



ARBOR  
ASSAYS

**DetectX<sup>®</sup>**

## **Histone Demethylase Fluorescent Activity Kit**

**Catalog Number K010-F1**

**Sample Types Validated:**

**LSD1-and *Jumonji*-type Demethylases**

**Patents Pending**

**Please read this insert completely prior to using the  
product.**

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

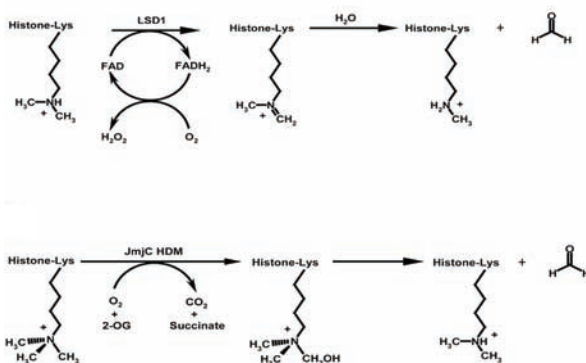
**[www.ArborAssays.com](http://www.ArborAssays.com)**

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## BACKGROUND

Formaldehyde is a common by-product formed in the oxidative demethylation of proteins, nucleic acids, and biological small molecules. Examples of formaldehyde-producing enzymes include DNA demethylases, histone demethylases (HDMs), and cytochrome P450 enzymes that demethylate drugs and other xenobiotic compounds<sup>1-6</sup>. HDMs catalyze the site-specific demethylation of methyl-lysine residues in histones to dynamically regulate chromatin structure, gene expression, and potentially other genomic functions. Lysine-specific HDMs were first discovered in 2004 and are currently among the most actively studied formaldehyde-producing enzymes<sup>7</sup>. At present, there are two known classes of HDMs: the flavin adenine dinucleotide (FAD)-dependent Lysine Specific Demethylase 1 (LSD1) family and the Fe(II)-dependent Jumonji C (JmjC) family. Although the LSD1 and JmjC HDMs employ different cofactors and catalytic mechanisms (see below), both produce formaldehyde as a byproduct of the demethylation reaction. Despite their biological importance, HDMs have proven difficult to quantitatively assay owing to their relatively low turnover numbers, hindering our understanding of their kinetic properties, substrate specificities, and reaction mechanisms.



1. Wolfe, AP, Jones, PL, and Wade, PA "DNA Demethylation." PNAS. 1999 96:15894-15896.
2. Parta, SK, et al., "Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development" Cancer Metastasis Rev. 2008 27(2):315-324.
3. Walsky, RL and Obach, RS, "Validated assays for human cytochrome P450 activities". Drug Metab Disp., 2004 32(6):647-660.
4. Hollenberg, PF, "Mechanisms of Cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism", FASEB J 1992 6(2):686-694.
5. Klose, RJ and Zhang, Y, "Regulation of histone methylation by demethylination and demethylation", Nature Mol. Biol. Rev. 2007 8:307-318.
6. Takeuchi, T, Watanabe, Y, Takano-Shimizu, T and Kondo, S., "Roles of jumonji and jumonji Family Genes in Chromatin Regulation and Development", Dev. Dynamics 2006 235:2449-2459.
7. Shi, Y., et al., "Histone demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1" Cell 2004, 119:941-953.

## ASSAY PRINCIPLE

The DetectX® Demethylase Activity kit is designed to quantitatively measure the enzymatic activity of formaldehyde-producing enzymes such as Histone Demethylases. The kit is unique in that the product of these enzymatic demethylation reactions, formaldehyde, is quantitated directly by a fluorescent product. No separation or washing is required. The kit has been validated for both LSD1 and JMJD2A histone Demethylases (HDMs).

The kit provides optimized buffers for the HDMs, LSD1 and JMJD2A, a stable formaldehyde standard, the Formaldehyde Detection Reagent (FDR) and two 96 well plates for detecting the generated fluorescent signal. The kit allows any enzymatic reaction generating formaldehyde to be measured. The end user will have to provide the demethylase system and any cofactors, etc. necessary for activity, along with any test inhibitors or activators. The kit allows end users to produce HDM activity in many *in vivo* and *in vitro* systems and then determine the activity by measuring formaldehyde generation.

For *in vitro* studies, the HDM reaction should be carried out in our supplied buffers using optimized reaction conditions for the demethylation. For HDM samples in cell lysates, we include a specially formulated Cell Lysis Buffer, X050-100ML, that has been shown not to interfere with formaldehyde detection. Cell lysis buffers containing SDS and Triton X-100 inhibit the formaldehyde signal reaction and should not be used.

Following the formaldehyde generating reaction, the reaction can be stopped by addition of a suitable inhibitor. The FDR is then added to all the wells. If calibration to formaldehyde is needed (for cross lab comparisons) then a formaldehyde standard curve generated from the supplied standard should be run.

After a short incubation at 37°C for 30 minutes, the fluorescent product is read at 510 nm in a fluorescent plate reader with excitation at 450 nm. The demethylase activity is determined based upon formaldehyde production, after making a suitable correction for any dilution of the sample, using software available with most fluorescence plate readers. We have provided two 96 well plates for measurement but this assay is adaptable for higher density plate formats. The end user should ensure that their HTS black plate is suitable for use with these reagents prior to running samples.

## RELATED PRODUCTS

### REAGENTS

Tranylcypromine LSD1 Inhibitor (10mM)

Catalog Number X042-1EA

#### **AbX™ Epigenetic Antibodies:**

LSD1 Polyclonal Antibody

Catalog Number A003-100UL

Histone H3 Polyclonal Antibody

Catalog Number A004-100UL

MonoMe Lys<sup>4</sup> Histone H3 Polyclonal Antibody

Catalog Number A005-100UL

DiMe Lys<sup>4</sup> Histone H3 Polyclonal Antibody

Catalog Number A006-100UL

TriMe Lys<sup>4</sup> Histone H3 Polyclonal Antibody

Catalog Number A007-100UL



## DEMETHYLASE REACTION CONDITIONS

We have ensured that the DetectX® Demethylase Assay detects the activity of the 2 types of demethylase systems, the LSD1-type and the *Jumonji*-type. Below we have listed the *in vitro* conditions we used in validating this fluorescent detection system, its compatibility with the LSD1 and JMJD2A reactions and the ability to quantitate the formaldehyde produced by both enzymatic reactions. For HDM samples in cell lysates, we offer as an optional reagent, a specially formulated Cell Lysis Buffer, X050-100ML, that has been shown not to interfere with formaldehyde detection. Cell lysis buffers containing SDS and Triton X-100 inhibit the formaldehyde signal reaction and should not be used.

**It should be noted that most HDM enzyme reactions should be compatible with the formaldehyde readout system. As HDMs are relatively slow turnover enzymes the amount of formaldehyde produced is low and only plate readers that are capable of measuring low fluorescent signals and having adjustable gain or filter settings may be compatible.**

### Typical LSD1 Enzyme Reaction

To duplicate wells, 50  $\mu$ L of either formaldehyde standards, LSD1 enzyme or a blank all made using the LSD1-type Assay Buffer and 50  $\mu$ L of a specific 1-21 sequence of Histone H3 with the dimethylated lysine at amino acid 4 ( $H^3K^4\text{-Me}_2$ ) dissolved in LSD1-type Assay Buffer were added. The plate was sealed and incubated at 30°C for one hour. At the end of the incubation, 25  $\mu$ L of the FDR was added to each well, the plate was resealed and incubated at 37°C for 30 minutes.

### Typical JMJD2A Enzyme Reaction

To duplicate wells, 50  $\mu$ L of 2mM ascorbate, 100 $\mu$ M  $\text{FeSO}_4$  and 25  $\mu$ L of either formaldehyde standards, JMJD2A enzyme or a blank all made using the *Jumonji*-type Assay Buffer were added, followed by 25  $\mu$ L of a specific 1-24 sequence of Histone H3 with the trimethylated lysine at amino acid 9 ( $H^3K^9\text{-Me}_3$ ) containing 2mM alpha-ketoglutarate dissolved in *Jumonji*-type Assay Buffer. The plate was sealed and incubated at 30°C for 30 minutes. To stop the reaction, 5  $\mu$ L of 4 mM deferoxamine in *Jumonji*-type Assay Buffer was added. 25  $\mu$ L of the FDR was then added to each well, the plate was resealed and incubated at 37°C for 30 minutes.

## REACTION OVERVIEW

1. **Carry out demethylating enzyme reaction.**
2. **Stop the reaction (optimal), add FDR.**
3. **Incubate at 37°C for 30 minutes, read signal.**
4. **Calibrate to Formaldehyde generated.**

## SUPPLIED COMPONENTS

<b>Black Half Area 96 Well Plate</b>	2 plates	Catalog Number X037-2EA
See: <a href="http://www.ArborAssays.com/resources/lit.asp">http://www.ArborAssays.com/resources/lit.asp</a> for plate dimension data.		
<b>LSD1-type Assay Buffer</b>	60 mL	Catalog Number X038-60ML
A phosphate buffer containing detergents and stabilizers.		
<b>JMJD2A-type Assay Buffer</b>	60 mL	Catalog Number X039-60ML
A HEPES buffer containing stabilizers.		
<b>Formaldehyde Standard</b>	0.5 mL	Catalog Number C001-500UL
2,000 $\mu$ M formaldehyde solution in a special stabilizing solution. Outer container has formaldehyde absorbing pad. The standard is stable if kept tightly sealed.		
<b>KEEP TIGHTLY SEALED</b>		
<b>DetectX<sup>®</sup> Formaldehyde Reagent</b>	5 mL	Catalog Number C002-5ML
Special formulation of reagents to detect formaldehyde in solution. Contains $\leq$ 0.09% sodium azide as a preservative.		
<b>Demethylase Cell Lysis Buffer (CLB)</b>	100 mL	Catalog Number X050-100ML
A Tris based buffer containing detergents. <b>Store Frozen</b> as this buffer contains no preservatives.		
<b>Plate Sealers</b>	2 Each	Catalog Number X002-1EA

## STORAGE INSTRUCTIONS

**All components of this kit should be stored at 4°C, except the CLB which should be stored at -20°C, until the expiration date of the kit.**



## OTHER MATERIALS REQUIRED

Supply of distilled or deionized water free of formaldehyde.

Repeater pipet with disposable tips capable of dispensing 25  $\mu$ L.

Incubators capable of accurately maintaining 30°C and 37°C.

Demethylase enzyme samples. A source of LSD1-type or *Jumonji*-type demethylase, along with any cofactors, enzyme substrates, inhibitors, and/or activators.

Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 450 nm. Set plate parameters for a 96-well Corning Costar 3694 plate. See: <http://www.ArborAssays.com/resources/lit.asp> for plate dimension data.

Software for converting raw relative fluorescent unit (FLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

## PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

Formaldehyde is a toxic, volatile, reactive chemical that can form adducts with proteins and nucleic acids. It reacts with oxygen to form formic acid and so should be kept sealed and only used in well-ventilated laboratories. For disposal, we suggest discarding all excess standards and samples in a 10% aqueous solution of sodium bisulfite, such as Sigma catalog number 13438.

Some of the components of this kit contain sodium azide as a preservative, which may react with lead or copper plumbing to form potentially explosive complexes. When disposing of reagents always flush with large volumes of water to prevent azide build-up.

## SAMPLE TYPES

Histone demethylases diluted in the assay buffers provided are compatible with this assay.

For HDM samples in cell lysates, we include a specially formulated Cell Lysis Buffer, X050-100ML, that has been shown not to interfere with formaldehyde detection. **Cell lysis buffers containing SDS and Triton X-100 inhibit the formaldehyde signal reaction and should not be used.**

**NOTE:** Cell lysates made in CLB can be measured in the assay directly. The standards for the formaldehyde standard curve should be made in CLB.

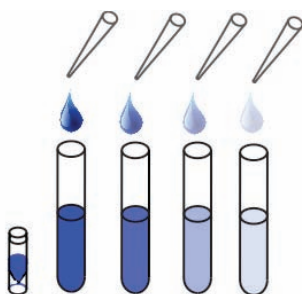
## REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine activity. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

### Standard Preparation

Label seven glass test tubes as #1 through #7. Pipet 450  $\mu\text{L}$  of Assay Buffer containing all cofactors and additives into tube #1 and 250  $\mu\text{L}$  into tubes #2-#7. Add 50  $\mu\text{L}$  of the Formaldehyde stock solution to tube #1 and vortex completely. Add 250  $\mu\text{L}$  of tube #1 to tube #2 and vortex completely. Repeat these serial dilutions for tubes #3 through #7. The concentration of formaldehyde in tubes 1 through 7 will be 200, 100, 50, 25, 12.5, 6.25 and 3.125  $\mu\text{M}$ .

### Use all Standards within 1 hour of preparation



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
<b>Assay Buffer Volume (<math>\mu\text{L}</math>)</b>	<b>450</b>	250	250	250	250	250	250
<b>Addition</b>	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
<b>Volume of Addition (<math>\mu\text{L}</math>)</b>	<b>50</b>	250	250	250	250	250	250
<b>Final Conc (<math>\mu\text{M}</math>)</b>	200	100	50	25	12.5	6.25	3.125

**NOTE:** Cell lysates made in CLB can be measured in the assay directly. The standards for the formaldehyde standard curve should be made in CLB.

# ASSAY PROTOCOL

Demethylase reaction volume should be no more than 100  $\mu\text{L}$  in each well including all cofactors, inhibitors and activators diluted into the kit Assay Buffer or CLB for cell lysates.

## Demethylase Reaction

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification.
2. Set up the appropriate demethylase reaction in one of the supplied buffers.
3. Pipet 100  $\mu\text{L}$  standards or samples plus all cofactors and inhibitors into duplicate wells in the black plate.
4. Pipet 100  $\mu\text{L}$  Assay Buffer or CLB plus all cofactors and inhibitors into duplicate wells as a Zero standard.
5. Carry out demethylation reaction and preferably stop reaction at an appropriate time.

## Formaldehyde Detection

6. Add 25  $\mu\text{L}$  of the DetectX<sup>®</sup> Formaldehyde Detection Reagent to each well using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
8. Incubate at 37°C for 30 minutes. Room temperature incubation will yield approximately 75% of the fluorescent signal generated with 37°C incubation.
9. Set plate parameters for a 96-well Corning Costar 3694 plate.  
See: <http://www.ArborAssays.com/resources/lit.asp> for plate dimension data.  
Read the fluorescent signal from each well in a plate reader capable of reading the fluorescent signal at 510 nm with excitation at 450 nm. Please contact your plate reader manufacturer for suitable filter sets.
10. Use the plate reader's built-in 4PLC software capabilities to calculate formaldehyde concentrations for each sample.

## CALCULATION OF RESULTS

Average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The sample activity obtained should be multiplied by the dilution factor to obtain neat sample values.

### TYPICAL DATA - LSD1 ASSAY

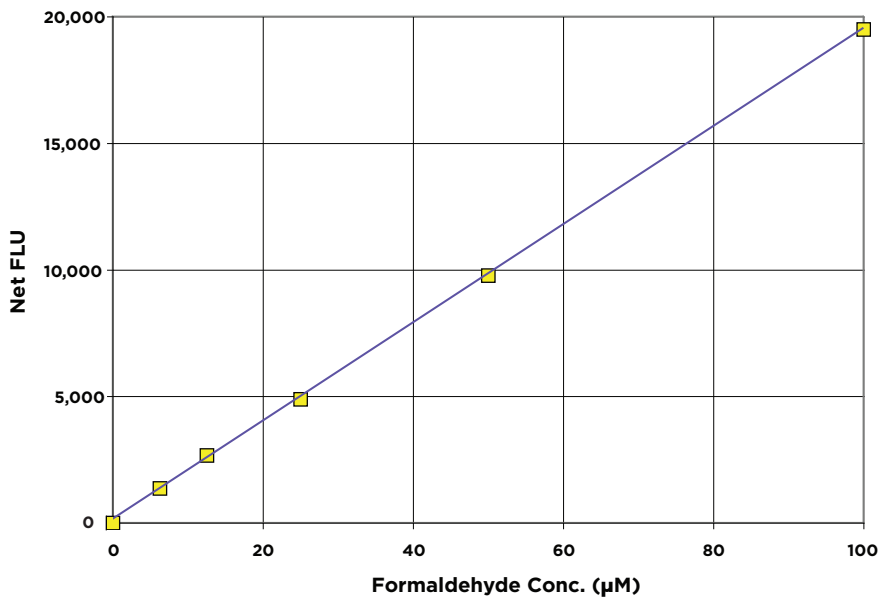
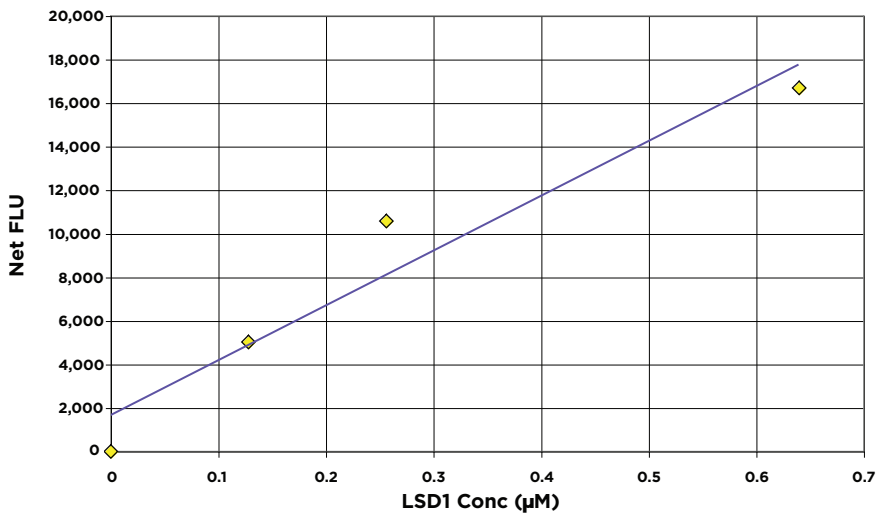
LSD1 Conc. ( $\mu\text{M}$ )	Mean FLU	Net FLU
Zero	1,827	0
0.64	18,522	16,695
0.256	12,410	10,584
0.128	6,849	5,022

### TYPICAL DATA - JMJD2A ASSAY

JMJD2A Conc. ( $\mu\text{M}$ )	Mean FLU	Net FLU
Zero	3,028	0
10	17,973	14,945
5	10,719	7,691
2.5	4,528	1,500

**Always run your own standard curve for calculation of results.  
Do not use this data.**

## Typical LSD1 and Formaldehyde Standard Curves



**Always run your own standard curves for calculation of results. Do not use this data.**

## LSD1 Interference Study

The following additives were added to the LSD1 enzyme reaction with its 1-21 methylated peptide substrate to test for interference with the signal generation.

### Organic Solvents

5% of the organic solvents methanol, N,N-dimethylformamide and dimethylsulfoxide had no negative effect on the generation of fluorescence in the presence of 0.256  $\mu\text{M}$  LSD1.

### Detergents

0.005% SDS inhibited the enzymatic generation of formaldehyde by 89%. 0.1% or less Triton X-100 and 0.01% or less Tween 20 had no effect on signal generation.

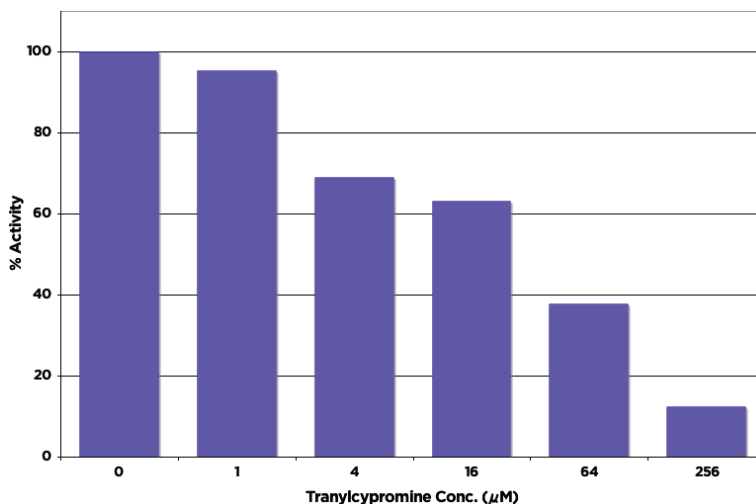
### Preservatives

0.09% sodium azide in the assay buffer reduced the signal by 20%. 0.09% Kathon inhibited the signal by 91.5%, 0.005% gentamicin had no negative effect on the signal generation.

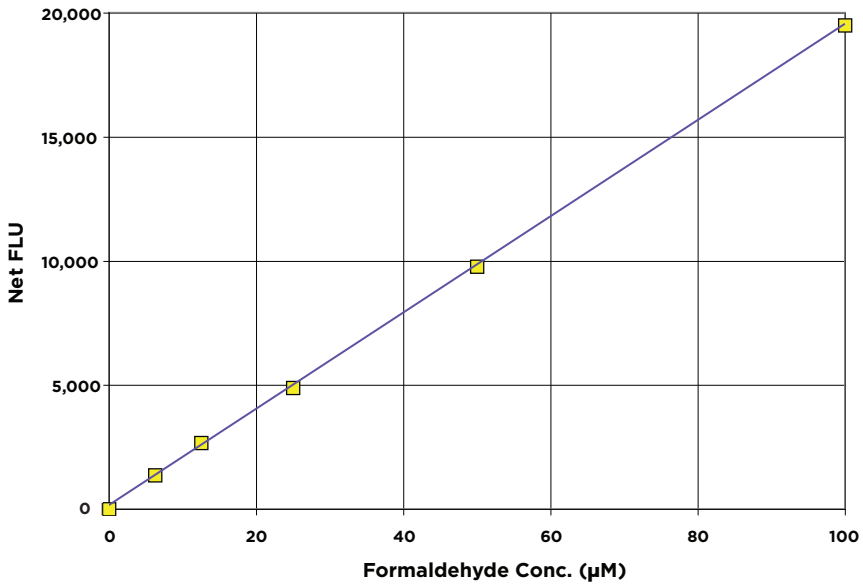
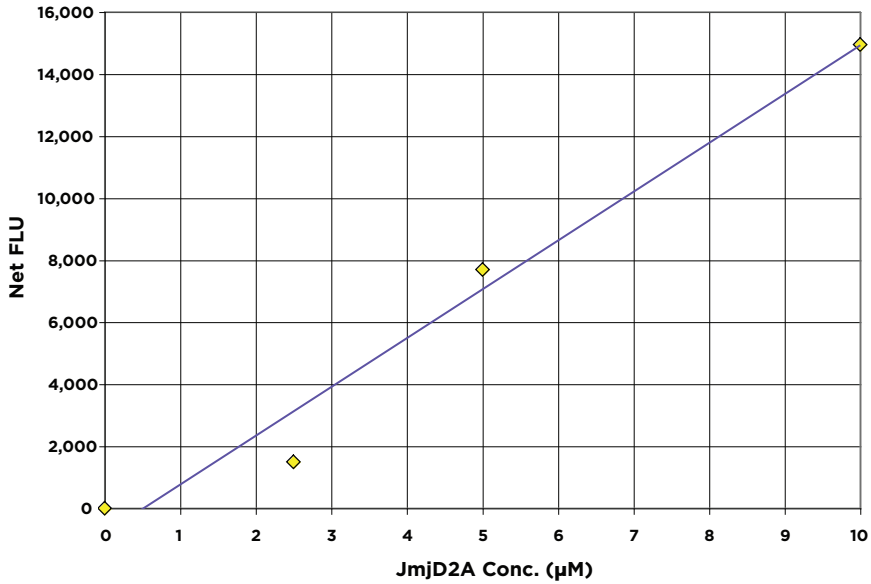
## Inhibition of LSD1 Activity

The LSD1 inhibitor, Tranylcypromine, was added to LSD1 enzyme reaction with its 1-21 methylated peptide substrate at concentrations ranging from 256  $\mu\text{M}$  to 1  $\mu\text{M}$ .

**Tranylcypromine Inhibition Data**



## Typical JMJD2A and Formaldehyde Standard Curves



**Always run your own standard curves for calculation of results. Do not use this data.**

## JMJD2A Interference Study

The following additives were added to the JMJD2A enzyme reaction with its 1-24 methylated peptide substrate to test for interference with the signal generation.

### Organic Solvents

5% of the organic solvents methanol, N,N-dimethylformamide and dimethylsulfoxide had no negative effect on the generation of fluorescence in the presence of JMJD2A.

### Detergents

0.005% SDS inhibited the enzymatic generation of formaldehyde by 7.1%. 0.1% Triton X-100 inhibited the signal by 4.2%. 0.01% or less Tween 20 had no effect on signal generation.

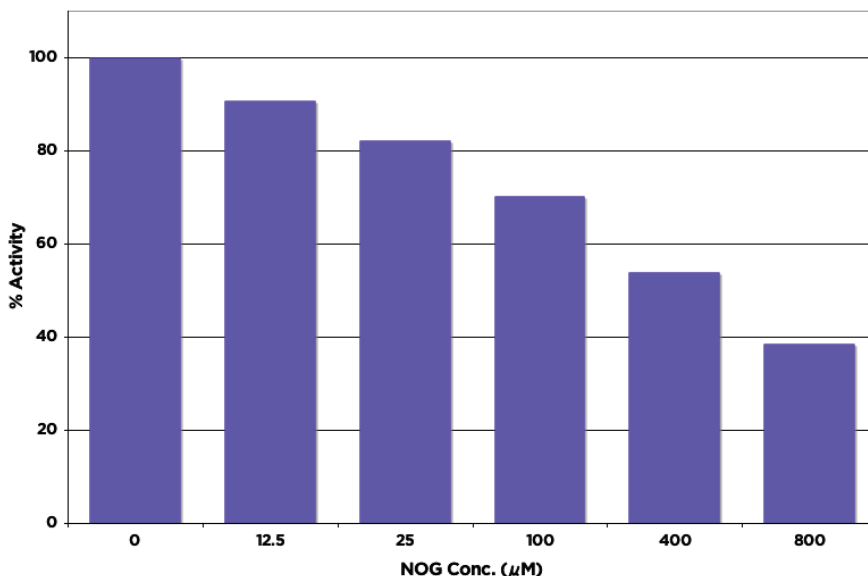
### Preservatives

0.09% sodium azide in the assay buffer reduced the signal by 35.3%. 0.09% Kathon inhibited the signal by 56.9, 0.005% gentamicin had no negative effect on the signal generation.

## Inhibition of JMJD2A Activity

The JMJD2A inhibitor, N-Oxalylglycine, was added to the JMJD2A enzyme reaction with its 1-24 methylated peptide substrate at concentrations ranging from 800  $\mu\text{M}$  to 12.5  $\mu\text{M}$ .

**NOG Inhibition Data**



## LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

## CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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