Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric)

Base Catalog # P-3081

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is suitable for measuring activity or inhibition of total JMJD2 using nuclear extracts or JMJD2 subtypes (JMJD2A-JMJD2F) 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 JMJD2s from recombinant proteins or isolated from cell/tissues.

Input Material: Input materials can be nuclear extracts or purified JMJD2 enzymes. The amount of nuclear extracts for each assay can be 0.5 µg to 20 µg with an optimal range of 5 to 10 µg. The amount of purified enzymes can be 2 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Internal Control: The JMJD2 assay standard (demethylated histone H3-K9) is provided in this kit for the quantification of JMJD2 enzyme activity. Because JMJD2 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.

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KIT CONTENTS

Component	48 Assays Cat. #P-3081-48	96 Assays Cat. #P-3081-96	Storage Upon Receipt
JB1 (10X Wash Buffer)	14 ml	28 ml	4°C
JB2 (JMJD2 Assay Buffer)	4 ml	8 ml	RT
JB3 (JMJD2 Substrate, 50 µg/ml)*	60 µl	120 µl	–20°C
JB4 (JMJD2 Assay Standard, 50 µg/ml)*	10 µl	20 µl	–20°C
JB5 (Capture Antibody, 1000 µg/ml)*	5 µl	10 µl	4°C
JB6 (Detection Antibody, 400 µg/ml)*	6 µl	12 µl	–20°C
JB7 (Fluoro Developer)*	10 µl	20 µl	–20°C
JB8 (Fluoro Enhancer)*	10 µl	20 µl	4°C
JB9 (Fluoro Dilutor)	4 ml	8 ml	RT
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
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 **JB3**, **JB4, JB6** and **JB7** at –20°C away from light; (2) Store **JB1**, **JB5**, **JB8, Co-factor 1, Co-factor 2, Co-factor 3** and **8-Well Assay Strips** at 4°C away from light; (3) Store remaining components (**JB2**,**JB9** and **Adhesive Covering Film**) at room temperature.

Note: (1) Check if JB1 (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
- ☐ Fluorescence microplate reader capable of reading fluorescence at 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™ JMJD2 Demethylase Activity/Inhibition 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™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric) and methods of use contain proprietary technologies by EpigenTek.

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-K9 seems to be of particular significance, as it is associated with repression regions of the genome. H3-K9 methylation was considered irreversible until the identification of a large number of histone demethylases indicating that demethylation events play an important role in histone modification dynamics. So far at least 2 classes of H3-K9 specific histone demethylase, JMJD1(JHDM2), and JMJD2 (JHDM3) have been identified. The JMJD1 family, including JMJD1A, JMJD1B, and JMJD1C can remove di- and mono-methylation from H3-K9 while the JMJD2 family, including JMJD2A, JMJD2B, JMJD2C, and JMJD2D, JMJD2E, and JMJD2F can remove tri-methylation from H3-K9 and H3-K36. JMJD2 demethylases are Jumonji domain proteins and catalyze the removal of methylation by using a hydroxylation reaction with iron and a-ketoglutarate required as cofactors.

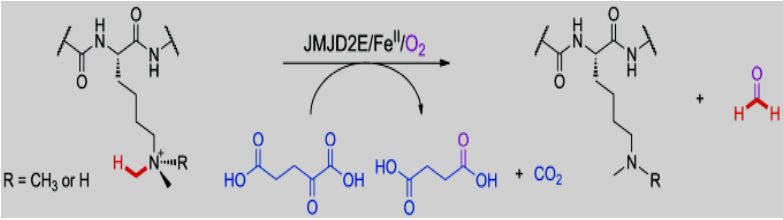


Fig 1. Histone H3-K9 demethylation reaction catalyzed by JMJD2 demethylase. (Hopkinson et al: Chembiochem, 4: 506-510, 2010)

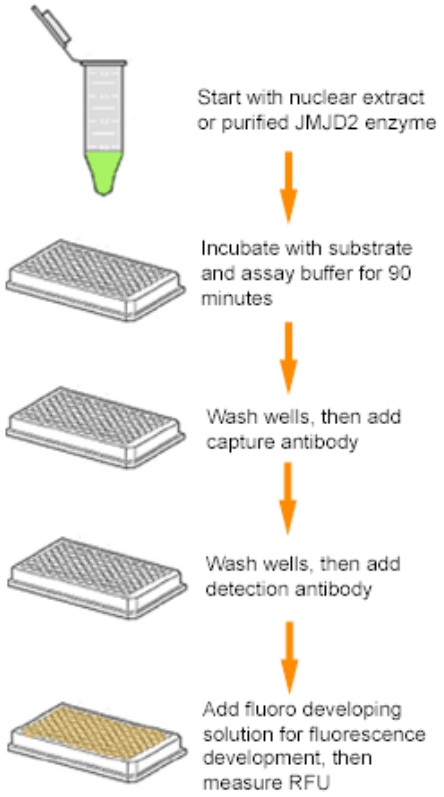
JMJD2 demethylases are found to have potential oncogenic functions. For example, JMJD2A is amplified in prostate cancer and JMJD2C overexpression is observed in oesophageal carcinoma. Detection of activity and inhibition of JMJD2 would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing, as well as benefiting cancer diagnostics and therapeutics.

There are only a couple of methods used for detecting JMJD2 activity and inhibition. These methods are based on the measurement of formaldehyde release, a by-product of JMJD2 enzymatic reaction, and have significant weaknesses: (1) Large amounts (at µg level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues cannot be used; (3) Redox-sensitive JMJD2 inhibitiors are not suitable for testing with these methods; (4) High interference by SDS, DMSO, thiol-containing chemicals, and ions, which are often contained in enzyme solutions, tested compound solvents, and assay buffers; and (5) Less accuracy than a direct measurement of JMJD2-converted demethylated product. The Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is designed to address these issues. Compared to a formaldehyde release-based method, this kit has the following advantages:

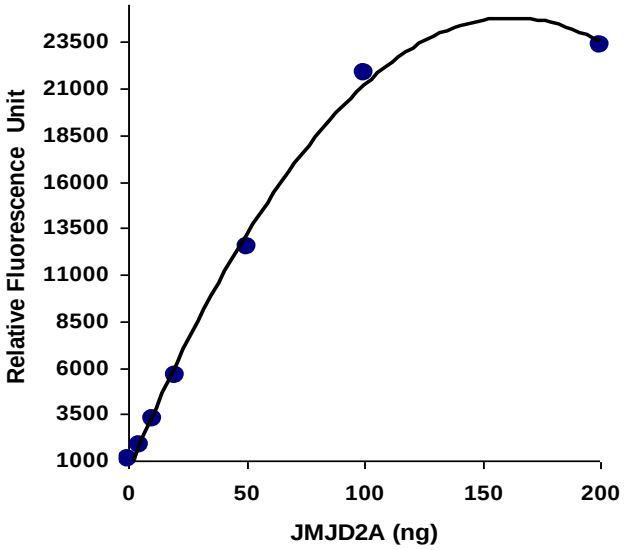
- 3-hour fluorometric procedure in a 96 stripwell microplate format allows for either manual or high throughput analysis.
- Directly measures JMJD2 activity via a straightforward detection of JMJD2-converted demethylated products, rather than by-products, thus eliminating assay interference caused by 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 and purified JMJD2 can be used, which allows for the detection of inhibitory effects of JMJD2 inhibitors in vivo and in vitro.
- Sensitivity is up to 2,000 times higher than formaldehyde release-based JMJD2 assays, allowing activity to be fluorometrically detected from as low as 2 ng of purified JMJD2 enzyme.
- Demethylated H3-K9 standard is included, allowing specific activity of JMJD2 to be quantified.
- Accurate, reliable, and consistent with extremely low background signals. High throughput analysis can be completed within 3 hours.

PRINCIPLE & PROCEDURE

In this assay, a tri-methylated histone H3-K9 substrate is stably coated onto microplate wells. Active JMJD2s bind to the substrate and remove methyl groups from the substrate. The JMJD2-demethylated products can be recognized with a specific antibody. The ratio or amount of demethylated products, which is proportional to enzyme activity, can then be fluorometrically measured by reading the fluorsecence in a fluorescent microplate spectrophometer at 530 excitation and 590 emission. The activity of the JMJD2 enzyme is in turn proportional to the fluorescent intensity measured.



Schematic procedure of Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric)



Demonstration of high sensitivity of the JMJD2 activity assay achieved by using recombinant JMJD2 with the Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Fluorometric).

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 ug with an optimal range of 5 to 10 µg. The amount of purified enzymes can be between 2 ng and 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 JMJD2 Storage: Nuclear extract or purified JMJD2 enzyme should be stored in aliquots at –80°C until use.

1. Working Buffer and Solution Preparation

a. Prepare **Diluted JB1** 1X Wash Buffer:

48-Assay Kit: Add 13 ml of **JB1** 10X Wash Buffer to 117 ml of distilled water and adjust pH to 7.2-7.5.

96-Assay Kit: Add 26 ml of **JB1** 10X Wash Buffer to 234 ml of distilled water and adjust pH to 7.2-7.5.

This **Diluted JB1** 1X Wash Buffer can now be stored at 4°C for up to six months.

b. Prepare **CJB2** Completed Assay Buffer**:**

Add **Co-factor 1**, **Co-factor 2**, and **Co-factor 3** to **JB2** assay buffer at a ratio of 1:100 for each Co-factor (i.e., add 1 µl of each Co-factor to 100 µl of **JB2** for a total of total of 103 µl).

c. Prepare **Diluted JB5** Capture Antibody Solution:

Dilute **JB5** Capture Antibody with **Diluted JB1** 1X Wash Buffer at a ratio of 1:1000 (i.e., add 1 µl of capture antibody to 1000 µl of 1X Wash Buffer). 50 µl of **Diluted JB5** are required for each assay well.

d. Prepare **Diluted JB6** Detection Antibody Solution:

Dilute **JB6 D**etection Antibody with **Diluted JB1** 1X Wash Buffer at a ratio of 1:2000 (i.e., add 1 µl of detection antibody to 2000 µl of 1X Wash Buffer). 50 µl of **Diluted JB6** are required for each assay well.

e. Prepare **Diluted JB4** Standard Solution:

Suggested Standard Curve Preparation: First, dilute **JB4** Assay Standard with **JB2** Assay Buffer to 5 ng/µl by adding 1 µl of **JB4** to 9 µl of **JB2**. Then, further prepare five concentrations by combining the 5 ng/µl **Diluted JB4** with **JB2** into final concentrations of 0.2, 0.5, 1.0, 2.0, and 5.0 ng/µl according to the following dilution chart:

Tube	JB4 (5 ng/µl)	JB2	Resulting JB4 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

f. Prepare **Fluorescence Development Solution**:

Add 1 µl of **JB7** Fluoro Developer and 1 µl of **JB8** Fluoro Enhancer to each 500 µl of **JB9** Fluoro Dilutor.

*Note: Keep each of the diluted solutions except **Diluted JB1** 1X Wash Buffer on ice until use. Any remaining diluted solutions other than **Diluted JB1** 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 **CJB2** and 1 µl of **JB3** to each blank well.

c. Standard Wells: Add 49 µl of **CJB2** and 1 µl of **Diluted JB4** standard solution to each standard well with a minimum of five wells, each at a different concentration between 0.2 and 5 ng/µl (based on the dilution chart in Step 1e; see Table 3 on Page 9 as an example).

d. Sample Wells Without Inhibitor: Add 44 to 48 µl of **CJB2**, 1 µl of **JB3**, 1 to 5 µl of your nuclear extract or 1 to 5 µl of purified JMJD2 enzyme. Total volume should be 50 µl per well.

e. Sample Wells With Inhibitor: Add 40 to 43 µl of **CJB2**, 1 µl of **JB3**, 1 to 4 µl of nuclear extract or 1 to 4 µl of purified JMJD2 enzyme, and 5 µl of inhibitor solution. Total volume should be 50 µl per well.

*Note: (1) Follow the suggested well setup diagrams; (2) It is recommended to use 5 µg to 10 µg of nuclear extract per well or 10 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitor 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 **JB2** at a 1:10 ratio (e.g., add 0.5 µl of inhibitor to 4.5 µl of **JB2**) 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 the strip-well microplate with **Adhesive Covering Film** to avoid evaporation and incubate at 37°C for 60 to 120 min.

*Note: (1) The incubation time may depend on intrinsic JMJD2 activity. However, in general, 60-90 min incubation is suitable for active purified JMJD2 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 µl of the Diluted JB1 1X Wash Buffer each time.

3. Antibody Binding & Signal Enhancing

a. Add 50 µl of the **Diluted JB5** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

b. Remove the **Diluted JB5** solution from each well.

c. Wash each well three times with 150 µl of the **Diluted JB1** each time.

d. Add 50 µl of the **Diluted JB6** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted JB6** solution from each well.

f. Wash each well four times with 150 µl of the **Diluted JB1** 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 µl of **Fluorescence Development Solution** to each well and incubate at room temperature for 2 to 4 min away from light. The **Fluorescence Development 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 microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

5. JMJD2 Activity Calculation

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

b. Calculate JMJD2 activity or inhibition using the following formulas:

For simple calculation:

$$\textit{JMJD2 Activity (OD/min/mg)} = \frac{\textit{(Sample RFU – Blank RFU)}}{\textit{(Protein Amount (µg)* x min**)}} \text{ x } 1000$$

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

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)

$$\text{JMJD2 activity} = \frac{(6800 - 800)}{(5 \times 120)} \times 1000 = 10000 \text{ RFU/min/mg}$$

For accurate or specific activity calculation:

1. Generate a standard curve and plot RFU value versus amount of **JB4** 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 JMJD2-converted demethylated product using the following formulas:

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

$$\textit{JMJD2 Activity (ng/min/mg)} \quad \frac{\textit{Demethylated Product (ng)}}{\textit{(Protein Amount (µg) x min*)}} \text{ x } 1000$$

***** 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 WORKING BUFFER AND SOLUTION SETUP

Table 2. Approximate amount of required buffers and solutions for defined assay wells.

Reagents	1 well	1 strip (8 wells)	2 strips (16 wells)	6 strips (48 wells)	12 strips (96 wells)
Diluted JB1	2.5 ml	20 ml	40 ml	120 ml	240 ml
JB2	50 µl	400 µl	800 µl	2400 µl	4800 µl
JB3	1 µl	8 µl	16 µl	50 µl	120 µl
JB4	NA	NA	1 µl (optional)	2 µl	2 µl
Diluted JB5	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted JB6	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 3. The suggested strip-well plate setup for JMJD2 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	JB4 0.2 ng	JB4 0.2 ng	Sample	Sample	Sample	Sample
C	JB4 0.5 ng	JB4 0.5 ng	Sample	Sample	Sample	Sample
D	JB4 1.0 ng	JB4 1.0 ng	Sample	Sample	Sample	Sample
E	JB4 2.0 ng	JB4 2.0 ng	Sample	Sample	Sample	Sample
F	JB4 5.0 ng	JB4 5.0 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 in this User Guide for storage of **JB4** (JMJD2 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 JB6** is too long.	The incubation time at Step 3d should not exceed 45 min.
	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 JMJD2 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 JMJD2 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 fluorescent 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 n 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).

RELATED PRODUCTS

Nuclear Extract Preparation

OP-0002-1 EpiQuik™ Nuclear Extraction Kit

Histone Demethylase Activity/Inhibition Assy

P-3077 EpiQuik™ Histone Demthylase (H3-K9 Specific) Activity/Inhibition Fast Assay Kit

P-3078 Epigenase™ LSD1 Demethylase Activity/Inhibition Assay Kit (Colorimetric)

P-3079 Epigenase™ LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric)

P-3080 Epigenase™ JMJD2 Demethylase Activity/Inhibition Assay Kit (Colorimetric)

P-3082 Epigenase™ JARID Demethylase Activity/Inhibition Assay Kit (Colorimetric)

P-3083 Epigenase™ JARID Demethylase Activity/Inhibition Assay Kit (Fluorometric)