Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric)

Base Catalog # P-4035

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric) is suitable for measuring the activity or inhibition of total HDAC enzyme using nuclear extracts or purified HDAC isoforms (HDACs 1-11) from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including, but not limited to cultured cells and fresh and frozen tissues. Class III HDACs (SIRTs 1-7) can also be detected after adding NAD+ co-factors to the assay. Nuclear extracts can be prepared by using your own successful method. For your convenience and the best results, EpigenTek offers a nuclear extraction kit (Cat # OP-0002) optimized for use with this kit. Nuclear extracts can be used immediately or stored at –80°C for future use. Purified enzymes can be active HDACs from recombinant proteins or isolated from cell/tissues.

Input Material: Input materials can be nuclear extracts or purified HDAC enzymes. The amount of nuclear extracts for each assay can be 0.5 µg to 20 µg with an optimal range of 5-10 µg. The amount of purified enzymes can be 5 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Internal Control: The HDAC assay standard (deacetylated histones) is provided in this kit for quantification of HDAC enzyme activity. Because HDAC activity can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and samples, change gloves immediately.

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KIT CONTENTS

Component	48 Assays Cat. #P-4035-48	96 Assays Cat. #P-4035-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
HO1 (HDAC Assay Buffer)	4 ml	8 ml	RT
HO2 (HDAC Substrate, 50 µg/ml)*	60 µl	120 µl	–20°C
HO3 (HDAC Assay Standard, 50 µg/ml)*	10 µl	20 µl	–20°C
HO4 (Capture Antibody, 1000 µg/ml*)	5 µl	10 µl	4°C
HO5 (Detection Antibody, 400 µg/ml)*	6 µl	12 µl	–20°C
HI (HDAC Inhibitor TSA, 100 µM)*	20 µl	40 µl	–20°C
FD (Fluoro Developer)*	10 µl	20 µl	–20°C
FE (Fluoro Enhancer)*	10 µl	20 µl	4°C
DB (Dilution Buffer)	4 ml	8 ml	RT
8-Well Assay Strips (With Frame)	6	12	4°C
Adhesive Covering Film	1	1	RT

** Spin the solution down to the bottom prior to use.*

SHIPPING & STORAGE

The kit is shipped in three parts: the first part at ambient room temperature and the second and third parts on frozen ice packs at 4°C. Upon receipt: (1) Store **HO2**, **HO3**, **HO5**, **HI** and **FD** at –20°C away from light; (2) Store **WB**, **HO4**, **FE**, and **8-Well Assay Strips** at 4°C away from light; and (3) Store remaining components (**HO1**, **DB**, and **Adhesive Covering Film**) at room temperature away from light.

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

*Note: (1) Check if **WB** (10X Wash Buffer) contains salt precipitates before use. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette or multiple-channel pipette
- ☐ Multiple-channel pipette reservoirs
- ☐ Aerosol resistant pipette tips
- ☐ Fluorescence microplate reader capable of reading fluorescence at $530_{ex}/590_{em}$ nm
- ☐ 1.5 ml microcentrifuge tubes
- ☐ Incubator for 37°C incubation

- ☐ Distilled water
- ☐ Nuclear extract or purified enzymes
- ☐ Parafilm M or aluminium foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric) is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric) and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

Acetylation of epsilon amino group of specific lysine residues contained in core histones is one of the most robust epigenetic marks and is essential for the regulation of multiple cellular processes. The acetylation of histones by histone acetyltransferases (HATs) seems to be of particular significance, as it is associated with active regions of the genome. In contrast, histone deacetylation by histone deacetylases (HDACs) leads to transcriptional repression. So far, at least 4 classes of HDACs are identified. Class I HDACs include 1, 2, 3, and 8. Class II HDACs are comprised of 4, 5, 6, 7, 9, and 10. Class III enzymes, known as sirtuins, require NAD+ cofactors and include SIRTs 1-7. The Class IV enzyme, which contains only HDAC11, has features of both Class I and II.

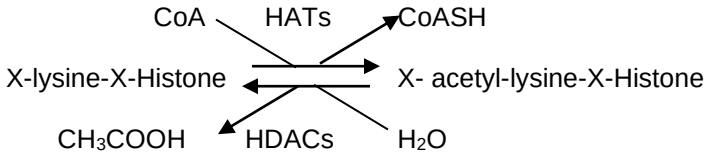


Fig 1. Histone acetylation and deacetylation catalyzed by HATs and HDACs.

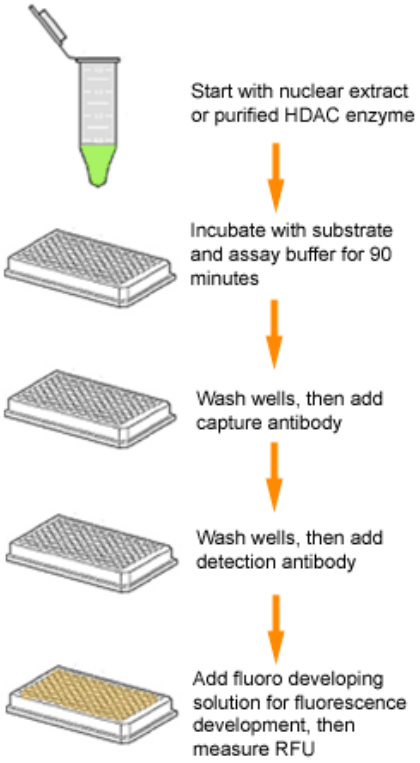
HDACs are tightly involved in cell cycle regulation, cell proliferation, and in the development of human diseases including cancer, cardiovascular and pulmonary diseases, and neurological diseases. For example, in various tumor cells, HDAC enzymes are found to be over-expressed. In chronic obstructive pulmonary disease (COPD), HDACs are observed to be decreased. In Huntington's disease, HDAC4 is significantly involved. The detection of activity and inhibition of HDACs is important in elucidating mechanisms of epigenetic regulation of gene activation and silencing, as well as benefiting cancer diagnostics and therapeutics.

There are only a couple of methods used for detecting HDAC activity/inhibition. These methods are based on the measurement of the deacetylated histone cleavage by lysyl endopeptidase or trypsin, and have significant weakness: (1) Nuclear extracts from cell/tissues can not be used because of interfering by lysyl endipeptidases from extracts; (2) Trypsin-sensitive HDAC inhibitiors are not suitable for testing with these methods, as trypsin digestion can lead to false positives when trypsin inhibitors are present in the compound library; (3) High interference by DMSO and thiol-containing chemicals, which are often contained in enzyme solution or tested compound solvents; and (4) Less accuracy than direct measurement of HDAC-converted deacetylated products. These problems were averted with EpigenTek's earlier EpiQuik™ HDAC Activity/Inhibition Assay Kit, a popular assay method for HDAC activity and inhibition. The Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric) is a further refinement of its predecessor -- this latest method retains the simplicity, rapidness, high throughput capability, and non-radioactivity featured in the previous version, while offering the following advantages:

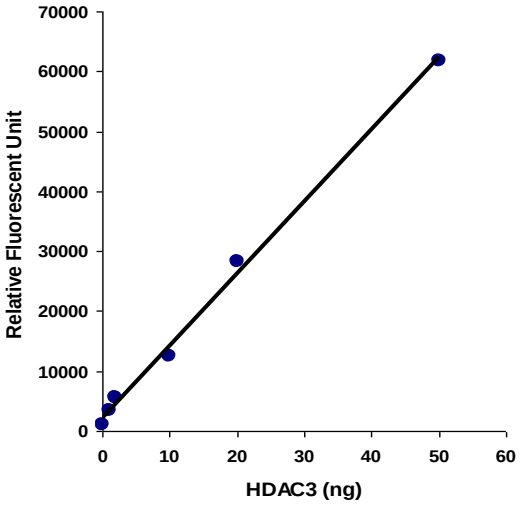
- Improved kit composition enables background signals to be very low, which allows the assay to be more accurate, sensitive, reliable, and consistent.
- Innovative fluorometric assay measures HDAC activity/inhibition by directly detecting HDAC-converted deacetylated products, rather than trypsin-based peptide cleavage, thus eliminating assay interference caused by DMSO, thiol-containing chemicals, trypsin, and cellular lysyl endipeptidases.
- Both cell/tissue extracts and purified HDAC enzyme can be used, which allows for the detection of inhibitory effects of HDAC inhibitor in vivo and in vitro.
- Novel assay principle allows high sensitivity to be achieved. The activity can be detected from as low as 0.5 ng of purified HDAC enzyme, which is about 10-fold higher than that obtained by trypsin-based peptide cleavage assays.
- A deacetylated histone standard is included, which allows the specific activity of HDACs to be quantified.
- Trichostatin A (TSA,) a potent inhibitor of class I and class II HDAC activity, is included as the positive control for the HDAC inhibition.
- Strip-well microplate format makes the assay flexible and quick: manual or high throughput analysis can be completed within 3.5 hours.

PRINCIPLE & PROCEDURE

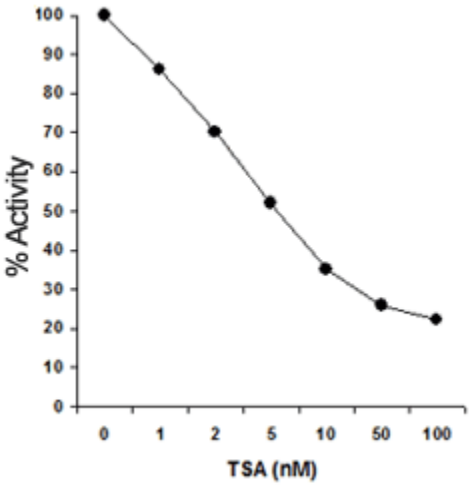
The Epigenase™ HDAC Activity/Inhibition Assay Kit contains all reagents necessary for the measurement of HDAC activity/inhibition. In this assay, an acetylated histone HDAC substrate is stably coated onto the microplate wells. Active HDACs bind to the substrate and removes acetyl groups from the substrate. The HDAC-deacetylated products can be recognized with a specific antibody. The ratio or amount of deacetylated products, which is proportional to the enzyme activity, can then be fluorometrically measured by reading the fluorescence in a fluorescent microplate reader at $530_{ex}/590_{em}$. The activity of the HDAC enzyme is proportional to the fluorescent intensity measured.



Schematic procedure of the Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric)



Demonstration of high sensitivity of an HDAC activity assay achieved by using recombinant HDAC3 with the Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric).



Demonstration of the inhibitory effect of an HDAC inhibitor detected by the Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Fluorometric). HDAC3 concentration: 20 ng/well.

ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Input Amount: The amount of nuclear extracts for each assay can be between 0.5 µg and 20 µg with an optimal range of 5 to 10 µg. The amount of purified enzymes can be between 5 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extraction: You can use your method of choice for preparing nuclear extracts. EpigenTek also offers a nuclear extraction kit (Cat # OP-0002) optimized for use with this kit.

Nuclear Extract or Purified HDAC Enzyme Storage: Nuclear extract or purified HDAC enzyme should be stored in aliquots at –80°C until use.

1. Working Buffer & Solution Preparation

a. Prepare **Diluted WB** 1X Wash Buffer:

48-Assay Kit: Add 13 ml of **WB** 10X Wash Buffer to 117 ml of distilled water and adjust pH to 7.2-7.5.

96-Assay Kit: Add 26 ml of **WB** 10X Wash Buffer to 234 ml of distilled water and adjust pH to 7.2-7.5.

This **Diluted WB** 1X Wash Buffer can now be stored at 4°C for up to six months.

b. Prepare **Diluted HO4** Capture Antibody Solution:

Dilute **HO4** Capture Antibody with **Diluted WB** 1X Wash Buffer at a ratio of 1:1000 (ex: add 1 µl of **HO4** to 1000 µl of **Diluted WB** 1X Wash Buffer). 50 µl of **Diluted HO4** will be required for each assay well.

c. Prepare **Diluted HO5** Detection Antibody Solution:

Dilute **HO5** Detection Antibody with **Diluted WB** 1X Wash Buffer at a ratio of 1:2000 (ex: add 1 µl of **HO5** Detection Antibody to 2000 µl of **Diluted WB** 1X Wash Buffer). 50 µl of **Diluted HO5** will be required for each assay well.

d. Prepare **Diluted HO3** Standard Solution:

First, dilute **HO3** Assay Standard with **HO1** Assay Buffer to 5 ng/µl by adding 1 µl of **HO3** to 9 µl of **HO1**. Then, further prepare five concentrations by combining the 5 ng/µl diluted **HO3** with **HO1** into final concentrations of 0.2, 0.5, 1, 2, and 5 ng/µl according to the following dilution chart:

Tube	HO3 (5 ng/µl)	HO1	Resulting HO3 Concentration
1	1.0 µl	24.0 µl	0.2 ng/µl
2	1.0 µl	9.0 µl	0.5 ng/µl
3	1.0 µl	4.0 µl	1.0 ng/µl
4	2.0 µl	3.0 µl	2.0 ng/µl
5	4.0 µl	0.0 µl	5.0 ng/µl

e. Prepare **Fluorescence Development Solution**:

Add 1 µl of **FD** Fluoro Developer and 1µl of **FE** Fluoro Enhancer to every 500 µl of **DB** Dilution Buffer.

Note: *Keep each of diluted solutions except* ***Diluted WB*** *1X Wash Buffer on ice until use. Any remaining diluted solutions other than* ***Diluted WB*** *should be discarded if not used within the same day.*

2. Enzymatic Reaction

a. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive controls) to ensure that the signal generated is validated. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

b. Blank Wells: Add 49 µl of **HO1** and 1 µl of **HO2**

c. Standard Wells: Add 49 µl of **HO1** and 1 µl of **Diluted HO3** to each standard well with a minimum of five wells, each at different concentrations between 0.2 to 5 ng/µl (based on the dilution chart in Step 1d; see Table 2 as an example).

d. Sample Wells Without Inhibitors: Add 45 to 48 µl of **HO1**, 1 µl of **HO2**, and 1 to 4 µl of nuclear extracts or 1 to 4 µl of purified HDAC enzyme. Total volume should be 50 µl/well.

e. Sample Wells With Inhibitor: Add 40 to 43 µl of **HO1**, 1 µl of **HO2**, 1 to 4 µl of nuclear extracts or 1 to 4 µl of purified HDAC enzyme, and 5 µl of inhibitor solution. Total volume should be 50 µl/well.

*Note: (1) Follow the suggested well setup diagrams; (2) It is recommended to use 2 µg to 10 µg of nuclear extract per well or 10 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with **HO1** at a 1:10 ratio (e.g., add 0.5 µl of inhibitor to 4.5 µl of **HO1**), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less. The HDAC inhibitor, TSA (**HI**), included in the kit can be used as a control inhibitor.*

f. Tightly cover strip-well microplate with **Adhesive Covering Film** to avoid evaporation and incubate at 37°C for 60-90 min.

*Note: (1) The incubation time may depend on intrinsic HDAC activity. However, in general, 60 min incubation is suitable for active purified HDAC enzymes and 90 min incubation is required for nuclear extracts; (2) The **Adhesive Covering Film** can be cut to the required size to cover the strips based on the number of strips to be used.*

g. Remove the reaction solution from each well. Wash each well three times with 150 µl of the **Diluted WB** 1X Wash Buffer each time.

3. Antibody Binding & Signal Enhancing

a. Add 50 µl of the **Diluted HO4** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

b. Remove the **Diluted HO4** solution from each well.

c. Wash each well three times with 150 µl of the **Diluted WB** each time.

d. Add 50 µl of the **Diluted HO5** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted HO5** solution from each well.

f. Wash each well four times with 150 µl of the **Diluted WB** each time.

Note: Ensure any residual wash buffer in the wells is removed as much as possible at each wash step. The wash can be carried out by simply pipetting the washing buffer into the wells and then pipetting the buffer out from the wells (discard the buffer).

4. Signal Detection

a. Add 50 µl of **Fluorescence Development Solution** to each well and incubate at room temperature. Continue to monitor the development (approximately 2-4 minutes) until the **Fluorescence Development Solution** turns pink in the presence of sufficient deacetylated products.

b. Read the fluorescence on a fluorescence microplate reader within 2 to 10 min at $530_{EX}/590_{EM}$.

If the strip well frame does not fit the microplate reader, transfer the solution to a standard 96-well microplate and read the RFU on a fluorescence microplate reader at $530_{EX}/590_{EM}$ nm.

5. HDAC Activity Calculation

a. Calculate the average duplicate readings for the sample wells and blank wells.

b. Calculate HDAC activity or inhibition using the following formulas:

For simple calculation:

$$\textit{HDAC Activity (RFU/min/mg)} = \frac{\textit{(Sample RFU– Blank RFU)}}{\textit{(Protein Amount (µg)* x min**)}} \times 1000$$

* Protein amount (µg) added into the reaction at step 2d.
** Incubation time (minutes) at step 2f.

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Example calculation:

Average RFU of sample is 6800
Average RFU of blank is 800
Protein amount is 5 µg
Incubation time is 60 minutes (1 hour)
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$$\text{HDAC activity} = \frac{(6800 - 800)}{(5 \times 60)} \times 1000 = 20000 \text{ RFU/min/mg}$$

For accurate or specific activity calculation:

1. Generate a standard curve and plot RFU value versus amount of **HO3** at each concentration point.
2. Determine the slope as RFU/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of HDAC-converted deacetylated product using the following formulas:

$$\textit{Demethylated product (ng)} = \frac{\textit{(Sample RFU – Blank RFU)}}{\textit{Slope}}$$

$$HDAC\ Activity\ (ng/min/mg) = \frac{Deacetylated\ Product\ (ng)}{(Protein\ Amount\ (\mu g) \times min^*)} \times 1000$$

* Incubation time (minutes) at Step 2f.

For inhibition calculation:

$$Inhibition\ \% = \left[1 - \frac{Inhibitor\ Sample\ RFU - Blank\ RFU}{No\ Inhibitor\ Sample\ RFU - Blank\ RFU}\right] \times 100\%$$

SUGGESTED BUFFER AND SOLUTION SETUP

Table 1. Approximate amount of required buffers and solutions for defined assay wells based on the protocol.

Reagents	1 well	1 strip (8 wells)	2 strips (16 wells)	6 strips (48 wells)	12 strips (96 wells)
Diluted WB	2.5 ml	20 ml	40 ml	120 ml	240 ml
HO1	50 µl	400 µl	800 µl	2400 µl	4800 µl
HO2	1 µl	8 µl	16 µl	50 µl	120 µl
HO3	N/A	N/A	1 µl (optional)	2 µl	2 µl
Diluted HO4	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted HO5	50 µl	400 µl	800 µl	2400 µl	4800 µl
Fluorescence Development Solution	0.05 ml	0.4ml	0.8 ml	2.4 ml	4.8 ml

SUGGESTED STRIP WELL SETUP

Table 2. The suggested strip-well plate setup for HDAC activity/inhibition assay in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	HO3 0.1 ng	HO3 0.1 ng	Sample	Sample	Sample	Sample
C	HO3 0.5 ng	HO3 0.5 ng	Sample	Sample	Sample	Sample
D	HO3 1.0 ng	HO3 1.0 ng	Sample	Sample	Sample	Sample
E	HO3 2.0 ng	HO3 2.0 ng	Sample	Sample	Sample	Sample
F	HO3 5 ng	HO3 5 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in both the positive control and sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake.
	The well is incorrectly washed before enzyme reaction	Ensure the well is not washed prior to adding the positive control and sample.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Incorrect fluorescence reading.	Check if appropriate fluorescence wavelength ($530_{EX}/590_{Em}$) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and the cap is tightly capped after each opening or use.
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance in this User Guide for storage of **HO3** (HDAC Assay Standard).
High background present in the blank wells	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by sample or standard.	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips.
	Incubation time with **Diluted HO5** is too long.	The incubation time at Step 3d should not exceed 2 hours.
	Over development of fluorescence.	Decrease the development time in Step 4a.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for HDAC protein extraction. For the best results, it is advised to use EpigenTek's Nuclear Extraction Kit (Cat. No. OP-0002). Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in Step 2. The sample can be titrated to determine the optimal amount to use in the assay.

	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80°C, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of HDAC contained in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared nuclear extracts or purified enzymes.
Uneven fluorescence development	Insufficient washing of the wells.	Ensure the wells are washed according to the guidance of washing and residue washing buffer is removed as much as possible.
	Delayed fluorescence development in the wells.	Ensure fluorescence development solution is added sequentially and is consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).

RELATED PRODUCTS

Nuclear Extract Preparation
OP-0002 EpiQuik™ Nuclear Extraction Kit

HDAC Activity/Inhibition Assay
P-4034 Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Colorimetric)
P-4036 Epigenase™ SIRT Activity/Inhibition Assay Kit (Colorimetric)
P-4037 Epigenase™ SIRT Activity/Inhibition Assay Kit (Fluorometric)

HDAC Level Quantification
P-4005 EpiQuik™ HDAC1 Assay Kit
P-4006 EpiQuik™ HDAC2 Assay Kit
P-4007 EpiQuik™ HDAC8 Assay Kit