ab113459 – KDM1/LSD1 Activity Quantification Kit (Colorimetric)

Instructions for Use

For the measurement of activity/inhibition of KDM1/LSD1 using nuclear extracts or purified enzymes from a broad range of species

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

Version 7 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 H3K4 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. LSD1 can remove di- and mono-methylation from H3K4 by using an amine oxidase reaction. LSD1 is associated with complexes that function as both transcriptional inactivators and activators. It demethylates mono-/di-methyl H3K4 when associated with the Co-REST complex at neuronal genes, or mono-/di-methyl H3-K9 when associated with the androgen receptor.

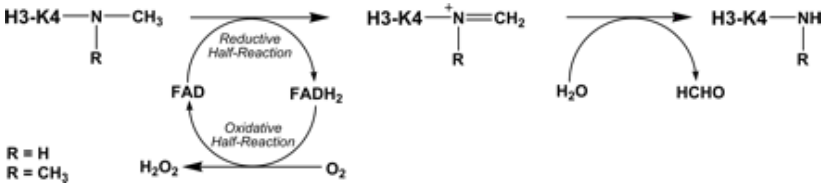


Figure 1. Histone H3-K4 demethylation reaction catalyzed by LSD1.

LSD1 is found to be pivotal in development and differentiation. For example, this enzyme is required to induce skeletal muscle differentiation, and mutation of drosophila LSD1 results in tissue-specific defect in development through disrupting H3K4 methylation. LSD1 is also shown to participate in regulation of chromatin remodeling, cell death and global DNA methylation. More importantly, LSD1 is found to be involved in some pathological processes such as cancer and inflammatory diseases. For example, expression of LSD1 is observed in cancer and LSD1 triggers MYC and E2F-mediated transcription in cancer cells. Detection of activity and inhibition of LSD1 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 LSD1 activity/inhibition. These methods are based on the measurement of H_2O_2 or formaldehyde release, a by-product of LSD1 enzymatic reaction and have significant weaknesses including: (1) Large amount (at µg level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues cannot be used; (3) Redox-sensitive LSD1 inhibitiors are not suitable for testing with these methods; (4) Highly interfered by DMSO and thiol-containing chemicals, which are often contained in enzyme solution or tested compound solvents; and (5) Less accuracy than direct measurement of LSD1-converted demethylated product. These problems were averted with ab113457 a popular assay method for LSD1 activity/inhibition.

ab113459 retains the simplicity, rapidness, high throughput, and non-radioactivity featured in the previous version, and has the following advantages:

- Strip-well microplate format makes the assay flexible and quick: manual or high throughput analysis that can be completed within 3 hours.
- Enhanced kit composition enables background signals to be extremely low, which allows the assay to be more accurate, sensitive, reliable, and consistent.
- Innovative colorimetric assay directly measures LSD1 activity by a straightforward detection of LSD1-converted demethylated product, rather than by-products. Thus it eliminates assay interferences caused by thiol-containing chemicals such as DTT, GSH, and 2-mercaptoethanol.
- Both cell/tissue extracts and purified LSD1 can be used, which allows for the detection of inhibitory effects of LSD1 inhibitor *in vivo* and *in vitro*.
- Novel assay principle allows high sensitivity to be achieved. The activity can be detected from as low as 5 ng of purified LSD1

enzyme, which is about 20 fold higher than that obtained by H_2O_2/formaldehyde release-based LSD1 assays.

- Demethylated H3K4 standard is included, which allows the specific activity of LSD1 to be quantified.

The KDM1/LSD1 Activity Quantification Assay Kit (Colorimetric) contains all reagents necessary for the measurement of KDM1/LSD1 activity/inhibition. In this assay, di-methylated histone H3K4 LSD1 substrate is stably coated onto the strip wells. Active LSD1 binds to the substrate and removes methyl groups from the substrate. The LSD1-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 colorimetric microplate reader at a wavelength of 450 nm. The activity of LSD1 enzyme is proportional to the optical density intensity measured.

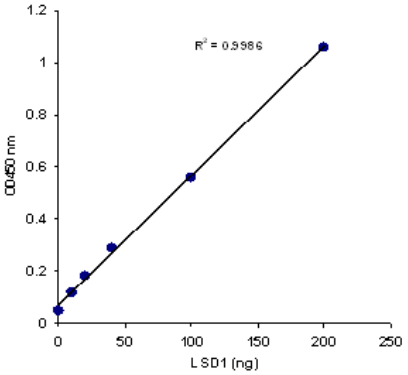


Figure 2. Demonstration of high sensitivity of KDM1/LSD1 activity assay achieved by using recombinant KDM1/LSD1 with ab113459.

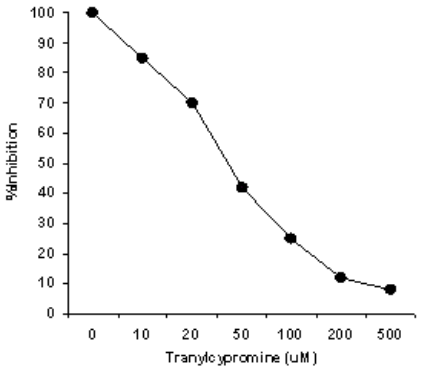


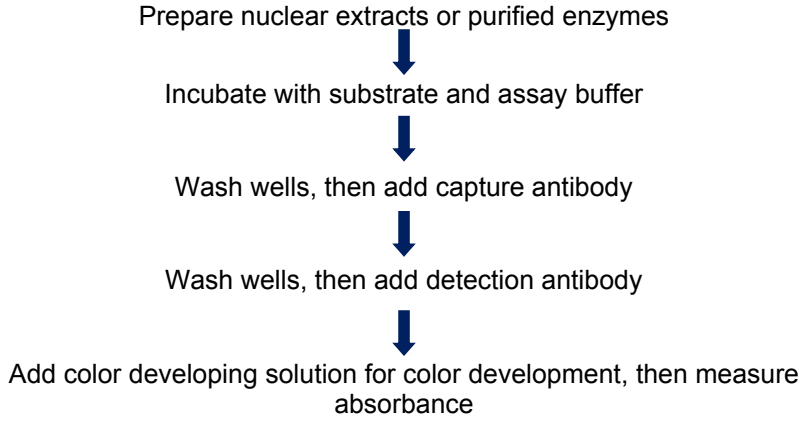
Figure 3. Demonstration of inhibitory effects of a KDM1/LSD1 inhibitor detected by ab113459.

ab113459 is suitable for measuring LSD1 activity/inhibition using nuclear extracts or 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 LSD1 from recombinant proteins or isolated from cell/tissues.

The LSD1 assay standard (demethylated histone H3K4) is provided in this kit for quantification of LSD1 enzyme activity. Because LSD1 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.

Δ Note: This kit may cross react with LSD2.

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.

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

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
LSD1 Assay Buffer	4 mL	8 mL	RT
LSD1 Substrate, 50 µg/mL*	60 µL	120 µL	-20°C
LSD1 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
LSD1 Inhibitor Tranylcypromine, 1 mM*	20 µL	40 µL	4°C
Developer Solution*	5 mL	10 mL	4°C
Stop Solution*	5 mL	10 mL	RT
8-well assay strips (with 1 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
- 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

Prepare Diluted 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

Prepare 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

Prepare 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 this Diluted Detection Antibody will be required for each assay well.

9.4 Diluted Assay Standard Solution

Suggested Standard Curve Preparation: First, dilute LSD1 Assay Standard with LSD1 Assay Buffer to 5 ng/µL by adding 1 µL of LSD1 Assay Standard to 9 µL of LSD1 Assay Buffer. Then, further prepare five concentrations by combining the 5 ng/µL diluted LSD1 Assay Standard with LSD1 Assay Buffer into final concentrations of 0.2, 0.5, 1, 2, and 5 ng/µL according to the following dilution table:

Tube	5ng/µL LSD1 Assay Standard (µL)	LSD1 Assay Buffer (µL)	Resulting LSD1 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 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.*

9.5 **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 (3 strips)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL
LSD1 Assay Buffer	50 µL	400 µL	800 µL	2400 µL
LSD1 Substrate	1 µL	8 µL	16 µL	50 µL
LSD1 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
Developer Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL
Stop solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be 0.5 µg – 20 µg with optimized range of 5-10 µg. The amount of purified enzymes can be 5 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 LSD1 enzyme should be stored at –80°C until use.

11. PLATE PREPARATION

The suggested strip-well plate setup for the LSD1 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 LSD1 Assay Buffer and 1 µL of LSD1 Substrate to each blank well.

12.1.3 Standard Wells: For a standard curve, add 49 µL of LSD1 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 chart in Section 9).

12.1.4 Sample Wells Without Inhibitor: Add 45 µL - 48 µL LSD1 Assay Buffer, 1 µL of LSD1 Substrate, and 1 - 4 µL of your nuclear extracts or 1 - 4 µL of your purified LSD1 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 LSD1 Assay Buffer, 1 µL of LSD1 Substrate, 1 to 4 µL of your nuclear extracts or 1 to 4 µL of your purified LSD1 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 LSD1 Assay Buffer at a 1:10 ratio (e.g., add 0.5 µL of inhibitor to 4.5 µL of LSD1 Assay Buffer), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

The LSD1 inhibitor, Tranylcypromine included in the kit can be used as a 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 LSD1 activity. In general, 60-90 min incubation is suitable for active purified LSD1 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 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 methylated DNA.

12.3.2 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.*

13. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Calculate LSD1 activity or inhibition using the following formulae:

$$\text{LSD1 activity (OD/min/mg)} = \frac{\text{Sample OD} - \text{Blank OD}}{\text{(Protein Amount (µg)* x min**)}} \times 1000$$

*Protein amount (µg) added into the reaction at step 12.1.4.
**Incubation time (minutes) at step 12.1.6.

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)

LSD1 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 LSD1 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 LSD1-converted demethylated product using the following formulae:

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

$$\text{LSD1 activity (ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{\text{(Protein Amount (µg) x min*)}} \times 1000$$

*Incubation time (minutes) at step 12.1.6.

For inhibition calculation:

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

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 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 LSD1 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 LSD1 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 LSD1 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).

15. NOTES

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