

Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric)

Base Catalog # P-3085

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) is suitable for measuring activity/inhibition of JMJD3/UTX using nuclear extracts or JMJD3/UTX using purified enzymes from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues. 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 JMJD3 or UTX from recombinant proteins or isolated from cell/tissues.

Starting Materials: Input materials can be nuclear extracts or purified JMJD3 and UTX enzymes. The amount of nuclear extracts for each assay can be 1 μg to 20 μg with an optimized range of 5-10 μg . The amount of purified enzymes can be 10 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Internal Control: The JMJD3/UTX assay standard (demethylated histone H3-K27) is provided in this kit for quantification of JMJD3 and UTX enzyme activity. Because JMJD3 and UTX 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 sample, change gloves immediately.

KIT CONTENTS

Component	48 Assays Cat. #P-3085-48	96 Assays Cat. #P-3085-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
JE1 (JMJD Assay Buffer)	4 ml	8 ml	RT
JE2 (JMJD Substrate, 50 µg/ml)*	60 µl	120 µl	-20°C
JE3 (JMJD Assay Standard, 50 µg/ml)*	10 µl	20 µl	-20°C
JE4 (Capture Antibody, 1000 µg/ml*)	5 µl	10 µl	4°C
JE5 (Detection Antibody, 400 µg/ml)*	6 µl	12 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
Co-factor 1*	30 µl	60 µl	4°C
Co-factor 2*	30 µl	60 µl	4°C
Co-factor 3*	30 µl	60 µl	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 part on frozen ice packs at 4°C. Upon receipt: (1) Store **JE2, JE3, JE5** and **FD** at -20°C away from light; (2) Store **WB, JE4, FE, Co-factor 1, Co-factor 2, Co-factor 3** and **8-Well Assay Strips** at 4°C away from light; (3) Store remaining components (**JE1, DB, and Adhesive Covering Film**) at room temperature away from light.

*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.*

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

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette or multiple-channel pipette
- ☐ Multiple-channel pipette reservoirs
- ☐ Aerosol resistant pipette tips
- ☐ Microplate reader capable of reading fluorescence 530ex/590em
- ☐ 1.5 ml microcentrifuge tubes

- ☐ Incubator for 37°C incubation
- ☐ Distilled water
- ☐ Nuclear extract or purified enzymes
- ☐ Parafilm M or aluminum foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) is tested against predetermined specifications to ensure consistent product quality. Epigenetek 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: Epigenetek 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. Thus, only use the User Guide that was supplied with the kit when using that kit.

Usage Limitation: The Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) and methods of use contain proprietary technologies by Epigenetek.

A BRIEF OVERVIEW

Lysine histone methylation is one of the most robust epigenetic marks, and is essential for the regulation of multiple cellular processes. The methylation of H3-K27 seems to be of particular significance, as it is associated with repression regions of the genome. H3-K27 methylation was considered irreversible until the identification of a large number of histone demethylases indicated that demethylation events play an important role in histone modification dynamics. So far at least 2 classes of H3-K27 specific histone demethylase, JMJD3 (KDM6B), and UTX (KDM6A) have been identified. The JMJD3 and UTX can remove di- and tri-methylation from H3-K27. JMJD3 and UTX demethylases are JmjC-domain-containing proteins and catalyze the removal of methylation by using a hydroxylation reaction with a required iron and a-ketoglutarate as cofactors.



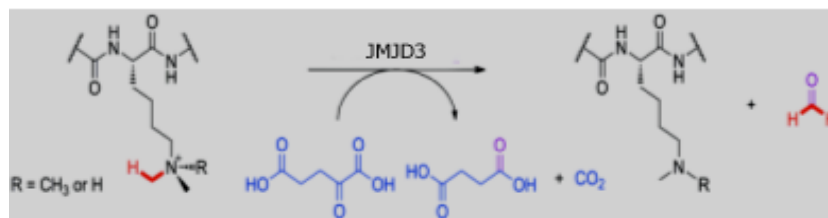


Fig 1. Histone H3-K27 demethylation reaction catalyzed by JMJD3 or UTX demethylase.

JMJD3 and UTX demethylases are found to have potential oncogenic functions. For example, JMJD3 is amplified in prostate cancer and UTX mutation was found in multiple cancer types including kidney cancer and multiple myeloma. Detection of activity and inhibition of JMJD3/UTX would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing and benefiting cancer diagnostics and therapeutics.

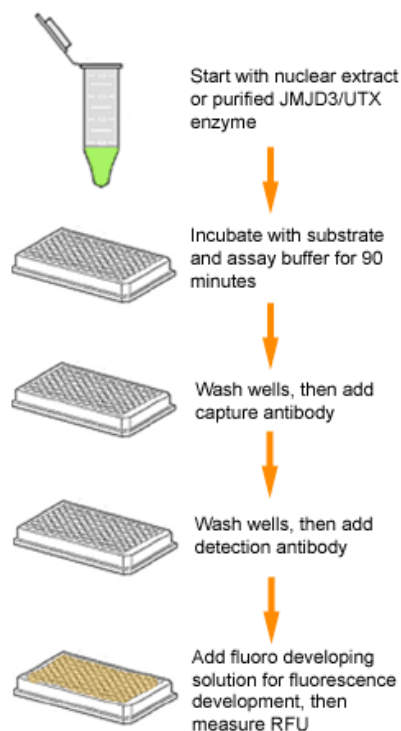
Currently, there are very few methods used for the detection of JMJD3/UTX activity/inhibition. The common method is based on the measurement of formaldehyde release, a by-product of JMJD3/UTX enzymatic reaction but has significant weaknesses: (1) Large amount (at μg level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues cannot be used; (3) Redox-sensitive JMJD3/UTX inhibitors are not suitable for testing with these methods; (4) Highly interfered by SDS, DMSO, thiol-containing chemicals, and ions, which are often contained in enzyme solution, tested compound solvents, and assay buffers; and (5) Less accuracy than direct measurement of JMJD3/UTX-converted demethylated products. We have now added the Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) to address this issue. Compared to formaldehyde release-based method, this kit has the following advantages:

- 3 hour colorimetric procedure in a 96 stripwell microplate format allows for either manual or high throughput analysis.
- Directly measures JMJD3 or UTX activity via a straightforward detection of JMJD3/UTX-converted demethylated products, rather than by-products, thus eliminating assay interference caused by thiol-containing chemicals such as DTT, GSH and 2-mercaptoethanol, or caused by detergents/ions such as tween-20, SDS, triton X-100, Fe, and Na.
- Both cell/tissue extracts containing JMJD3/UTX demethylases and purified JMJD3 or UTX proteins can be used, which allows for the detection of inhibitory effects of JMJD3/UTX inhibitors in vivo and in vitro.
- Sensitivity is up to 200 times higher than formaldehyde release-based JMJD3/UTX assays, allowing activity to be detected from as low as 10 ng of purified JMJD3 and UTX enzyme.
- Demethylated H3-K27 standard is included, allowing specific activity of JMJD3/UTX to be quantified.
- Accurate, reliable, and consistent with extremely low background signals.

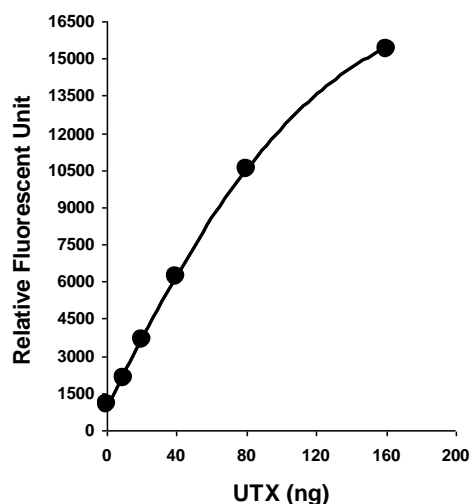
PRINCIPLE & PROCEDURE

The Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric) contains all the reagents necessary for the measurement of JMJD3/UTX activity/inhibition. In this assay, trimethylated histone H3-K27 substrate is stably coated onto strip wells. Active JMJD3/UTX binds to the substrate and removes methyl groups from the substrate. The JMJD3/UTX-demethylated products are recognized using a specific antibody. The ratio or amount of demethylated products,

which is proportional to enzyme activity, can then be fluorometrically measured by reading the fluorescence in a microplate reader. The activity of JMJD3/UTX enzyme is proportional to the RFU intensity measured.



Schematic procedure of the Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric).



Demonstration of high sensitivity of JMJD3/UTX activity assay achieved by using UTX recombinant protein with Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Fluorometric).

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 1 µg to 20 µg with an optimal range of 5-10 µg. The amount of purified enzymes can be 10 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

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

Nuclear Extract or Purified JMJD3/UTX Storage: Nuclear extracts or purified JMJD3/UTX enzymes should be stored in aliquot at -80°C until use.

1. Working Buffer & Solution Preparation

- 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 **CJE1** Completed Assay Buffer:

Add **Co-factor 1**, **Co-factor 2**, and **Co-factor 3** to **JE1** Assay Buffer at a ratio of 1:100 (i.e., add 1 µl of each Co-factor to 100 µl of assay buffer for a total of 103 µl).

c. Prepare **Diluted JE4** Capture Antibody Solution:

Dilute **JE4** Capture Antibody with **Diluted WB** 1X Wash Buffer at a ratio of 1:1000 (i.e., add 1 µl of **JE4** to 1000 µl of **Diluted WB**). 50 µl of **Diluted JE4** will be required for each assay well.

d. Prepare **Diluted JE5** Detection Antibody Solution:

Dilute **JE5** Detection Antibody with **WB** 1X Wash Buffer at a ratio of 1:2000 (i.e. add 1 µl of **JE5** to 2000 µl of **Diluted WB**). 50 µl of **Diluted JE5** will be required for each assay well.

e. Prepare **Diluted JE3** Standard Solution:

Suggested Standard Curve Preparation: First, dilute **JE3** Assay Standard with **JE1** Assay Buffer to 10 ng/µl by adding 1 µl of **JE3** to 4 µl of **JE1**. Then, further prepare five concentrations by combining the 10 ng/µl **Diluted JE3** with **JE1** into final concentrations of 0.5, 1, 2, 5 and 10 ng/µl according to the following dilution chart:

Tube	JE3 (10 ng/µl)	JE1	Resulting JE3 Concentration
1	1.0 µl	19.0 µl	0.5 ng/µl
2	1.0 µl	9.0 µl	1.0 ng/µl
3	1.0 µl	4.0 µl	2.0 ng/µl
4	2.0 µl	2.0 µl	5.0 ng/µl
5	4.0 µl	0.0 µl	10.0 ng/µl

f. Prepare **FD** Fluoro-Developer Solution:

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

*Note: Keep each of the 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 control) 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 **CJE1** and 1 µl of **JE2** to each blank well.

- c. Standard Wells: Add 49 μ l of **CJE1** and 1 μ l of **Diluted JE3** to each standard well with a minimum of five wells, each at a different concentration between 0.5 and 10 ng/ μ l (based on the dilution chart in Step 1e; see Table 2 in the "Suggested Strip Well Setup" section as an example).
- d. Sample Wells Without Inhibitor: Add 45 to 48 μ l of **CJE1**, 1 μ l of **JE2**, 1 to 4 μ l of your nuclear extracts or 1 to 4 μ l of your purified JMJD3/UTX enzyme. Total volume should be 50 μ l per well.
- e. Sample Wells With Inhibitor: Add 40 to 43 μ l of **CJE1**, 1 μ l of **JE2**, 1 to 4 μ l of your nuclear extracts or 1 to 4 μ l of purified JMJD3/UTX enzyme, and 5 μ l of inhibitor solution. Total volume should be 50 μ l per well.

*Note: (1) Follow the diagrams under the "Suggested Working Buffer & Solution Setup" section; (2) It is recommended to use 2 μ g to 10 μ g of nuclear extract per well or 20 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitor to be added into the sample wells can be varied (ex: 1 μ M to 1000 μ M). However, the final concentration of the inhibitors before adding to the wells should be prepared with **JE1** at a 1:10 ratio (ex: add 0.5 μ l of inhibitor to 4.5 μ l of **JE1**) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less. The Jumonji demethylase general inhibitor, N-Oxalylglycine can be used as the control inhibitor.*

- f. Tightly cover strip plate with **Adhesive Covering Film** to avoid evaporation and incubate at 37°C for 60-120 min.

*Note: (1) The incubation time may depend on intrinsic JMJD3/UTX activity. However, in general, 60-90 min incubation is suitable for active purified JMJD3/UTX enzyme and 90-120 min incubation is required for nuclear extract; (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 μ l of the **Diluted WB 1X Wash Buffer** each time.

3. Antibody Binding & Signal Enhancing

- a. Add 50 μ l of the **Diluted JE4** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 60 min.
- b. Remove the **Diluted JE4** solution from each well.
- c. Wash each well three times with 150 μ l of the **Diluted WB** each time.
- d. Add 50 μ l of the **Diluted JE5** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 min.
- e. Remove the **Diluted JE5** solution from each well.
- f. Wash each well four times with 150 μ l 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.

4. Signal Detection

- a. Add 50 μ l of **FD Fluoro-Developer Solution** to each well and incubate at room temperature for 2 to 4 min away from light. **FD Fluoro-Developer Solution** will turn pink in the presence of sufficient demethylated products.

- b. Read the fluorescence on a fluorescence microplate reader within 2 to 10 min at 530ex/590em nm.

Note: If the strip-well plate frame does not fit in the fluorescence microplate reader, transfer the solution to a standard 96-well microplate.

5. JMJD3/UTX Activity Calculation

- a. Calculate the average duplicate readings for the sample wells and blank wells.
b. Calculate JMJD3/UTX activity or inhibition using the following formulas:

For simple calculation:

$$\text{JMJD3/UTX Activity (RFU/min/mg)} = \frac{(\text{Sample RFU} - \text{Blank RFU})}{(\text{Protein Amount } (\mu\text{g}) \times \text{min}^{**})} \times 1000$$

* Protein amount added into the reaction at step 2d.

** Incubation time at step 2f (in minutes).

Example calculation:

Average RFU of sample is 6800

Average RFU of blank is 800

Protein amount is 5 μg

Incubation time is 2 hours (120 min)

$$\frac{(6800 - 800)}{(5 \times 120)} \times 1000 = 10000 \text{ RFU /min/mg}$$

For accurate or specific activity calculation:

1. Generate a standard curve and plot RFU value versus amount of **JE3** at each concentration point.
2. Determine the slope as RFU/ng (ex: use Excel statistics for slope calculation), then calculate the amount of JMJD3/UTX-converted demethylated product using the following formulas:

$$\text{Demethylated product (ng)} = \frac{(\text{Sample RFU} - \text{Blank RFU})}{\text{Slope}}$$

$$\text{JMJD3/UTX Activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{(\text{Protein Amount } (\mu\text{g}) \times \text{min}^*)} \times 1000$$

* Incubation time at step 2f (in minutes).

For inhibition calculation:

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

SUGGESTED WORKING BUFFER AND SOLUTION SETUP

Table 1. Approximate amount of required buffers and solutions for the 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
CJE1	50 µl	400 µl	800 µl	2400 µl	4800 µl
JE2	1 µl	8 µl	16 µl	50 µl	120 µl
JE3	NA	NA	1 µl (optional)	2 µl	2 µl
Diluted JE4	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted JE5	50 µl	400 µl	800 µl	2400 µl	4800 µl
Fluoro-Developer	0.05 ml	0.4 ml	0.8 ml	2.4 ml	4.8 ml

SUGGESTED STRIP WELL SETUP

Table 2. The suggested strip-well plate setup for JMJD3/UTX activity 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	JE3 0.5 ng	JE3 0.5 ng	Sample	Sample	Sample	Sample
C	JE3 1.0 ng	JE3 1.0 ng	Sample	Sample	Sample	Sample
D	JE3 2.0 ng	JE3 2.0 ng	Sample	Sample	Sample	Sample
E	JE3 5.0 ng	JE3 5.0 ng	Sample	Sample	Sample	Sample
F	JE3 10 ng	JE3 10 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 (530ex/590em) 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 on Page 2 of this User Guide for storage of JE3 (JMJD 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 JE5 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 JMJD3/UTX 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 JMJD3 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 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).
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RELATED PRODUCTS

Nuclear Extract Preparation

OP-002-1 EpiQuik™ Nuclear Extraction Kit

Histone Demethylase Activity/Inhibition Assay

P-3078 Epigenase™ LSD1 Activity/Inhibition Assay Ultra Kit
 P-3079 Epigenase™ LSD1 Activity/Inhibition Assay Ultra Kit (Fluorometric)
 P-3080 Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit
 P-3081 Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric)
 P-3082 Epigenase™ JARID Demethylase Activity/Inhibition Assay Kit
 P-3083 Epigenase™ JARID Demethylase Activity/Inhibition Assay Kit (Fluorometric)
 P-3084 Epigenase™ JMJD3/UTX Demethylase Activity/Inhibition Assay Kit (Colorimetric)

