyme from human, mouse, or rat samples.

Components
The kit is sufficient for 25 assays in 96 well plates.

HDAC Assay Buffer (Wide Mouth Bottle) Catalog Number EPI013A	9 mL
Extraction Buffer (Narrow Mouth Bottle) Catalog Number EPI013B	100 mL
HDAC Substrate (Amber Cap) Catalog Number EPI013C	100 µL
Developer (Orange Cap) Catalog Number EPI013D	500 µL
AMC Standard (1 mM) (Yellow Cap) Catalog Number EPI013E	100 µL
Rabbit HDAC3 Antibody (Red Cap) Catalog Number EPI013F	500 µL
Rabbit IgG (Control Antibody) (Green Cap) Catalog Number EPI013G	250 µL
Protein-A/G Sepharose® Beads (Blue Cap) Catalog Number EPI013H	650 µL
Positive Control (Jurkat Cell Lysate) (Violet Cap) Catalog Number EPI013I	1 vl

Reagents and equipment required but not provided.
- 96 well black plates for fluorometric assays.
- Fluorescence plate reader
- Rotary mixer
- Phosphate Buffered Saline (Catalog Number P5368 or equivalent)
- Protease Inhibitor Cocktail (Catalog Number P8340 or equivalent)
- CelLytic™ NuCLEAR™ Extraction Kit (Catalog Number NXTRACT or equivalent)
- Dounce homogenizer (Catalog Number D8938 or equivalent)

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

Storage/Stability
The kit is shipped on wet ice. Store the kit at –20 °C, protected from light. However, some components have different storage temperature once reconstituted. See Preparation Instructions for storage conditions of individual components.

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 – Store at –20 °C or 2–8 °C. Briefly warm to 37 °C before use.

Extraction Buffer – Thaw Extraction Buffer and add protease inhibitors as per manufacturer's instructions. Make fresh as needed and keep on ice while in use.

HDAC Substrate – Store at –20 °C.

Developer – Aliquot 250 µL into tubes and store at –20 °C. Keep on ice while in use. Use within 2 months.

AFC (7-amino-4-trifluoromethyl coumarin) Standard – Store at –20 °C.

Protein-A/G Sepharose Beads (50% slurry in 20% ethanol aqueous solution) – Store at 2–8 °C after thawing. Do not freeze.

Positive Control – Reconstitute with 25 µL of water. Mix gently by pipetting. Aliquot and store at –70 °C. Use within 2 months.

Phosphate Buffered Saline (PBS) – Chill PBS before use. Add protease inhibitors as per manufacturer's instructions. Make fresh as needed and keep on ice while in use.

Procedure

Sample Preparation

1. Cell Lysate
   a. Grow cells in 6 or 12 well plates, treat as desired.
   b. For adherent cells, remove medium and wash cells with PBS.
   c. For suspension cells, collect cells by centrifugation, wash cells with PBS at room temperature, and collect cells again by centrifugation.
   d. Remove the PBS, place the plate on ice, and add cold Extraction Buffer containing protease inhibitors. Add 125 µL/well (12 well plate) or 250 µL/well (6 well plate).
   e. Keep on ice for one minute.
   f. Scrape the cells and gently transfer the disrupted cell suspension into a cooled microcentrifuge tube.
   g. Mix on a rotary mixer at 2–8 °C for 30 minutes.
   h. Centrifuge at 10,000 × *g* for 10 minutes at 2–8 °C, then discard cell debris pellet.

2. Nuclear Extract
   Prepare nuclear extracts from 2×10^5 to 2×10^6 cells using CelLytic NuCLEAR Extraction Kit (Catalog Number NXTRACT) or other equivalent method.

3. Tissue Extract
   a. Use fresh or frozen (stored at –70 °C) tissue to prepare the tissue extract.
   b. Rinse tissue and transfer 25–50 mg of tissue to a chilled dounce homogenizer.
   c. For every 25 mg of tissue, add 500 µL of cold Extraction Buffer containing protease inhibitors and homogenize the tissue on ice with 10–15 strokes.
   d. Transfer the contents to a microfuge tube and add 500 µL of cold Extraction Buffer containing protease inhibitors to the same tube.
   e. Keep the tube on a rotary shaker at 2–8 °C for 30–60 minutes.
   f. Pass the lysate 3 times through a 25 gauge needle.
   g. Centrifuge at 10,000 × *g* for 10 minutes at 2–8 °C.
   h. Transfer the supernatant to a fresh tube.

   Note: Repeated freeze-thaw of sample will cause loss in HDAC activity.

4. Protein Estimation
   Determine the protein concentration using the Bradford Assay.

Immunoprecipitation

1. Antibody Binding
   a. Cell lysates and nuclear extracts (CL/NE) are in the linear range for HDAC activity when 50–100 μg of protein is used. Use 0.5–1 mg of tissue extract for the IP reaction. If samples have high HDAC levels, the amount used needs to be standardized.
   b. For each IP reaction (sample or control), add 50–100 μg CL/NE to a chilled tube on ice.
   c. Add 20 μL of HDAC3 Antibody to samples. Add 20 μL of Control Antibody to samples to prepare background controls.
   d. Bring the volume to 500 μL with PBS containing protease inhibitor.
   e. Incubate overnight at 2–8 °C on a rotary mixer.

2. Preparation of Protein-A/G Beads:
   a. Use wide bore pipette tips when pipetting beads.
   b. Wash the protein-A/G beads (25 μL of 50% slurry per reaction) 2 times with 1 mL of PBS, by centrifuging at 14,000 × *g* for 10 seconds and aspirating the supernatant between washes.
   c. Suspend as 50% slurry in PBS.

3. Bead Capture:
   a. After overnight incubation of the sample/ antibody mixture (step 1e), add 25 μL of the protein-A/G bead slurry (step 2c) to each tube and incubate for an hour at 2–8 °C.
   b. Collect the beads by centrifugation at 14,000 × *g* for 10 seconds at 2–8 °C.
   c. Wash beads 3 times with 1 mL of PBS, by centrifuging at 14,000 × *g* for 10 seconds and aspirating the supernatant between washes.
   d. Assay HDAC Activity immediately.

HDAC Activity Assay

1. Warm HDAC Assay Buffer to 37 °C prior to use. Mix enough reagents for the number of assays to be performed including sample and background control. For each assay, prepare 168 μL of the Reaction Mix according to the scheme in Table 1.

   Table 1.
   Reaction Mix

Reagent	Volume
HDAC Assay Buffer	164 μL
HDAC Substrate	4 μL
Total volume	168 μL

2. Add 168 μL of Reaction Mix to each sample and background control tube, mix gently, and incubate at 37 °C for two hours.

3. The Positive Control is used to measure total HDAC Activity. In a separate tube add 2–5 μL of Positive Control, 4 μL HDAC Substrate, and adjust the volume to 180 μL with HDAC Assay Buffer. Mix gently and incubate at 37 °C for two hours.

4. Add 20 μL of the Developer to each tube and mix. Incubate for 30 minutes at 37 °C.

5. Preparation of AFC Standards for Standard Curve– Dilute the 1 mM AFC Standard to 10 μM by adding 10 μL of 1 mM AFC Standard to 990 μL of water. Add 0, 10, 20, 30, 40, and 50 μL of diluted 10 μM AFC Standard into individual wells in a 96 well black plate. Adjust the volume to 100 μL/well with HDAC Assay buffer to generate 0, 100, 200, 300, 400, and 500 pmole/well of AFC Standard, respectively. Mix well.

6. Centrifuge the Positive Control, Background Control, and Sample tubes at 14,000 × *g* for 2 minutes at room temperature.

7. Transfer 100 μL of each reaction supernatant to individual wells in a black plate.

8. Read fluorescence at (λ_{ex} = 368/λ_{em} = 442 nm).

Results

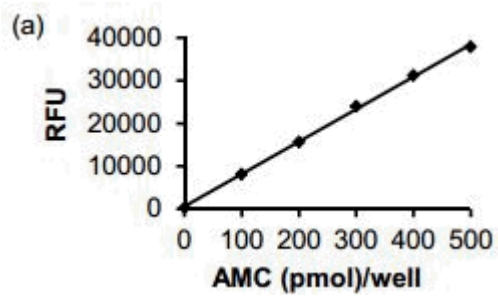
Calculations

Plot the AFC Standard Curve from the fluorescence measurements of the AFC Standards.

Note: A new standard curve must be set up each time the assay is run.

Figure 1.

AFC Standard Curve.



HDAC Activity

Subtract the Background Control reading from all Sample readings. Apply the corrected sample reading to the AFC Standard Curve to obtain B (pmole of AFC in the sample wells).

HDAC Activity = $2 \times B/TS$ = pmole/min/mg = milliunits

where:

B = AFC amount from the Standard Curve (pmole)

2 = Sample dilution factor*

T = Reaction time (120 minutes)

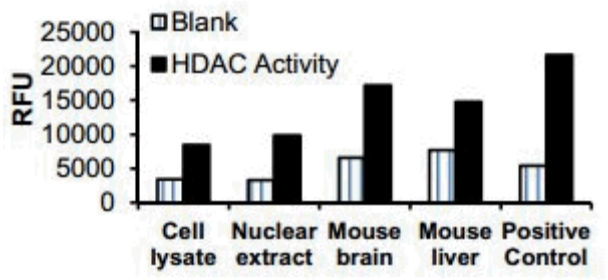
S = Sample amount (mg)

* Reaction Volume (Sample and Background Control) = 200 µL = ~12 µL beads + 168 µL reaction mix + 20 µL Developer. 100 µL of the reaction is used to measure the fluorescence and hence the sample dilution factor is 2.

Unit definition: One unit of HDAC is the amount of enzyme that generates one nanomole of deacetylated substrate/min/mg at 37 °C.

Figure 2.

HDAC3 IP Activity Assay of Jurkat cell lysate and Nuclear Extract, mouse brain and liver extract and HDAC Activity Assay of Positive Control. Assays were performed according to the kit protocol.



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