HDAC3 Immunoprecipitation (IP) & Activity Assay Kit

(Catalog # BN00608; 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. 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. Assay Genie's HDAC3 IP & Activity Assay Kit provides an antibody-based method to specifically immunoprecipitate the HDAC3 complex from cells & tissues and to measure HDAC3 activity fluorometrically. HDA3 is immunoprecipitated from cell or nuclear extract(s) using 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.

II. Application:

- Immunoprecipitation of HDAC3 complex from cell and nuclear extract(s)
- Measurement of HDAC3 activity of immunoprecipitated complex/purified enzyme
- Screening for activators or inhibitors of HDAC3

III. Sample Type: Human, Mouse or Rat

- Cell lysate, tissue extract and nuclear extract

IV. Kit Contents:

Components	BN00608	Cap Code	Part Number
HDAC Assay Buffer	9 ml	WM	BN00608-1
Extraction Buffer	100 ml	NM	BN00608-2
HDAC Substrate	100 µl	Amber	BN00608-3
Developer	500 µl	Orange	BN00608-4
AMC (7-amino-4-methyl coumarin) Standard (1 mM)	100 µl	Yellow	BN00608-5
Rabbit HDAC3 Antibody	500 µl	Red	BN00608-6
Rabbit IgG (Control Antibody)	250 µl	Green	BN00608-7
Protein-A/G Sepharose Beads (50% slurry in 20% ethanol/H_2O)	650 µl	Blue	BN00608-8
Positive Control (Jurkat Cell Lysate, lyophilized)	1 vial	Violet	BN00608-9

V. User Supplied Reagents and Equipment:

- 96-well white/black 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

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.
- **Extraction Buffer:** Thaw Extraction 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.
- **AMC 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 just before use. Keep on ice while in use.

VIII. HDAC3 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 Extraction Buffer containing protease inhibitor 125 µl/well if using a 12-well plate or 250 µl/well if using a 6-well plate. Keep on ice for one min. 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 30-60 min. Centrifuge at 10,000 g for 10 min. 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 # BN00534) or other equivalent method.

c. Tissue Extract: Use fresh or frozen (stored at -80ºC) tissue to prepare the tissue extract. Rinse tissue and transfer 25-50 mg of tissue to a prechilled dounce homogenizer. For every 25 mg of tissue, add 500 µl cold Extraction Buffer containing protease inhibitors and homogenize the tissue on ice with 10-15 strokes. Transfer the content to a microfuge tube and add 500 µl cold Extraction Buffer

containing protease inhibitors to the same tube. If volume exceeds the capacity of the tube, use a larger tube. Keep the tube on a rotary shaker at 4ºC for 30-60 min. Centrifuge at 10,000 x g for 10 min. at 4°C; transfer the supernatant to a clean tube. Use this tissue extract for IP-Activity Assay.

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 and Nuclear Extracts (CL/NE) are in the linear range for HDAC IP-activity when about 50-100 µg protein content is used. Use 0.5-1 mg of tissue extract for the IP reaction. 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 to a pre-chilled tube on ice. Add 20 µl HDAC3 Antibody to samples (S), & 20 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 supernatant 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 carefully 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 sample(s) and sample 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 tube (sample and background control) containing the beads, mix gently and incubate at 37°C for two hours.

b. Positive Control: 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. **Note: The positive control is used to measure total HDAC Activity.**

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

d. Standard Curve: Dilute the AMC Standard to 10 µM by adding 10 µl of 1 mM AMC Standard to 990 µl of dH_2O. Add 0, 10, 20, 30, 40 and 50 µl of the diluted 10 µM AMC 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, 100, 200, 300, 400, and 500 pmol/well of AMC 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 supernatant to individual wells in a white/black plate. Read fluorescence at Ex/Em = 368/442 nm.

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

Sample HDAC Activity = 2 x B/T x S = pmol/min/mg = mU

Where: **B** = AMC amount from the Standard Curve (pmol)
2 = sample dilution factor**
T = reaction time (120 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 and 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).

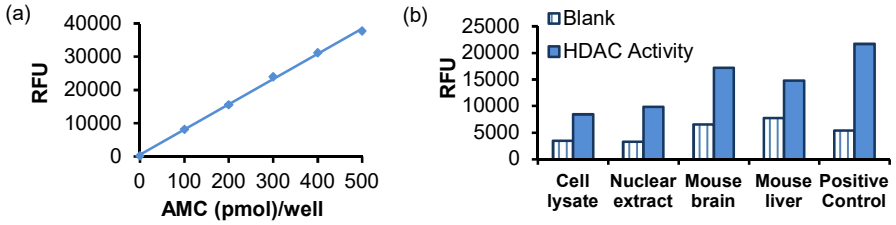

Figure: AMC Standard Curve (a). HDAC3 IP Activity Assay of Jurkat cell lysate and nuclear extract, mouse brain and liver extract and HDAC Activity Assay of Positive Control (b). Assays were performed according to the kit protocol.

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