HDAC2 Immunoprecipitation (IP) & Activity Assay Kit

(Catalog # BN00605; 25 assays; Store at -20°C)

I. Introduction:

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, especially in the heart and nervous system. It is also deeply involved in cellular proliferation, cell cycle and apoptosis. Assay Genie's HDAC2 IP & Activity Assay Kit provides an antibody-based method to specifically immunoprecipitate the HDAC2 complex from cells & tissues and to measure HDAC2 activity fluorometrically. HDAC2 is immunoprecipitated from cell or nuclear extract(s) using HDAC2 antibody followed by capturing the complex on protein-A/G beads. The immunoprecipitated complex is incubated 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.

II. Application:

- Immunoprecipitation of HDAC2 complex from cell, nuclear or tissue extract(s)
- Measurement of HDAC2 activity of immunoprecipitated complex/purified enzyme
- Screening for activators or inhibitors of HDAC2

III. Sample Type: Human, Mouse or Rat

- Cell Lysate and Nuclear Extract
- Tissue Extract

IV. Kit Contents:

Components	BN00605	Cap Code	Part Number
HDAC Assay Buffer	9 ml	WM	BN00605-1
Cell Lysis Buffer	100 ml	NM	BN00605-2
HDAC Substrate	100 µl	Amber	BN00605-3
Developer	500 µl	Orange	BN00605-4
AFC (7-amino-4-trifluoromethyl coumarin) Standard (1 mM)	100 µl	Yellow	BN00605-5
Rabbit HDAC2 Antibody	250 µl	Red	BN00605-6
Rabbit IgG (Control Antibody)	100 µl	Green	BN00605-7
Protein A/G Sepharose Beads (50% slurry in 20% ethanol/H_2O)	650 µl	Blue	BN00605-8
Positive Control (Jurkat Cell Lysate, lyophilized)	1 vial	Violet	BN00605-9

V. User Supplied Reagents and Equipment:

- 96-well white/black opaque plate with flat bottom
- Fluorescence plate reader
- Rotary mixer
- Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.
- Protease Inhibitor Cocktail
- Nuclear/Cytosol Fractionation Kit (BV cat. # BN00534 or equivalent)

VI. Storage and Handling:

Store kit at -20°C, protected from light. Avoid repeated freeze/thaw for all non-buffer components. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the experiment.

VII. Reagent Preparation and Storage Conditions:

- **HDAC Assay Buffer:** Store at -20°C or 4°C. Briefly warm to 37°C before use.
- **Cell Lysis Buffer:** Thaw cell lysis buffer and add protease inhibitors as per manufacturer's instruction. Make fresh as needed and keep on ice while in use.
- **HDAC Substrate:** Store at -20°C. **Note:** Use a fresh pipette tip each time.
- **Developer:** Aliquot 250 µl into tubes and store at -20°C. Keep on ice while in use. Use within 2 months.
- **AFC Standard:** Store at -20°C.
- **Protein-A/G Sepharose Beads:** Store at 4°C once you open the kit. **Do not freeze.**
- **Positive Control:** Reconstitute with 25 µl deionized water. Mix gently by pipetting. Aliquot and store at -80°C. Use within 2 months.
- **Phosphate Buffered Saline (PBS):** Chill PBS before use. Add Protease Inhibitors as per manufacturer's instruction. Make fresh as needed and keep on ice while is use.

VIII. HDAC2 IP & Activity Assay Protocol:

1. **Sample Preparation**:
   a. **Cell Lysate:** Grow cells in 6- or 12- well plates, treat as desired. For adherent cells, remove media and wash cells with PBS. Remove the PBS, place the plate on ice and add cold lysis buffer containing protease inhibitors 125 µl/well (12-well plate) or 250 µl/well (6-well plate). Keep on ice for one minute. Scrape the cells and gently transfer the disrupted cell suspension into a pre-cooled microcentrifuge tube. Mix on a rotary mixer at 4°C for 30 minutes. Centrifuge at 10,000 g for 10 minutes at 4°C; discard cell debris pellet. For Suspension Cells, collect cells by centrifugation, wash cells with PBS at room temperature and collect cells again by centrifugation. Remove the PBS carefully and prepare cell lysates as described above for adherent cells.
   b. **Nuclear Extract:** Prepare nuclear extracts from ~ 2 x 10^5 to 2 x 10^6 cells using Assay Genie's Nuclear/Cytosol Fractionation Kit (catalog # K266) or other equivalent method.
   c. **Tissue Extract:** Snap freeze dissected tissue and immediately grind to a fine powder using a mortar and pestle in a liquid N_2 bath. Transfer the ground tissue to a pre-weighed chilled tube. Weigh the powder (can be stored at this point at -80°C) and add 300 µl Lysis Buffer containing protease inhibitors per 5 mg tissue. Mix on a rocker at 4˚C for about an hour. Pass the lysate through a 25

gauge needle 3X. Collect the lysate and centrifuge at high speed at 4˚C for 5 minutes to remove debris. Transfer the supernatant to a fresh tube.

d. Protein Estimation: Determine the protein concentration using the Bradford Assay.

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

2. Immunoprecipitation

a. Antibody Binding: Cell lysates, Nuclear and Tissue extracts (CL/NE/TE) are in the linear range for HDAC IP-activity when about 50-100 µg protein content is used. If samples have high HDAC levels, you need to standardize the amount used for the IP. For each IP reaction (sample or background control), add 50-100 µg of CL/NE/TE to a pre-chilled tube on ice. Add 10 µl HDAC2 Antibody to samples [S], & 10 µl Control Antibody to the background control(s) [BC]. Bring the volume to 500 µl with PBS containing protease inhibitors. Incubate overnight at 4°C on a rotary mixer.

b. Preparation of Protein-A/G Beads: Use wide bore pipette tips when pipetting beads. Wash the Protein-A/G beads (25 µl of 50% slurry/reaction) 2X with 1ml PBS, centrifuge at 14000 g for ~10 seconds and aspirate the supernatant in between washes. Suspend as 50% slurry in PBS.

c. Bead Capture: After overnight incubation, add 25 µl of protein-A/G beads slurry from step 2b to each tube and incubate for an hour at 4°C. Collect the beads by centrifugation at 14,000 g for ~10 second at 4°C. Wash beads 3X with 1ml PBS, micro-centrifuging at 14,000 g for ~10 seconds and aspirating the supernatant in between washes. Assay HDAC Activity Immediately.

3. HDAC Activity Assay:

a. Reaction Mix Preparation: Warm HDAC Assay Buffer to 37°C prior to use. Mix enough reagents for the number of assays to be performed including samples & background control(s). For each reaction, prepare 168 µl Mix containing:

	Reaction Mix
HDAC Assay Buffer	164 µl
HDAC Substrate	4 µl

Add 168 µl Reaction Mix to each sample & background control tube, mix gently and incubate at 37°C for one hour.

b. Positive Control: In a separate tube add 2-5 µl of positive control, 4 µl HDAC Substrate, and adjust volume to 180 µl with HDAC Assay buffer. Mix gently and incubate at 37°C for one hour. **Note: The positive control is used to measure total HDAC Activity.**

c. Developer: Add 20 µl of the developer to each tube and mix. Incubate for 30 minutes at 37°C.

d. Standard Curve: Dilute the AFC Standard to 10 µM by adding 10 µl of 1 mM AFC Standard to 990 µl of dH_2O. Add 0, 20, 40, 60, 80, and 100 µl of diluted 10 µM AFC Standard into individual wells in a 96-well white/black plate and adjust the volume to 100 µl/well with HDAC Assay Buffer to generate 0, 200, 400, 600, 800, and 1000 pmol/well of AFC Standard respectively. Mix well.

e. Measurement: Centrifuge the tubes at 14,000 g for 2 minutes at room temperature. Transfer 100 µl of each reaction to individual wells in a white/black plate. Read fluorescence at Ex/Em = 380/500 nm.

f. Calculation: Plot the AFC Standard Curve. Subtract the Background Control reading from all Sample readings. Apply the corrected sample reading to the AFC Standard Curve to get B pmol of AFC in the sample wells.

Sample HDAC activity = 2 x B/TS = pmol/min/mg = mU

Where: **B** = AFC amount from the Standard Curve (pmol)
2 = sample dilution factor**
T = reaction time (60 min)
S = sample amount (mg)

Unit Definition: One unit of HDAC is the amount of enzyme that generates one nanomole of deacetylated substrate/min/mg at 37°C.
** (Reaction Volume = 200 µl; S & BC volume = ~12 µl beads + 168 µl reaction mix + 20 µl Developer. 100µl of the reaction is used to measure the fluorescence and hence the sample dilution factor is 2).

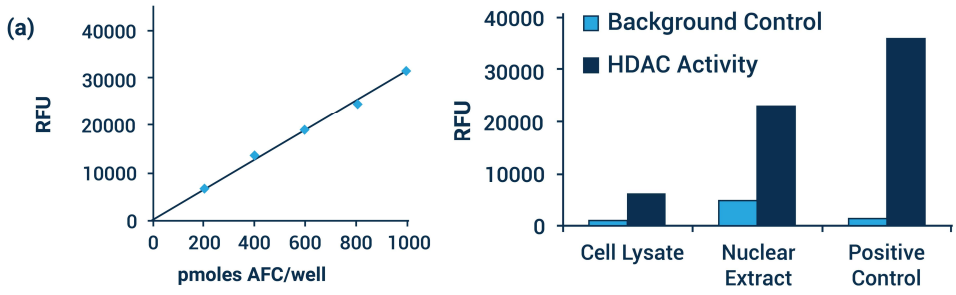

Figure: AFC standard curve (a). HDAC2 IP Activity Assay of HeLa cell lysate and nuclear extract and HDAC Activity Assay of Positive Control as per the kit protocol (b).

FOR RESEARCH USE ONLY! Not to be used on humans.