ab113464 – KDM5 JARID Activity Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of activity/inhibition of total KDM5/JARID using nuclear extracts or KDM5/JARID subtypes (JARID1A-JARID1D) using purified enzymes from a broad range of species

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

Version 1 Last Updated 5 February 2019

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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 H3-K4 seems to be of particular significance, as it is associated with active regions of the genome. H3K4 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 H3K4 specific histone demethylase, LSD1 (BHC110, KDM1) and JARIDs have been identified. The JARID family, except JARID2 (JARID1A, JARID1B, JARID1C and JARID1D), can remove tri-methylation from H3K4. JARID demethylases are Jumonji-domain proteins and catalyze the removal of methylation by using a hydroxylation reaction with a requirement of iron and alpha-ketoglutarate as cofactors.

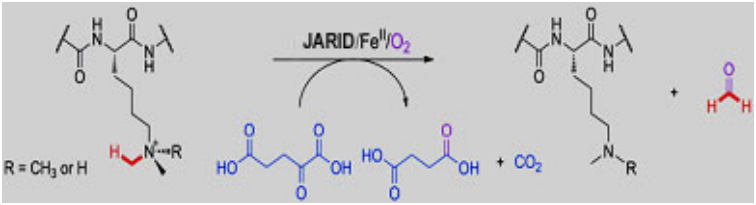


Figure 1. Histone H3-K4 demethylation reaction catalyzed by JARID demethylase.

JARIDs function as transcription repressors and might participate in different biological processes through recruitment to different chromosomal regions and differing enzymatic activities. JARID demethylases are also found to have potential oncogenic functions. For example, all 5 members of JARID are overexpressed in bladder cancer, and may promote cancer progression by regulating E2F expression. Increased JARID1A expression is observed in gastric cancer and its inhibition triggers senescence of malignant cells. Detection of activity and inhibition of JARID would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing and benefiting cancer diagnostics and therapeutics.

INTRODUCTION

Prior to this kit, there was only one method used for detecting JARID activity/inhibition. This method is based on the measurement of formaldehyde release, a by-product of JARID enzymatic reaction and has significant weaknesses: (1) a large amount (at µg level) of substrate and enzyme are required; (2) nuclear extracts from cell/tissues cannot be used; (3) redox-sensitive JARID inhibitiors are not suitable for testing with this method; (4) high interference 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 JARID-converted demethylated products. The KDM5/JARID Activity Quantification Assay Kit (Fluorometric) addresses all of 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 JARID activity via a straightforward detection of JARID-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 JARID proteins (including JARID1A, JARID1B, JARID1C, and JARID1D) can be used, which allows for the detection of inhibitory effects of JARID inhibitors in vivo and in vitro.
- Sensitivity is up to 2,000 times higher than formaldehyde release-based JARID assays, allowing activity to be colorimetrically detected from as low as 5 ng of purified JARID enzyme.
- Demethylated H3K4 standard is included, allowing specific activity of JARID to be quantified.
- Accurate, reliable, and consistent with extremely low background signals.

In ab113464 a tri-methylated histone H3K4 substrate is stably coated onto microplate wells. Active JARIDs bind to the substrate and remove methyl groups from the substrate. The JARID-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 fluorescence in a fluorescent microplate reader at 530 excitation and 590 emission. The activity of the JARID enzyme is in turn proportional to the relative fluorescent units measured.

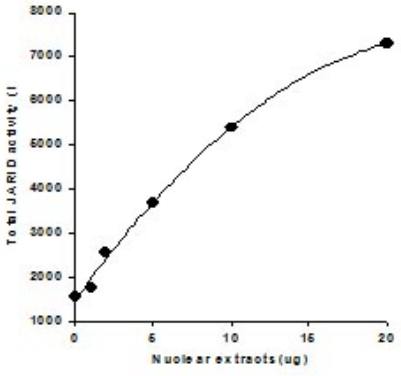
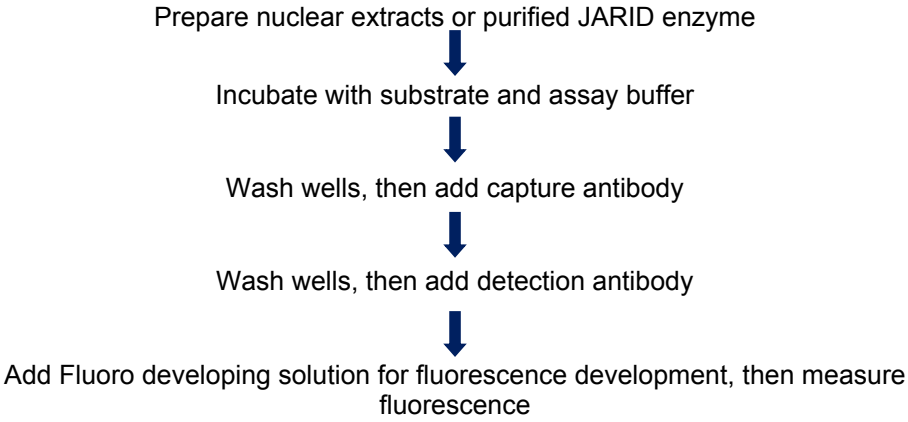


Figure 2. Demonstration of high sensitivity of the KDM5/JARID activity assay achieved by using A549 nuclear extracts with ab113464.

ab113464 is suitable for measuring activity or inhibition of total JARID using nuclear extracts or subtype JARID (JARID1A-JARID1D) purified enzymes from a broad range of species such as mammalians, plant, fungal, and bacterial types, in a variety of forms including cultured cells and fresh tissues. Input materials can be nuclear extracts or purified JARID enzymes. The amount of nuclear extracts for each assay can be 0.5 µg - 20 µg with an optimal 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.

The JARID assay standard (demethylated histone H3-K4) is provided in this kit for the quantification of JARID enzyme activity. Because JARID 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.

2. ASSAY SUMMARY



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

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.

5. MATERIALS SUPPLIED

Item	24 Tests	48 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
Assay Buffer	4 mL	8 mL	RT
Substrate, 50 µg/mL*	60 µL	120 µL	-20°C
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
Fluoro Developer*	10 µL	20 µL	-20°C
Fluoro Enhancer*	10 µL	20 µL	4°C
Fluoro Diluter*	4 mL	8 mL	RT
Co-factor 1	30 µL	30 µL	4°C
Co-factor 2	30 µL	30 µL	4°C
Co-factor 3	30 µL	30 µL	4°C
8-Well Assay Strip (with Frame)	6	12	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
- Fluorescence microplate reader capable of reading fluorescence at 530 excitation and 590 emission nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Plate seal
- 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 13 mL of 10X Wash Buffer to 117 mL of distilled water and adjust pH to 7.2-7.5. 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 Fluorescence Development Solution

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer to every 500 µL of Fluoro Diluter.

9.6 Suggested Standard Curve Preparation

Dilute 1 µL of Assay Standard in 9 µL of Assay Buffer for a final Standard concentration of 5 ng/µL. Prepare five concentrations by combining the 5 ng/µL Assay Standard with Assay Buffer into final concentrations of 0.2, 0.5, 1, 2 and 5 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	24.0	0.2
2	1.0	9.0	0.5
3	1.0	4.0	1.0
4	2.0	3.0	2.0
5	4.0	0.0	5.0

Note: *Keep each of the diluted solutions (except Diluted 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.*

9.7 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)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL
Assay Buffer	50 µL	400 µL	800 µL	2400 µL
Substrate	1 µL	8 µL	16 µL	50 µL
Assay Standard	N/A	N/A	1 µL (optional)	2 µL
Diluted Capture Antibody	50 µL	400 µL	800 µL	2400 µL
Diluted Detection Antibody	50 µL	400 µL	800 µL	2400 µL
Fluoro Developer	0.05 mL	0.4 mL	0.8 mL	2.4 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 JARID enzyme should be stored at –80°C until use.

11. PLATE PREPARATION

The suggested strip-well plate setup for the JARID 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 0.2 ng	Assay Standard 0.2 ng	Sample	Sample	Sample	Sample
C	Assay Standard 0.5 ng	Assay Standard 0.5 ng	Sample	Sample	Sample	Sample
D	Assay Standard 1.0 ng	Assay Standard 1.0 ng	Sample	Sample	Sample	Sample
E	Assay Standard 2.0 ng	Assay Standard 2.0 ng	Sample	Sample	Sample	Sample
F	Assay Standard 5.0 ng	Assay Standard 5.0 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

12. ASSAY PROCEDURE

12.1 **Enzymatic Reaction**

12.1.1 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).

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

12.1.3 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).

12.1.4 Sample Wells Without Inhibitor: Add 44 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 JARID enzyme to each sample well without inhibitor. Total volume should be 50 µl per well.

12.1.5 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 JARID 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.

12.1.6 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 JARID activity. In general, 60-90 min incubation is suitable for active purified JARID 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.*

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

12.2 **Antibody Binding & Signal Enhancing**

12.2.1 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.

12.2.2 Remove the Diluted Capture Antibody solution from each well.

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

12.2.4 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.

12.2.5 Remove the Diluted Detection Antibody solution from each well.

12.2.6 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.*

12.3 **Signal Detection**

12.3.1 Add 50 µl of Fluoro Developer to each well and incubate at room temperature for 2 - 4 min away from direct light. The Fluoro Developer will turn pink in the presence of sufficient demethylated products.

12.3.2 Read the fluorescence on a fluorescence microplate reader within 2 - 10 min at Ex/Em = 530/590 nm.

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

13. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Calculate JARID activity or inhibition using the following formulae:

$$\text{JARID activity (RFU/min/mg)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{(Protein Amount (µg)* x min**)}} \times 1000$$

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

Example calculation:

Average RFU of sample is 6800

Average RFU of blank is 800

Protein amount is 5 µg

Incubation time is 2 hours (120 minutes)

JARID activity = [(6800 – 800) / (5 x 120)] x 1000
= 10000 RFU/min/mg

For accurate or specific activity calculation:

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

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

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

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

*Incubation time (minutes) at step 11.1.6.

For inhibition calculation:

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

14. 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 fluorescence reading.	Check if appropriate fluorescent wavelength (Ex/Em = 530/590 nm filter) 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
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in Section C step 3.	Ensure a sufficient amount of standard is added.

Problem	Cause	Solution
	The standard is degraded due to improper storage conditions.	Follow the Storage guidance of this User Guide for storage instructions of (JARID 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.
	Incubation time with Diluted EJA6 is too long	The incubation time at section 12.2.4 should not exceed 45 min.
	Over development of color.	Decrease the development time in 12.3.1 and measure absorbance as quickly as possible.

Problem	Cause	Solution
No signal or weak signal only in sample wells	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in 12.1. 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 –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 JARID 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.
	Delayed fluorescence development in the wells.	Ensure fluorescence 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).

15. NOTES

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