ab156910 – KDM6A/KDM6B Activity Quantification Kit (Colorimetric)

Instructions for Use

For the measurement of activity/inhibition of KDM6A &KDM6B using nuclear extracts or purified enzymes from various samples

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Lysine histone methylation is one of the most robust epigenetic marks, and is essential for the regulation of multiple cellular processes. The methylation of H3K27 seems to be of particular significance, as it is associated with repression regions of the genome. H3K27 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 H3K27 specific histone demethylase, KDM6B (JMJD3) and KDM6A (UTX) have been identified. KDM6A (UTX) and KDM6B (JMJD3) demethylases are JmjC-domain-containing proteins and catalyze the removal of methylation (di- and tri-methylation from H3K27) by using a hydroxylation reaction with a required iron and a-ketoglutarate as cofactors.

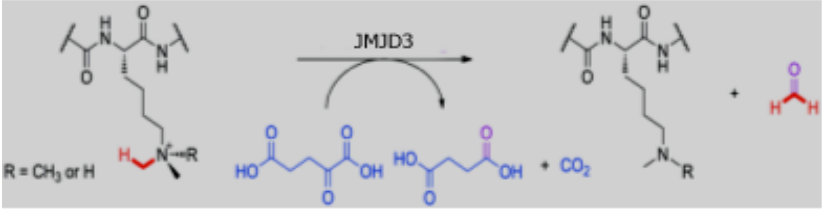

Figure 1. Histone H3-K27 demethylation reaction catalyzed by KDM6A/ KDM6B demethylase.

KDM6A and KDM6B demethylases are found to have potential oncogenic functions. For example, KDM6A (UTX) mutation was found in multiple cancer types including kidney cancer and multiple myeloma and KDM6B (JMJD3) is amplified in prostate cancer. Detection of activity and inhibition of KDM6B (JMJD3) would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing and benefiting cancer diagnostics and therapeutics.

There are only a couple of methods used for detecting KDM6A/KDM6B activity/inhibition. The common method is based on the measurement of formaldehyde release, a by-product of KDM6A/KDM6B enzymatic reaction which has significant weaknesses: (1) Large amounts of substrate and enzyme are required; (2) Nuclear extracts from cell or tissues cannot be used; (3) Redox-sensitive KDM6A/KDM6B inhibitors are not suitable for testing with these methods; (4) There is high interference by SDS, DMSO, thiol-containing chemicals, and ions, often contained in enzyme solutions and assay buffers; and (5) Less accuracy than direct measurement of KDM6A/KDM6B-converted demethylated products.

The KDM6A/KDM6B Activity Quantification Assay Kit (Colorimetric) has been created to address this issue. Compared to formaldehyde release-based method, this kit has the following advantages:

- 3 hour colorimetric procedure in a 96 strip well microplate format allows for either manual or high throughput analysis.
- Directly measures KDM6A (UTX) or KDM6B (JMJD3) activity via a straightforward detection of KDM6A/KDM6B-converted demethylated products, rather than by-products. This eliminates assay interference caused by thiol-containing chemicals such as DTT or 2-mercaptoethanol, or caused by detergents/ions such as tween-20, SDS, triton X-100, Fe, and Na.
- Cell/ tissue extracts containing KDM6A/KDM6B demethylases and purified KDM6A or KDM6B proteins can be used, which allows for the detection of inhibitory effects of KDM6A/KDM6B inhibitors *in vivo* and *in vitro.*
- Sensitivity is up to 200 times higher than formaldehyde release-based KDM6A/KDM6B assays, which allows detecting activity from as low as 10 ng of purified KDM6A and KDM6B.
- Demethylated H3K27 standard is included, allowing specific activity of KDM6A/KDM6B to be quantified.
- Accurate, reliable, and consistent with extremely low background signals.

INTRODUCTION

The KDM6A/KDM6B Activity Quantification Assay Kit (Colorimetric) is suitable for measuring activity/inhibition of KDM6A/ KDM6B using nuclear extracts or KDM6A (UTX) and KDM6B (JMJD3) 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. Nuclear extracts can be used immediately or stored at –80°C for future use. Purified enzymes can be active KDM6A/KDM6B from recombinant proteins or isolated from cell/tissues.

The KDM6A/KDM6B Activity Quantification Assay Kit (Colorimetric) contains all reagents necessary for the measurement of KDM6A/KDM6B activity/inhibition. In this assay, tri-methylated histone H3K27 substrate is stably coated onto strip wells. Active KDM6A/KDM6B binds to the substrate and removes methyl groups from the substrate. The KDM6A/KDM6B - 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 colorimetrically measured by reading the absorbance in a microplate reader. The activity of KDM6A/KDM6B enzyme is proportional to the OD intensity measured.

2. ASSAY SUMMARY

Start with nuclear extracts or purified KDM6A/KDM6B enzyme

↓

Incubate with substrate and assay buffer

↓

Wash wells, then add capture antibody

↓

Wash wells, then add detection antibody

↓

Add color developing solution for color development, then measure absorbance

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt away from light.

Observe the storage conditions for individual prepared components in sections 9 & 10.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if 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.

Check if a blue color is present in the Developer Solution, which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of Developer Solution required into a secondary container (tube or vial) before adding the Developer Solution into the assay wells.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
JMJD Assay Buffer	4 mL	8 mL	RT
JMJD Substrate, 50 µg/mL*	60 µL	120 µL	-20°C
JMJD Assay Standard, 50 µg/mL*	10 µL	20 µL	-20°C
Capture Antibody, 1000 µg/mL*	5 µL	10 µL	4°C
Detection Antibody, 400 µg/mL*	6 µL	12 µL	-20°C
Developer Solution	5 mL	10 mL	4°C
Stop Solution	5 mL	10 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.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Microplate reader capable of reading absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzymes
- Parafilm M or aluminium foil

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 1X Wash Buffer

Add the volume specified in the table below of 10X Wash Buffer to distilled water (pH to 7.2-7.5).

	Volume to Dilute (mL)	Volume distilled water (mL)	Total Volume (mL)
48 Tests	**13**	**117**	**130**
96 Tests	**26**	**234**	**260**

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

9.2 Diluted Capture Antibody Solution

Dilute Capture Antibody with 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 Capture Antibody will be required for each assay well.

9.3 Diluted Detection Antibody Solution

Dilute Detection Antibody with 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 Detection Antibody will be required for each assay well.

9.4 Completed Assay Buffer

Add Co-factor 1, Co-factor 2, and Co-factor 3 to 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 assay buffer for a total of 103 µL).

9.5 Suggested Standard Curve Preparation

Dilute 1 µL of Assay Standard in 4 µL of Assay Buffer for a final Standard concentration of 10 ng/µL. Prepare five concentrations by combining the 10 ng/µL Assay Standard with Assay Buffer into final concentrations of 0.5, 1, 2, 5 and 10 ng/µL according to the following dilution table:

Tube	10 ng/µL Assay Standard (µL)	Assay Buffer (µL)	Resulting Assay Standard concentration (ng/µL)
1	1.0	19.0	0.5
2	1.0	9.0	1.0
3	1.0	4.0	2.0
4	2.0	2.0	5.0
5	4.0	0.0	10.0

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

9.6 Suggested Working Buffer and Solution Setup

The table below shows the approximate amount of required buffers and solutions for defined assay wells, based on the protocol.

Reagents	1 well	8 wells (1 strip)	16 wells (2 strips)	48 wells (6 strips)	96 wells (12 strips)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL	240 mL
Completed Assay Buffer	50 µL	400 µL	800 µL	2400 µL	4800 µL
Substrate	1 µL	8 µL	16 µL	50 µL	120 µL
Assay Standard	N/A	N/A	1 µL (optional)	2 µL	2 µL
Diluted Capture Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Diluted Detection Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Developer Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL
Stop solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be 1 µg – 20 µg with optimized range of 5-10 µg. The amount of purified enzymes can be 10 ng – 500 ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extracts: You can use your own method of choice for preparing nuclear extracts. Nuclear extract or purified KDM6A/KMD6B enzyme should be stored at –80°C until use.

Suggested Strip Well Setup: The suggested strip-well plate setup for the KDM6A/KMD6B activity assay in a 48-assay format is shown in the table below. The controls and samples can be measured in duplicates.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	Assay Standard 0.5 ng	Assay Standard 0.5 ng	Sample	Sample	Sample	Sample
C	Assay Standard 1.0 ng	Assay Standard 1.0 ng	Sample	Sample	Sample	Sample
D	Assay Standard 2.0 ng	Assay Standard 2.0 ng	Sample	Sample	Sample	Sample
E	Assay Standard 5.0 ng	Assay Standard 5.0 ng	Sample	Sample	Sample	Sample
F	Assay Standard 10.0 ng	Assay Standard 10.0 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

11. ASSAY PROCEDURE

11.1 Enzymatic Reaction

11.1.1 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).

11.1.2 Blank Wells: Add 49 µl of Completed Assay Buffer and 1 µL of Substrate to each blank well.

11.1.3 Standard Wells: For a standard curve, add 49 µl of Completed Assay Buffer and 1 µL of Diluted Assay Standard solution to each standard well with a minimum of five wells, each at a different concentration between 0.2 – 5 ng/µL (based on the dilution table in Section 9).

11.1.4 Sample Wells Without Inhibitor: Add 45 µL - 48 µL Completed Assay Buffer, 1 µL of Substrate, and 1 - 4 µL of your nuclear extracts or 1 - 4 µL of your purified KDM6A/KDM6B enzyme to each sample well without inhibitor. Total volume should be 50 µl per well.

11.1.5 Sample Wells with Inhibitor: Add 40 µl - 43 µL of Completed Assay buffer, 1 µL of Substrate, 1 to 4 µL of your nuclear extracts or 1 to 4 µL of your purified KMD6A/KMD6B enzyme, and 5 µL of inhibitor solution. Total volume should be 50 µL per well.

***Note**: Follow the suggested well setup diagrams in Section 10. It is recommended to use 2 µg – 10 µg of nuclear extract per well or 10 ng – 100 ng of purified enzyme per well. The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 µM – 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with Assay Buffer at a 1:10 ratio (e.g. add 0.5 µL of inhibitor to 4.5 µL of Assay Buffer), 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.

11.1.6 Tightly cover the strip-well microplate with the Adhesive Covering Film to avoid evaporation, and incubate at 37°C for 60 - 120 min.

***Note**: The incubation time may depend on intrinsic KDM6A/KDM6B activity. In general, 60-90 min incubation is suitable for active purified KDM6A/KDM6B enzymes and 90-120 min incubation is required for nuclear extracts. The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.*

11.1.7 Remove the reaction solution from each well. Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

11.2 **Antibody Binding & Signal Enhancing**

11.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

11.2.2 Remove the Diluted Capture Antibody solution from each well.

11.2.3 Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

11.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

11.2.5 Remove the Diluted Detection Antibody solution from each well.

11.2.6 Wash each well with 150 µL of the 1X Wash Buffer each time for four times.

***Note**: Ensure any residual wash buffer in the wells is thoroughly removed as much as possible at each wash step.*

11.3 **Signal Detection**

11.3.1 Add 100 µL of Developer Solution to each well and incubate at room temperature for 1 - 10 min away from light. Begin monitoring color change in the sample wells and control wells. The Developer solution will turn blue in the presence of sufficient demethylated products.

11.3.2 Add 100 µL of Stop Solution to each well to stop enzyme reaction when color in the positive control wells turns medium blue. The color will change to yellow after adding Stop Solution and the absorbance should be read on a microplate reader within 2 - 10 min at 450 nm with an optional reference wavelength of 655 nm.

Note: *Most microplate readers have the capability to carry out dual wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice, once at 450 nm and once at 655 nm. Then, manually subtract the 655 nm ODs from 450 nm ODs. If the strip-well microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

12. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Calculate KDM6A/KDM6B activity or inhibition using the following formulae:

$$\text{KDM6A/KDM6B activity (OD/min/mg)} = \frac{\text{Sample OD} - \text{Blank OD}}{(\text{Protein Amount } (\mu g)^{*} \times \text{min}^{**})} \times 1000$$

*Protein amount (µg) added into the reaction at step 11.1.4.
**Incubation time (minutes) at step 11.1.6.

Example calculation:

Average OD450 of sample is 0.65
Average OD450 of blank is 0.05
Protein amount is 5 µg
Incubation time is 2 hours (120 minutes)
KDM6A/KDM6B activity = [(0.65 – 0.05) / (5 x 120)] x 1000
= 1 OD/min/mg

For accurate or specific activity calculation:

Generate a standard curve and plot OD value versus amount of Assay Standard at each concentration point.

Determine the slope as OD/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of KDM6A/KDM6B -converted demethylated product using the following formulae:

$$\text{Demethylated Product (ng)} = \frac{\text{Sample OD} - \text{Blank OD}}{\text{Slope}}$$

$$\text{KDM6A/KDM6B activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{\text{(Protein Amount (µg) x min*)}} \times 1000$$

*Incubation time (minutes) at step 11.1.6.

For inhibition calculation:

$$\text{Inhibition \%} = 1 - \left(\frac{\text{Inhibitor Sample OD – Blank OD}}{\text{No Inhibitor Sample OD – Blank OD}} \right) \times 100\%$$

Typical Results

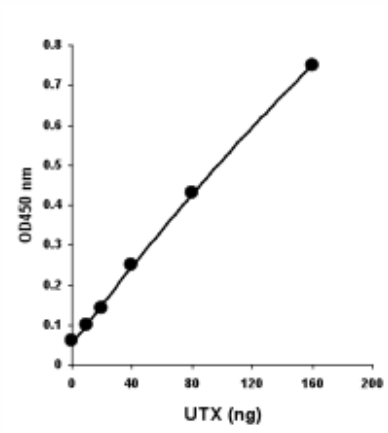

Figure 2. Demonstration of high sensitivity of KDM6A/KDM6B activity assay achieved by using KDM6B (UTX) recombinant protein with the KDM6A/KDM6B Activity Quantification Assay Kit (Colorimetric).

13. TROUBLESHOOTING

Problem	Cause	Solution
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 is followed correctly.
	Incorrect absorbance reading.	Check if appropriate absorbance wavelength (450 nm) is used.
No signal or weak signal in both the positive control and sample wells.	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and caps are tightly capped after each opening or use.

Problem	Cause	Solution
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in step 11.1.3.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Storage guidance in this User Guide for storage instructions of Assay Standard.
High Background Present for the Blank	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by sample or positive control.	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips.
High Background Present for the Blank	Over development of color.	Decrease the development time in step 11.3.1 and measure absorbance as quickly as possible.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for KDM6A/KDM6B protein extraction. Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.

Problem	Cause	Solution
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated. The sample can be titrated to determine the optimal amount to use in the assay.

Problem	Cause	Solution
No signal or weak signal only in sample wells	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80ºC, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of KDM6A/KDM6B 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 color development	Insufficient washing of the wells.	Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible.
Uneven color development	Delayed color development or delayed stopping of color development in the wells.	Ensure color development solution is added sequentially and consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).

14. NOTES

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