Current Protocols in Pharmacology

Chapter 3

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In Vitro Enzyme Assays for JmjC-domain-containing Lysine Histone Demethylases (JmjC-KDMs)

Running Title: Inhibition assays on lysine histone demethylases

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Significance Statement

Histone lysine methylation patterns play important roles in chromatin formation and transcriptional regulation. Histone N-methyl lysine demethylases (KDMs) remove methylation marks from histone tails so modulating gene expression. Some KDMs are current drug targets due to their roles in tumorigenesis. KDM mutations have also been linked to mental retardation, developmental defects and other pathologies. Consequently, there are significant efforts aimed at developing selective KDM inhibitors of both the lysine specific demethylases (LSD1) and JmjC-domain-containing KDM subfamilies. It is thus critical to develop efficient and robust assay protocols for KDMs, including those suitable for high-throughput screening.
ABSTRACT

Histone modifications, including lysine methylation marks on histone tails, modulate the accessibility of genes for transcription. Changes in methylation patterns in histone tails can modulate transcriptional activation or repression. The dynamic regulation of lysine methylation patterns is enabled by two distinct groups of enzymes: histone methyltransferases (KMTs) and demethylases (KDMs). The Jumonji C (JmJC) domain-containing lysine histone demethylases (JmJC-KDMs) modulate the methylation levels of histone tails by removing tri-, di, or mono-lysine methylation marks. JmJC-KDMs activities are dysfunctional in cancer and in other diseases, and some JmJC-KDMs are drug targets. Efforts have been made towards development of high-throughput assays for identification of selective small-molecule inhibitors of KDMs. The present unit describes protocols for mass spectrometry-based and formaldehyde dehydrogenase-coupled enzyme-based assays for the JmJC-KDMs.

Keywords: histone demethylase, JmJC KDMs, 2-oxoglutarate, oxygenase, epigenetics, mass spectrometry screening, transcriptional regulation.
INTRODUCTION

Post-translational modifications to the N-terminal tails of histones play important roles in many cellular functions and in the regulation of transcription (Hojfeldt, Agger, & Helin, 2013; Mosammaparast & Shi, 2010). The Nε-amino groups of histone tail lysines can be mono-, di- or tri-methylated. Tri-methylation of H3 lysine H3K4 and H3K36 is generally associated with transcriptional activation, while methylation of H3K9, H3K27, and H4K20 often results in transcriptional repression. In eukaryotes removal of the histone methylation marks is mediated via lysine histone demethylases (KDMs) from two distinct families: the flavin adenine dinucleotide (FAD)-dependent demethylases LSD1/2 (KDM1 family) and the Jumonji C (JmjC) domain containing demethylases (KDM2-7)(Klose, Kallin, & Zhang, 2006). The JmjC KDMs belong to the superfamily of Fe(II)/2-oxoglutarate (2OG)-dependent oxygenases, which use Fe(II) as a cofactor and O2 and 2OG as co-substrates. Conversion of 2OG to succinate and CO2 is coupled to oxidation of Nε-methylated lysine residues in histone lysine residues to hemiaminal intermediates, spontaneous decomposition of which results in formation of the corresponding demethylated lysine residue and formaldehyde (Figure 1). There are six human JmjC subfamilies of KDMs, most of which have multiple domains and manifest different sequence and Nε-methylation state selectivities towards their histone substrates (see (Hojfeldt et al., 2013; Kaniskan, Martini, & Jin, 2017; McAllister et al., 2016) for reviews on substrate specificity and inhibitors of KDMs).

Due to roles of KDMs in cancer development and progression and changes in histone methylation patterns associated with cardiovascular disease and mental retardation, JmjC-
KDMs are active drug targets (Cloos, Christensen, Agger, & Helin, 2008; Rotili & Mai, 2011). A number of small-molecule JmjC-KDM inhibitors have been developed, many of which contain metal-chelating groups and which are competitive with respect to 2OG (McAllister et al., 2016). A challenge in the development of JmjC-KDMs inhibitors is achieving selectivity over other human 2OG oxygenase and KDM subfamilies. Sensitive and robust assay platforms for screening inhibitor libraries are thus required. Here we describe two orthogonal assays which are routinely used for screening inhibitors against JmjC-KDMs: mass spectrometry (MS)-based end-point assays (Basic Protocol 1) and a fluorescence-based continuous assay (formaldehyde dehydrogenase (FDH) assay; Basic Protocol 2) which is amenable to a high-throughput plate reader format.

**STRATEGIC PLANNING**

Most JmjC-KDM assays have two elements: (i) the preparation of assay solutions and incubation of the JmjC-KDM with histone-derived substrates in the presence of cofactors/co-substrate and, where appropriate, an inhibitor; and (ii) analysis of the reaction progress, which can be carried out using different methodologies. In this Unit we describe JmjC-KDM assays employing MS-based determination of substrate/product ratios (matrix assisted laser desorption/ionization – time of flight MS (MALDI-TOF-MS) and LC-MS methods) and fluorescence-based enzyme-coupled formaldehyde detection (FDH coupled assay). We also make some general points on JmjC-KDM assay development and emphasize the need for appropriate controls.
The first consideration in assay development and optimization is normally choice of the assay reaction buffer and pH. It is important to use buffers compatible with the detection method. Low concentrations of JmjC-KDM in solution may require use of agents, e.g., high salt concentration or detergent (e.g., Tween 20) to stabilise the protein. These agents can interfere with crystallization of the MALDI matrix in MALDI-TOF-MS assays and should be avoided where possible. Matrix crystallization is relatively poor at high pH (Asakawa, Cai, & Liu, 2013). Thus the use of a volatile acid, e.g. formic acid, is preferred for reaction quenching, i.e., to halt the reaction by reducing the pH to improve matrix crystallization conditions. Therefore, the MS assays described in this unit are carried out in 50 mM HEPES and the reactions are quenched by 1% formic acid.

With the FDH coupled assays (Lizcano, Unzeta, & Tipton, 2000), interference with the coupled enzyme FDH must be avoided. Components of the assay mixture should therefore be tested with the FDH conditions prior to optimization for the JmjC-KDM reaction. Controls for inhibition of FDH activity by tested compounds should be carried out.

The stability of assay components is also a consideration in assay development. For example, thawed solutions of the JmjC-KDM need to be kept at 4 °C prior to the reaction (preferably as aliquots stored at -80 °C, that have been thawed immediately prior to use) to avoid enzyme inactivation. Preferably, the metal content of the JmjC KDM should be defined, especially if metal-affinity purification has been employed. Solutions of the redox-sensitive cofactors and co-substrates must be freshly prepared in appropriate buffers immediately before use in assays to avoid compromising results due to compound/cofactor degradation (Sakurai et al., 2010).
Therefore, sufficient time needs to be allocated to the preparation of stock solutions. Assay development with limited amounts of enzyme should involve practice in preparation of the necessary reagents, ideally using a more readily available 2OG oxygenase.

The conditions of the inhibitor screens needs to be selected based on knowledge of kinetic parameters of the JmjC-KDM (Copeland, 2005; Hopkinson, Tumber, Yapp, et al., 2013). Thus, after initial optimization of conditions (buffer, pH, temperature, additives), steady-state kinetics studies need to be performed. A scheme for assay development is given in Figure 2.

**BASIC PROTOCOL 1**

**MASS SPECTROMETRY-BASED JmjC-KDM ASSAYS**

This protocol describes the use of MALDI-TOF MS-based assays for JmjC-KDM inhibition screens. The procedure involves incubation of co-substrate mixtures with and without an inhibitor. The protocol uses the JmjC histone demethylase KDM4A as an example; however, it can be readily adopted for use with other JmjC-KDMs (for a list of JmjC-KDMs and appropriate constructs see www.thesgc.org/science/constructs (Rose et al., 2012)). Expression constructs for production of KDM4A (Ng et al., 2007) are available from Addgene (www.addgene.org) (Plasmid 38846). After the enzymatic reaction is quenched with 1% (v/v) aqueous formic acid the MALDI-TOF-MS or LC-MS analysis (e.g., using an Agilent RapidFire RF360 coupled to an Agilent 6530 Q-TOF MS) is performed to determine the ratio of modified/unmodified substrate (typically a histone fragment sequence). Initial time courses should be run to confirm the
activity, the appropriate concentration of the enzyme, and to determine the linear range and optimal reaction time for inhibitor assays. If appropriate and possible, active site titrations can be carried out to determine the concentration of active enzyme (Copeland, 2005). Note that the concentrations of the enzyme and substrates used will vary depending on the assay format (e.g., due to differences in assay sensitivities, H3 peptide optimized for specific LC column). In the example below, 2,4-pyridine dicarboxylic acid (2,4-PDCA), a known broad spectrum JmjC-KDM inhibitor (Rose et al., 2008), is used as a positive control (Figure 3, 4). Compounds libraries are typically stored in purified DMSO (concentrations between 10 mM and 100mM); the range of final assay concentrations of compound is recommended to be ≤ 1% DMSO (v/v). Higher DMSO concentrations can interfere with the enzyme activity as well as with MS analysis.

**Materials**

The assay uses the following components (King et al., 2010):

Recombinant KDM4A (residues 1-359) protein produced in *E. coli* and purified in-house as described (Ng et al., 2007); see www.thesgc.org/structures/2OQ7 for detailed expression and purification protocol. Store aliquots of stock solutions (200 μM) in 20mM HEPES (pH7.5) 500mM NaCl in 5% glycerol at -80°C (≥ 1 year)

H3(1-21) K9me3 peptide (ARTKQTAR-Kme3-STGGKAPRKQLA-NH₂, prepared by solid phase peptide synthesis or purchased (e.g. Anaspec, GL Biochem)

50 mM HEPES buffer, pH7.5 (Fisher Scientific BP310-1)
100mM Fe(II) stock and 100 μM working solutions: (NH₄)₂FeSO₄·6H₂O (Aldrich 203505)

L-ascorbic acid (Asc) (Sigma-Aldrich A92902)

Disodium 2-oxoglutarate (2OG) (Aldrich 75892)

2,4-pyridine dicarboxylic acid (2,4-PDCA) (Sigma 04473)

1% formic acid (w/w) (prepared from >96.0% formic acid Sigma-Aldrich 251564)

96-well polypropylene v-bottomed plates (such as Sigma M8185) for MALDI assay (384-well polypropylene v-bottomed plates (Greiner Bio One) for RapidFire MS assay)

MALDI 96-target plates (Waters, UK)

α-cyano-4-hydroxycinnamic acid (CHCA) MALDI matrix (Sigma C8982)

10x inhibitor in DMSO (10-fold concentration of the final test inhibitor concentration evaluated in the assay)

Aluminum plate seal (Greiner-Bio One 67690)

Equipment: MALDI-TOF-MS (Waters MALDI Micro MX) or RAPIDFIRE RF360 coupled to an Agilent 6530 Accurate Mass Q-TOF.

Protocol Steps:

Preparation of stock solutions and assay mixtures:
Step 1: Dissolve 1 mg peptide (prepared as a C-terminal amide) in MilliQ-water to a 10 mM concentration to obtain the stock solution.

*Aliquotted stock solutions should be stored at -20 °C for short-term storage or -80 °C for long-term storage. Dilute 10 mM solution with 50 mM HEPES buffer (pH 7.5) to obtain the 1 mM working solution.*

Step 2: Prepare 100 mM stock solutions of Asc in 50 mM HEPES buffer (pH 7.5), then dilute to 1 mM with 50 mM HEPES buffer (pH 7.5).

Step 3: Prepare 100 mM stock solutions of 2OG in 50 mM HEPES buffer (pH 7.5), then dilute to 1 mM with 50 mM HEPES buffer (pH 7.5).

Step 4: Prepare 100 mM stock solution of Fe(II) in 20 mM HCl; to obtain a working solution. Further dilute the 100 mM stock solution to 1 mM with Milli-Q water.

Step 5: Prepare the substrate mixture using working stock solutions of peptide, 2OG, Fe(II), Asc to achieve the concentrations shown in Table 1. Scale up for the required number of reactions.

Step 6: Prepare the enzyme mixture as in Table 1. Scale up for the required number of the reactions. Keep the enzyme mixture on ice until needed.

*Inhibition assay procedure:*

Step 7: Place aliquots of inhibitors (typically in up to 10% (v/v) DMSO in H₂O) in a 96-well plate.

Add the enzyme mixture (10 µL) and incubate for 15 min at room temperature. Include a control (10% (v/v) DMSO in H₂O instead of inhibitor) to determine the maximal enzyme activity.
A control is used to control for non-specific modifications to the peptide.

Step 8. Initiate the reaction by addition of the peptide mixture (12.5 μL).

Step 9: Add 25 μL of 1% (v/v) aqueous formic acid at defined quenching time point (usually 5-10 min).

Step 10: Seal the 96-well plate (e.g., aluminum plate seal) and store at -20 °C prior to MALDI-TOF-MS analysis (if the latter is performed on a different day). Otherwise proceed with the analysis.

MALDI-TOF-MS detection and analysis of the results:

Step 11. Spot 1 μL of CHCA matrix and then 1 μL of the sample onto MALDI target plate and mix by gentle pipetting on the plate. Allow sample spots to dry completely (10 – 15 min).

Step 12. Acquire MALDI spectra on MALDI-TOF-MS in the reflectron positive mode.

Step 13. Process MALDI spectra using MASSLYNX4.0 software to determine the relative intensities of the methylated and demethylated peptide peaks.

Step 14. Calculate the % demethylation using using the equation \( \% = \frac{l_2}{l_1+l_2} \times 100 \% \), where \( l_1 \) and \( l_2 \) are intensities of H3K9me3 and H3K9me2 peaks in the MALDI spectrum, respectively.

Step 15. Normalise the results to the control.

BASIC PROTOCOL 2

FDH-COUPLED DEMETHYLASE ASSAY
This protocol describes an inhibitor-screening assay using the FDH enzyme-coupled method. The assay is based on indirect determination of demethylation rates by coupling the reaction coproduct, formaldehyde, with a formaldehyde dehydrogenase (FDH) -catalyzed reaction. Formaldehyde is oxidised to formic acid by FDH, with simultaneous reduction of the β-nicotinamide adenine dinucleotide hydrate (NAD⁺) cosubstrate to NADH. The fluorescence of NADH (excitation 355 nm, emission 460 nM) can be monitored in real time using a plate reader (King et al., 2010; Lizcano et al., 2000; Rose et al., 2008).

**Materials**

The assay uses the following components (King et al., 2010):

Recombinant KDM4A (residues 1-359) protein produced in *E.coli* and purified in-house as described (Ng et al., 2007); see www.thescgc.org/structures/2OQ7 for detailed expression and purification protocol. Aliquots of stock solutions (200 μM) stored in 20mM HEPES (pH7.5) 500mM NaCl in 5% glycerol at -80°C.

FDH assay buffer: 50 mM HEPES (pH 7.5) + 0.01% (v/v) Tween 20 (Fisher Scientific BP310-1)

Fe(II) stock and working solutions: (NH₄)₂FeSO₄*6H₂O (Aldrich 203505)

L-ascorbic acid (Asc) (Sigma-Aldrich A92902)

H3(1-21)K9me3 peptide (ARTKQTAR-Kme3-STGGKAPKQLA-NH₂), prepared by solid phase peptide synthesis or purchased (e.g. Anaspec, GL Biochem)

Disodium 2-oxoglutarate (2OG) (Aldrich 75892)
Formaldehyde dehydrogenase from *Pseudomonas putida* (Sigma F1879)

NAD+: β-Nicotinamide adenine dinucleotide hydrate (Sigma-Aldrich N7004)

Inhibitors: 10x in water or in 10% DMSO (2,4-pyridine dicarboxylic acid (2,4-PDCA) (Sigma 04473))

Plates: 384 well microplate, black, µClear (Greiner Bio-One Ltd 781906)

Equipment: PHERAstar FS (BMG Labtech) with fluorescence filters (Ex 355nm / Em 460nM)

**Protocol steps:**

Step 1. Prepare the enzyme and substrate mixture as required (scale to the number of wells required and add a slight excess to account for pipetting losses) *(Table 2)*

Step 2. Place aliquots of the inhibitors in a 384-well plate. Include DMSO in control wells (no inhibitor control (100% activity, high value) and no-enzyme control (0% activity, low value).

Step 3. Add the enzyme mixture (10 µL) and incubate for 15 min at room temperature.

Step 4. Initiate the reaction by adding the substrate mixture (12.5 µL) and measure fluorescence (355ex/460em) at room temperature over time *(Figure 5)*.

Step 5. Measure the initial rate to calculate the slope (RFU/sec) and convert to µM/sec using formaldehyde standard curve with FDH (this changes with setup and instrument; see Support Protocol 1).

**SUPPORT PROTOCOL 1**
Determination of formaldehyde standard curve

This protocol describes determination of formaldehyde standard curve characterizing activity of FDH in the reaction of formaldehyde oxidation. This standard curve is required for quantification of formaldehyde produced in JmjC-KDM-catalyzed demethylation reaction and further determination of the initial rates of the demethylation reaction.

Materials

FDH assay buffer: 50 mM HEPES (pH 7.5) + 0.01% (v/v) Tween 20 (Fisher Scientific BP310-1)

Formaldehyde dehydrogenase from Pseudomonas putida (Sigma F1879)

NAD+: β-Nicotinamide adenine dinucleotide hydrate (Sigma-Aldrich N7004)

Formaldehyde solution 36.5-38% in H₂O (Sigma F8775)

Plates: 384 well microplate, black, µClear (Greiner Bio-One Ltd 781906)

Equipment: PHERAstar FS (BMG Labtech)

Protocol Steps:

Step 1. Prepare formaldehyde 10x stock solutions at different dilutions.

Step 2. Prepare FDH mixture and substrate mixture using the guidance provided in Table 3, scaled to the number of the required reactions.

Step 3. Transfer 12.5 μL of FDH mixture to 384-well plate and mix with 12.5 μL of the substrate mixture.
Step 4. Measure the fluorescence (355ex/460em) at room temperature over 20 min.

Step 5. Plot the RFU after 20 min against the formaldehyde concentration used to obtain the formaldehyde standard curve.

Step 6. Fit with linear function \((y=b*x+a)\) to obtain the relation between RFU and formaldehyde concentration.

**REAGENTS AND SOLUTIONS**

*Preparation of the buffer solution*

Dissolve HEPES to 0.5 M in Milli-Q water (18.2 MΩ cm at 25 °C), adjust pH to 7.5 with NaOH; filter through 0.2 μm filter. Dilute to 50 mM with Milli-Q water, add Tween 20 (for FDH-coupled assays).

*Preparation of the peptide solution*

Dissolve 1 mg of peptide in Milli-Q water (18.2 MΩ cm at 25 °C) to 10 mM, aliquot and store at -20 °C. Working stock 500 μM is prepared in the reaction buffer before the assay.

*Preparation of formaldehyde solutions*

Make aliquoted stock solutions of 60 units/1mL in FDH assay buffer, aliquot (50 – 100 μL) and store at -80 °C

*Preparation of formaldehyde solutions*

Dissolve formaldehyde in Milli-Q water to 4 % (w/w). Prepare a series of dilutions for the assay.
Preparation of CHCA matrix solution

Dissolve 5 mg of CHCA MALDI matrix in 500 μL of dilution solvent (70/30 (v/v) acetonitrile/water with 0.1% (v/v) TFA) to obtain saturated solution (10 mg/mL). Note: the matrix solution is light sensitive and should not be stored exposed to light.

COMMENTARY

Background Information

Biochemical assays that are used to identify potential inhibitors in the early stages of drug discovery can be classified based on the assay format and the detection method. Several assay methods have been developed to assess JmJC-KDM activity / inhibition. These include direct detection of histone peptide demethylation using MS methods (LC-MS, MALDI-TOF-MS, RapidFire) (Chen et al., 2007; Hutchinson et al., 2012; Ng et al., 2007; Whetstine et al., 2006), formaldehyde detection employing coupling with FDH (Lizcano et al., 2000; Sakurai et al., 2010), immunodetection with bulk histones using methylation status and site-specific antibodies (R. J. Klose et al., 2006; Tsukada et al., 2006), 2OG turnover assays (NMR(Hopkinson, Hamed, Rose, Claridge, & Schofield, 2010), [14C]-radioactive assays (Loenarz et al., 2010)), and antibody based assays employing luminescence detection (AlphaScreen) (Kawamura et al., 2010; Rose et al., 2012). The reported JmJC-KDM assays have their advantages and disadvantages, and the choice of assay is often dependent on the throughput and sensitivity required, reagent availability, access to appropriate equipment (NMR, MS), cost / development time issues, as well as the questions that are being asked. Continuous assays, such as FDH assays, are useful for detailed
kinetics, whereas MS-based assays are discontinuous, but substrate and product methylation states can be directly resolved. To ensure that the results display the correct ranking of the inhibitor potencies, it is preferable to employ an assay capable of providing information on both the kinetics of the enzymatic reaction and, if even in modified format, the inhibitor mode of action.

Peptides representing histone fragments containing the modifications of interest are widely used as model substrate for KDMs in inhibition assays. Therefore, MS methods have broad application for determination of the peptide demethylation status in the enzymatic reaction and direct analysis and quantification of peptide modification. These assays are preferred to assays monitoring co-substrate turnover, since they directly provide information on KDM activity by monitoring -14 Da mass shift(s). MALDI-TOF-MS and RapidFire have been extensively used for both kinetic studies on histone demethylases and inhibitor screening (Hopkinson, Tumber, & Yapp, 2013; Johansson et al., 2016; King et al., 2010).

**Critical Parameters**

Concentrations of the enzyme and the substrate are critical parameters that can affect results. IC\textsubscript{50} values depend on the enzyme concentration and conditions of the assay (Copeland, 2005). Thus, the IC\textsubscript{50} differences obtained using the MALDI-TOF MS and RapidFire MS methods can, in part, be attributed to the differences in KDM4A concentrations (2 μM and 0.15 μM, respectively). Substrate concentration of greater 2 times the K\textsubscript{M} should be normally be used to ensure saturating conditions. Lower substrate concentrations may be preferred for assays where the inhibitors are intended to compete for binding with the substrate for binding (i.e., at
the $K_M$ of the substrate). Concentrations of cofactors can also affect assay results. Therefore, the redox-sensitive Fe(II) and Asc concentrations need to be freshly prepared, optimized and kept at saturating levels.

For the FDH-coupled assay, it is crucial to check that the inhibitor does not inhibit the FDH enzyme activity. This can be confirmed by adding the highest concentration of the test inhibitor to the FDH reaction at a single formaldehyde concentration (recommendation 10 $\mu$M) (Sakurai et al., 2010) to test if the fluorescence signal is reduced. If FDH is inhibited, another assay format is required to assess KDM inhibition activity. Careful consideration should be given to choice of buffer since buffers containing primary alcohols (e.g., Tris) can be turned over by FDH.

**Troubleshooting**

Altered stability of the JmjC-KDM in solution (including by inhibitor binding) can alter KDM activity. KDM activity can be assessed by comparison of the results with control samples. Where available, potent inhibitors (e.g. where IC$_{50}$ is comparable to enzyme concentration used in the assays) should be used to carry out active site titrations (Copeland, 2005).

Instrument related issues (e.g., absence of the instrument signal or high signal-to-noise ratio) need to be resolved according to the instrument manufacturer procedures.

MS spectra of the no-enzyme controls need to be thoroughly analyzed to identify peptide purity, as well as possibility of non-specific modifications to the peptide, that can interfere with the observed results. Note non-enzymatic oxidation of some residues can be sometimes observed under the assay conditions for 2OG oxygenases (e.g., of methionine to methionine...
sulfoxide) (Jaakkola et al., 2001) and needs to be controlled for, including by using no-enzyme controls (in some circumstances substitution of a redox-sensitive residue may be required). Low peptide ion abundance in the MS spectrum can be improved by optimization of sample preparation procedure (e.g., choice of MALDI matrix, matrix co-crystallisation conditions) or spectra acquisition parameters (e.g., optimisation of the MALDI laser energy).

**Understanding Results**

Comparison of IC$_{50}$ values determined by different methods would most likely show higher values for MS-based methods compared to the FDH method due to higher concentrations of the enzyme required to observe modifications of the peptides. $K_i$ values should thus be determined where possible / appropriate. See prior reports (Chowdhury et al., 2011; Rose et al., 2012; Tarhonskaya et al., 2017) for further examples of IC$_{50}$ and $K_i$ value determination for 2OG competitive inhibitors of JmjC-KDMs.

**Time Considerations**

The assays typically take around 1-2 hours including preparation of the reagent solutions.

**ACKNOWLEDGEMENTS**

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LITERATURE CITED


Figure 1. Demethylation of methylated lysines by KDMs. Demethylation occurs via unstable hemiaminal intermediate, resulting in formaldehyde production and the demethylated product.

R = H or CH₃.
Figure 2. KDM assay development scheme.
Figure 3. KDM4A MALDI-TOF mass spectrometry assay. (A) An example of MALDI spectrum of KDM4A assay with H3(1-21)K9me3 in the presence and absence of an inhibitor (2,4-PDCA). (B) KDM4A time course of H3(1-21)K9me3 demethylation. (C) IC₅₀ determination from a 7-point, 2-fold dilution series of 2,4-PDCA, using the conditions in Table 1.
Figure 4. KDM4A RapidFire Mass Spectrometry Assay. Peptide substrate changes were monitored by electrospray ionization using a RapidFire RF360 connected to an Agilent QTOF. (A) IC\textsubscript{50} determination from an 11-point, 3-fold dilution series of 2,4-PDCA. Conditions: KDM4A 0.15\textmu M and histone H3(1-15)K9me3 10 \textmu M. (B) Mass spectrum of Histone H3(1-15)K9Me3 peptide from a KDM4A enzyme reaction in the presence of 100 \textmu M 2, 4-PDCA showing presence of the trimethyl peptide substrate (+3 charge state: m/z 510.954). (C) Mass spectrum from a KDM4A enzyme reaction in the presence of 0.5\% (v/v) DMSO showing depletion of the trimethyl peptide substrate and appearance of dimethyl peptide product (+3 charge state: m/z 506.282).
Figure 5: KDM4A Formaldehyde Dehydrogenase (FDH) Assay. (A) Enzyme progress curves for KDM4A monitoring demethylation of H3-(1-21)-K9Me3. KDM4A (150 nM) was incubated in the presence of 100 μM (○), 0.4 μM (△), 0.14 μM (▼) or 0.046 μM 2,4-PDCA (◇) and 0.5% DMSO control (□). The enzyme reaction was initiated by plate reader injection of substrate and the reaction monitored in real time in a BMG Labtech Pherastar plate reader (355 nM Excitation/450 nM Emission). (B) IC50 determination from an 11-point and 3-fold titration (100 μM – 0.0017 μM) of 2,4-PDCA.
Table 1. Example of Conditions for MALDI-TOF-MS JmjC-KDM assays.

<table>
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<tr>
<th></th>
<th>Stock concentration</th>
<th>Volume, μL</th>
<th>Final concentration</th>
</tr>
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<tbody>
<tr>
<td><strong>Enzyme</strong></td>
<td>KDM4A</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>mixture</strong></td>
<td></td>
<td></td>
<td>2 μM</td>
</tr>
<tr>
<td><strong>Buffer</strong></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Inhibitor</strong></td>
<td>x10 in 10% DMSO</td>
<td></td>
<td>X1</td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
<td>Peptide H3K9me3 (1-21)</td>
<td>500 μM</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>mixture</strong></td>
<td></td>
<td></td>
<td>50 μM</td>
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<tr>
<td>Fe(II)</td>
<td>100 μM</td>
<td>2.5</td>
<td>10 μM</td>
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<tr>
<td>Ascorbate</td>
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<td>2.5</td>
<td>100 μM</td>
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<tr>
<td>2OG</td>
<td>100 μM</td>
<td>2.5</td>
<td>10 μM</td>
</tr>
<tr>
<td><strong>Buffer</strong></td>
<td></td>
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</table>
Table 2. Conditions for FDH assay for the JmjC-KDM activity

<table>
<thead>
<tr>
<th></th>
<th>Stock concentration</th>
<th>Volume, μL</th>
<th>Final concentration</th>
</tr>
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<tbody>
<tr>
<td><strong>Enzyme mix</strong></td>
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<tr>
<td>KDM4A</td>
<td>1.5</td>
<td>2.5</td>
<td>0.15 μM</td>
</tr>
<tr>
<td>FDH</td>
<td>6 units/100 μL</td>
<td>2.5</td>
<td>0.025 units/assay</td>
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<tr>
<td><strong>Buffer</strong></td>
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<tr>
<td><strong>Inhibitor</strong></td>
<td>X10 in 10% DMSO</td>
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<td>X1</td>
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<tr>
<td><strong>Substrate mix</strong></td>
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</tr>
<tr>
<td>Peptide</td>
<td>500 μM</td>
<td>2.5</td>
<td>50 μM</td>
</tr>
<tr>
<td>H3K9me3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(II)</td>
<td>100 μM</td>
<td>2.5</td>
<td>10 μM</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>1 mM</td>
<td>2.5</td>
<td>100 μM</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>5 mM</td>
<td>2.5</td>
<td>500 μM</td>
</tr>
<tr>
<td>2OG</td>
<td>100 μM</td>
<td>2.5</td>
<td>10 μM</td>
</tr>
</tbody>
</table>
Table 3. Concentrations of the assay components for determination of the formaldehyde standard curve.

<table>
<thead>
<tr>
<th></th>
<th>Stock concentration</th>
<th>Volume, μL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FDH mix</strong></td>
<td>FDH</td>
<td>6 units/100 μL</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Substrate mix</strong></td>
<td>Formaldehyde x10</td>
<td>2.5</td>
<td>x1</td>
</tr>
<tr>
<td></td>
<td>NAD⁺ 5 mM</td>
<td>2.5</td>
<td>500 μM</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>7.5</td>
<td></td>
</tr>
</tbody>
</table>