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MULTI SPECIES

DetectX®

Histone Demethylase
Fluorescent Activity Kit

2 Plate Kit Catalog Number K010-F1

Sample Types Validated:

LSD1- and *Jumonji*-type Demethylases

Covered under US Patent numbers 8,173,386 & 8,765,396

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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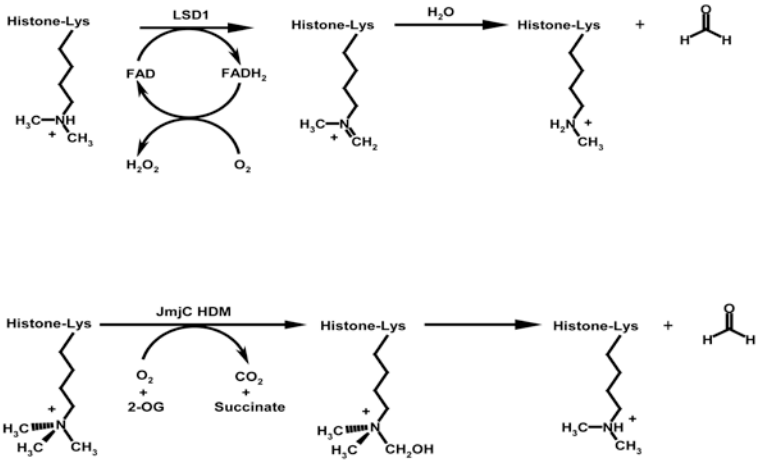
BACKGROUND

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[1-6]. 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[7]. 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.



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3. Walsky, RL and Obach, RS, “Validated assays for human cytochrome P450 activities”. Drug Metab Disp., 2004 32(6):647-660.
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5. Klose, RJ and Zhang, Y, “Regulation of histone methylation by demethylimination 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.
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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	Catalog No.
Tranylcypromine LSD1 Inhibitor (10µmol)	X042-1EA

AbX™ Antibodies	Catalog No.
DiMe Lys4 Histone H3 Polyclonal Antibody	A006-100UL
Histone H3 Polyclonal Antibody	A004-100UL
LSD1 Polyclonal Antibody	A003-100UL
MonoMe Lys4 Histone H3 Polyclonal Antibody	A005-100UL
TriMe Lys4 Histone H3 Polyclonal Antibody	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 provide 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 µL of either formaldehyde standards, LSD1 enzyme or a blank all made using the LSD1-type Assay Buffer and 50 µL of a specific 1-21 sequence of Histone H3 with the dimethylated lysine at amino acid 4 (H^3K^4-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 µL 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 µL of a solution of 2 mM ascorbate, 100 µM $FeSO_4$ and 25 µL of either formaldehyde standards, JMJD2A enzyme or a blank all made using the *Jumonji*-type Assay Buffer were added, followed by 25 µL of a specific 1-24 sequence of Histone H3 with the trimethylated lysine at amino acid 9 (H^3K^9-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 µL of 4 mM deferoxamine in *Jumonji*-type Assay Buffer was added. 25 µL 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

Black Half Area 96 Well Plate
See: www.ArborAssays.com/resources/#general-info for plate dimension data.
2 plates Catalog Number X037-2EA

LSD1-type Assay Buffer
A phosphate buffer containing detergents and stabilizers.
60 mL Catalog Number X038-60ML

JMJD2A-type Assay Buffer
A HEPES buffer containing stabilizers.
60 mL Catalog Number X039-60ML

Formaldehyde Standard
2,000 µM formaldehyde solution in a special stabilizing solution. Outer container has formaldehyde absorbing pad. The standard is stable if kept tightly sealed. **KEEP TIGHTLY SEALED.**
500 µL Catalog Number C001-500UL

DetectX® Formaldehyde Reagent
Special formulation of reagents to detect formaldehyde in solution. Contains 0.09% sodium azide as a preservative.
5 mL Catalog Number C002-5ML

Demethylase Cell Lysis Buffer
A Tris based buffer containing detergents. **Store Frozen** as this buffer contains no preservatives.
100 mL Catalog Number X050-100ML

Plate Sealers
2 Each Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C, except the cell lysis buffer 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 µL.

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: www.ArborAssays.com/resources/#general-info for plate dimension data.

The sensitivity of fluorescent assays is dependant on the capabilities of the plate reader. If your plate reader has adjustable gain you can modify the signals obtained from the assay by increasing or decreasing the gain settings, by changing the aperture settings for monochromator based readers, or by changing the band pass width of the emission and/or excitation filters on some readers. Please review the plate reader manual for details.

Signals expressed in this insert are Relative Fluorescent Units (RFU) and were obtained on our plate readers. The RFU numbers you obtain may be different from these, but the assay results should be similar.

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.**

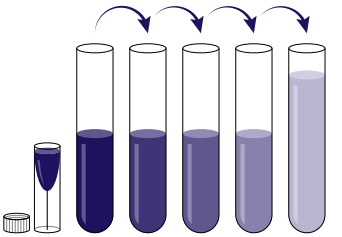
NOTE: Cell lysates made in cell lysis buffer can be measured in the assay directly. The standards for the formaldehyde standard curve should be made in cell lysis buffer.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Standard Preparation

Label glass test tubes as #1 through #7. Pipet 450 µL of Assay Buffer containing all cofactors and additives into tube #1 and 250 µL into tubes #2-#7. Add 50 µL of the Formaldehyde stock solution to tube #1 and vortex completely. Add 250 µL 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 µM.



Use all Standards within 2 hours of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer Volume (µL)	**450**	250	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	**50**	250	250	250	250	250	250
Final Conc (µM)	200	100	50	25	12.5	6.25	3.125

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine activity.

Demethylase reaction volume should be no more than 100 µL in each well including all cofactors, inhibitors and activators diluted into the kit Assay Buffer or cell lysis buffer 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, set plate parameters for a 96-well Corning Costar 3694 plate.
   See: www.ArborAssays.com/resources/#general-info for plate dimension data.

2. Set up the appropriate demethylase reaction in one of the supplied buffers.

3. Pipet 100 µL standards or samples plus all cofactors and inhibitors into duplicate wells in the black plate.

4. Pipet 100 µL Assay Buffer or cell lysis buffer 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 µL of the DetectX® 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. Cover the plate with the plate sealer and press to seal adequately.

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. 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. **This assay requires a plate reader with efficient fluorescence optics. Please refer to page 7 for details on increasing sensitivity.**

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.

Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-histone-demethylase-fluorescent-activity-kit.assay

TYPICAL DATA - LSD1 ASSAY

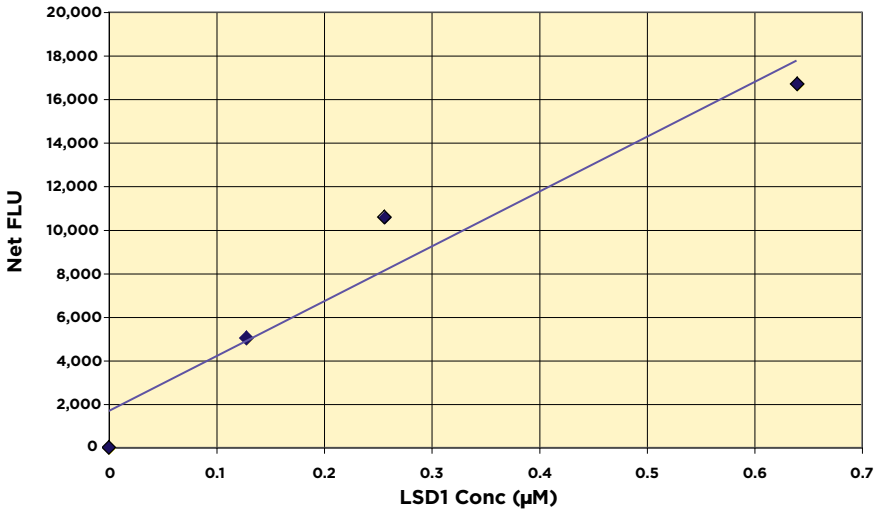
LSD1 Conc. (µM)	Mean FLU	Net FLU
Zero	1,827	0
0.64	18,522	16,695
0.256	12,410	10,584
0.128	6,849	5,022

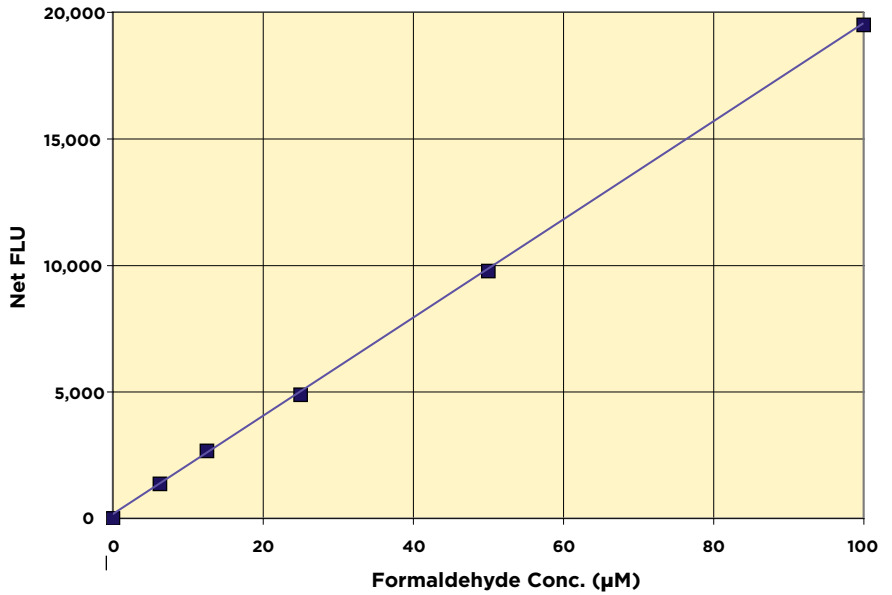
TYPICAL DATA - JMJD2A ASSAY

JMJD2A Conc. (µM)	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

The organic solvents methanol, N,N-dimethylformamide and dimethylsulfoxide, each at 5%, had no negative effect on the generation of fluorescence in the presence of 0.256 µM LSD1.

Detergents

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

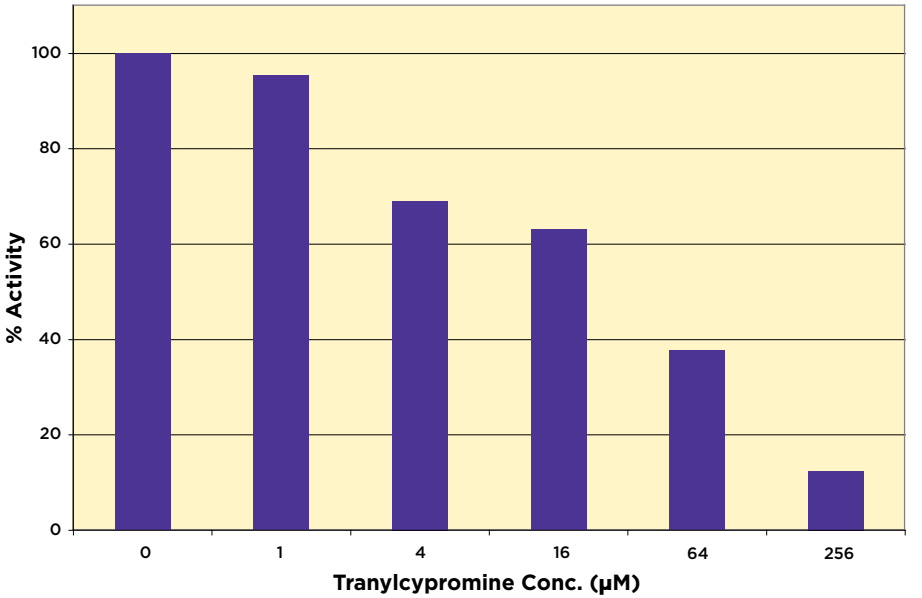
Preservatives

Sodium azide in the assay buffer at 0.09% reduced the signal by 20%. Kathon at 0.09% inhibited the signal by 91.5%. Gentamicin at 0.005% had no negative effect on the signal generation.

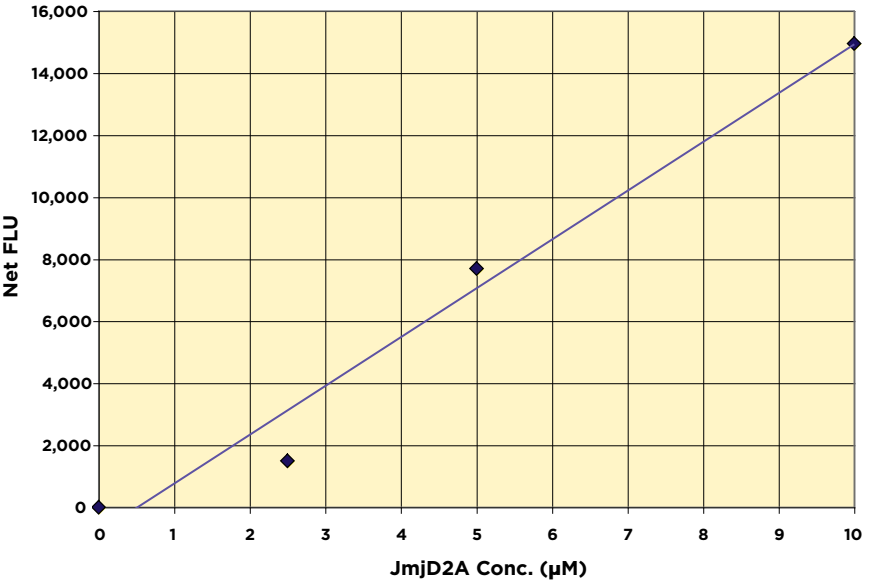
Inhibition of LSD1 Activity

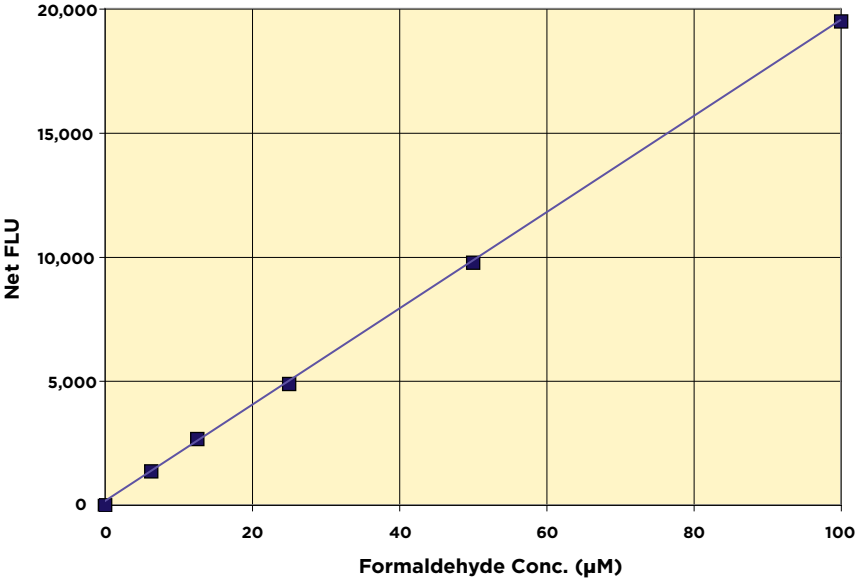
The LSD1 inhibitor Tranylcypromine was added to LSD1 enzyme reaction with its 1-21 methylated peptide substrate at concentrations ranging from 256 µM to 1 µM.

Tranycypromine 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

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

Detergents

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

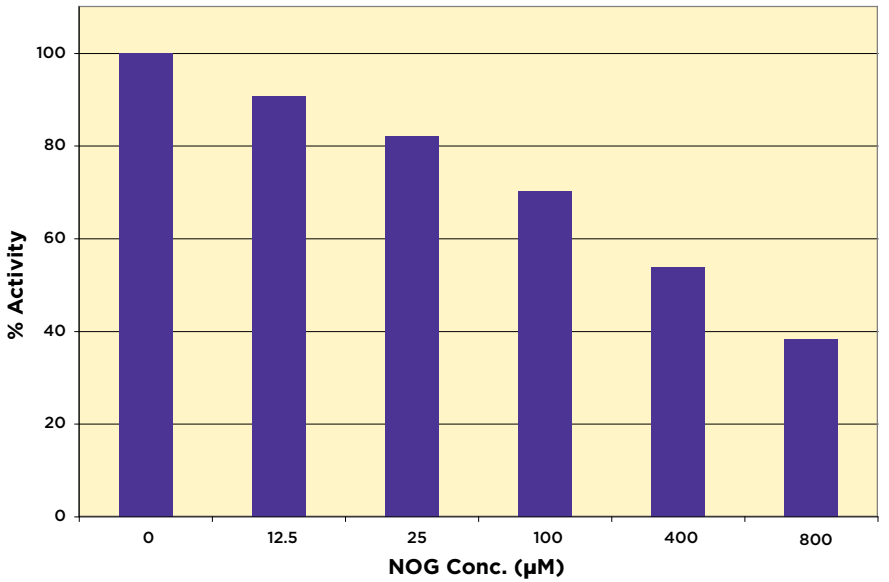
Preservatives

Sodium azide in the assay buffer at 0.09% reduced the signal by 35.3%. Kathon at 0.09% inhibited the signal by 56.9%. Gentamicin at 0.005% had no negative effect on the signal generation.

Inhibition of JMJD2A Activity

The JMJD2A inhibitor N-Oxalylglycine (NOG) was added to the JMJD2A enzyme reaction with its 1-24 methylated peptide substrate at concentrations ranging from 800 µM to 12.5 µM.

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

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Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

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