Enabling Discovery in Life Science®

LSD1 Fluorimetric Drug Discovery Kit

A CELLestial® Red Hydrogen Peroxide Assay System

Instruction Manual
BML-AK544

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✦*LSD1 Fluorimetric Drug Discovery Kit – BML-AK544*✦
A CELLestial® Red Hydrogen Peroxide Assay System

BACKGROUND

The mono, di- and trimethylation of particular lysine residues in histone tails (e.g. histone H3 lysine-4 (H3K4), H3K9, H3K27, H3K36, H4K20) are implicated, along with a variety of other post-translational modifications (e.g. lysine acetylation) in the transmission of heritable epigenetic information and the control of chromatin structure and DNA transcription (see review[1]). Due to early studies showing very slow turnover of lysine methyl groups[2], it was thought until recently that lysine methylation might be a 'permanent' or irreversible mark.

LSD1 (aka KDM1, Lysine-specific histone demethylase 1; AOF2), a flavin-containing amine oxidase homolog and component of various corepressor complexes, was the first enzyme demonstrated to be capable of lysine demethylation[3]. LSD1 catalyzes the oxidative demethylation of mono- and dimethylated H3K4 (H3K4Me2), producing hydrogen peroxide and formaldehyde in the process[3-5]. H3K4 methylation is considered a transcription-activating chromatin mark and, *in vivo*, LSD1 is frequently found in association with the transcriptional corepressor protein CoREST and HDACs 1 or 2[5]. However, in association with the androgen receptor in prostate or prostate tumor cells, LSD1 performs the gene-activating demethylation of H3K9[6]. LSD1 can demethylate K370Me2 of p53, blocking its interaction with the 53BP1 coactivator and repressing p53-activated gene expression[7], while p53 can recruit LSD1 to chromatin to perform the repressive demethylation of H3K4Me2[8]. In *C. elegans*, LSD1-mediated reversal of the H3K4Me2 modification has been shown to play an important role in epigenetic reprogramming of the germline, a result that suggests its activity may be required for the induction of pluripotency in stem cells[9]. Deletion of the LSD1 gene in embryonic stem cells leads to decreasing DNA methylation, an effect attributed to loss of a stabilizing demethylation performed by LSD1 on DNA methyltransferase I (Dnmt1)[10]. All told, there is increasing evidence of LSD1's importance in epigenetic and transcriptional regulation and of its roles in processes ranging from embryogenesis to carcinogenesis.

LSD1 is inhibited by a number of established monoamine oxidase inhibitor drugs[6, 11], including tranylcypromine[11, 12]. That and the fact that its expression is elevated in a number of cancers may make it a promising target for drug development[5, 13]. Enzo Life Sciences' *LSD1 Fluorometric/Colorimetric Drug Discovery Kit* provides active human recombinant LSD1 together with a sensitive, convenient assay system suitable for high-throughput screening.

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PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

ASSAY DESCRIPTION

The *LSD1 Fluorimetric Drug Discovery Kit* provides human recombinant LSD1 and all the necessary reagents for measuring its activity in a sensitive, real-time fluorescent assay. LSD1 catalyzed demethylation of the Histone H3 Dimethyl Lysine-4 Peptide (H3K4Me2 Peptide; Cat. # BML-P256) generates hydrogen peroxide. A fluorescent signal is generated via the horseradish peroxidase (HRP) catalyzed reaction of the hydrogen peroxide with the CELLestial® Red Peroxidase Substrate (BML-KI565). Although fluorescence detection will be more sensitive (Excitation in range of 530-570 nm; Emission ca. 590 nm), the CELLestial® Red peroxidation product may also be detected by following absorbance (see "Detection by Absorbance at 563 nm"). Included in the kit is a clear ½-volume 96-well microplate (80-2404), which may be used in either detection mode, and a black ½-volume microplate (80-2409) for fluorescence measurements. Also included are a stabilized H_2O_2 Stock Solution for preparing standard curves and the LSD1 inhibitor tranylcypromine, as a control for inhibitor screening/drug discovery work.

COMPONENTS OF BML-AK544

BML-SE544-0050 LSD1 (KDM1) (human, recombinant)
FORM: Solution in 6 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.2, 82 mM NaCl, 1.6 mM KCl and 40% v/v glycerol.
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 50 µg

BML-P256-0500 Histone H3 Dimethyl Lysine-4 Peptide (H3K4Me2 Peptide; Histone H3 residues 1-21, MW=2281.5)
FORM: Solid
STORAGE: -70°C
QUANTITY: 0.5 mg, Net Peptide

BML-KI564-0001 Horseradish Peroxidase (HRP) Concentrate (50x)
FORM: Lyophilized Solid. Dissolve in 200 µl of Assay Buffer.
STORAGE: -70°C
QUANTITY: 200 µl after reconstitution in Assay Buffer

BML-KI565-0001 CELLestial® Red Peroxidase Substrate (100x)
FORM: Solid. Dissolve in 100 µl DMSO to prepare 100x stock solution.
STORAGE: -70°C
QUANTITY: 100 µl after reconstitution in DMSO.

BML-KI566-0020 LSD1/HRP Assay Buffer
FORM: Buffered Solution.
STORAGE: -70°C
QUANTITY: 20 ml

BML-KI567-0040 H_2O_2 Stock Solution (3.0%; 0.88 M)
FORM: Solution
STORAGE: -70°C
QUANTITY: 40 µl

BML-KI568-0200 Dimethyl Sulfoxide (DMSO)
FORM: Pure liquid.
STORAGE: -70°C
QUANTITY: 200 µl

BML-KI569-0010 Tranylcypromine (LSD1 Inhibitor; MW=182.2)
FORM: White solid.
STORAGE: -70°C
QUANTITY: 10 mg

80-2410 ½-Volume Microplates
1 clear and 1 black, 96 well
STORAGE: Room temperature.

NOTES ON STORAGE AND HANDLING

Store the unopened kit and, after opening, all components except the microplates and instruction booklet at -70°C for the highest stability.

Unless the whole kit is to be used in a single day's experiment, it would be worthwhile, after the initial thawing of the LSD1 (BML-SE544) and reconstitution of the HRP (BML-KI564), to divide each of the two enzymes among several tubes as single-use aliquots. To retain maximum enzymatic activity, snap freeze the aliquots in liquid nitrogen or a dry ice/ethanol bath and store at -70°C Defrost frozen enzyme aliquots quickly, in a RT water bath or by rubbing between fingers, then immediately store on an ice bath.

The 100x stock of the Cellestial Red Peroxidase Substrate (BML-KI565) will be prepared, at room temperature, by dissolving the contents of the vial in 100 µl of DMSO (BML-KI568). This stock solution will then be diluted 50x to prepare '2x Substrate Solution(s)'. As soon as possible, place the remaining 100x stock at -70°C. Thaw at room temperature for subsequent use.

See "Preparing Reagents For Assay", below, for additional instructions.

OTHER MATERIALS REQUIRED

- Microplate reading fluorometer capable kinetic mode readings and of excitation at a wavelength in the range 530-570 nm and detection of emitted light at 590 nm.
- Pipettor or multi-channel pipettor capable of pipetting 2-100 µl accurately
- Ice bucket to keep reagents cold until use.
- Microplate warmer and/or other temperature control device (optional)
- Reservoir or spare microplate for "2x Substrates" (optional)

ASSAY PROCEDURES

Some Things To Consider When Planning Assays:

1. The *LSD1 Drug Discovery Kit* (BML-AK544) assay is designed to be performed with 0.5 µg LSD1 per well in a volume of 100 µl. Enough LSD1 and other reagents are included to do assays in all 96 wells of one of the ½-area microplates provided.
2. There is enough H3K4Me2 Peptide (BML-P256) to do an entire plate of assays at [H3K4Me2] = 20 µM. Since the K_m of the H3K4Me2 peptide in this system is ~120 µM (see "Application Examples", Fig. 3), 20 µM is a suitable condition for drug discovery assays.
3. The most accurate LSD1 activity data will be obtained from kinetic readings which can provide the slopes of the initial, linear phase of the reaction progress curve. At room temperature (23°C) this occurs over approximately the first 5 min. of the reaction (see "Application Examples", Fig. 2). It is therefore recommended the number of wells assayed at one time be limited to those that can be initiated by mixing in a relatively short time (e.g. 30 sec or less to start 1 or 2 columns with an 8-channel multipettor).
4. In order to insure the choice of suitable fluorimeter wavelength and gain settings, it is recommended that an H_2O_2 standard curve be set up and read prior to doing LSD1 activity assays ("H_2O_2 Standard Curve Protocol").

Preparing Reagents For Assay:

1. Remove the kit from -70°C freezer. Place the CELLestial® Red Peroxidase Substrate (BML-KI565), DMSO (BML-KI568), H_2O_2 Stock Solution (BML-KI567) and LSD1/HRP Assay Buffer (BML-KI566) at room temperature. Rapidly thaw the LSD1 (BML-SE544) and place it, the HRP (BML-KI564), the H3K4Me2 Peptide (BML-P256) and the Tranylcypromine (BML-KI569) on ice. When the H_2O_2 Stock Solution has thawed, place it on ice.
2. Prepare a 0.5 mM stock of the H3K4Me2 Peptide (BML-P256) by adding 438 µl of LSD1/HRP Assay Buffer to the 0.5 mg net peptide in the vial and vortexing well. Return vial to ice.
3. Prepare a 100 mM stock solution of Tranylcypromine (BML-KI569) by dissolving the 10 mg of solid with 0.55 ml of water. Tranylcypromine is an irreversible inhibitor of LSD1 and an inhibition experiment is described in a later section ("Application Examples", "Concentration Dependence of Tranylcypromine Inhibition"). The tranylcypromine stock solution may be stored frozen, at -70°C, for up to 3 months.
4. LSD1 will be used at 0.5 µg per well. Prepare enough of a 0.1 µg/µl dilution of LSD1 (BML-SE544) in LSD1/HRP Assay buffer to provide 5 µl per well and store on ice. (*See "NOTES ON STORAGE AND HANDLING" regarding aliquoting, freezing and storage of the unused portion of the undiluted LSD1.*)
5. Prepare the 50x Horseradish Peroxidase (HRP) stock by dissolving the contents of the HRP vial (BML-KI564) in 200 µl of Assay Buffer and store on ice. The assays will require 2 µl of this 50x stock per well. (*See "NOTES ON STORAGE AND HANDLING" regarding aliquoting, freezing and storage of the unused portion of the HRP.*)
6. Prepare the 100x stock of CELLestial® Red Peroxidase Substrate by dissolving the contents of the vial (BML-KI565) in 100 µl of the thawed, room temperature DMSO. This will be diluted 50x in preparation of 2x substrate solution(s). (*See "NOTES ON STORAGE AND HANDLING" regarding freezing and storage of the unused portion of the 100x stock of CELLestial® Red Peroxidase Substrate*.)
7. Prepare 2x Substrate solution(s), at room temperature, shortly before beginning the assay. Each assay well will require 50 µl. Each 50 µl will contain 1 µl of 100x CELLestial® Red Peroxidase Substrate and twice the desired final concentration of the H3K4Me2 Peptide. For example, to prepare 1 ml of 2x Substrates for a final [H3K4Me2] of 20 µM, mix 900 µl of Assay Buffer (room temperature), 80 µl of 0.5 mM H3K4Me2 (step 2) and 20 µl of 100x CELLestial® Red Peroxidase Substrate.

Store unused 0.5 mM H3K4Me2 Peptide and 100x CELLestial® Red Peroxidase Substrate at -70°C.

(NOTE: If inhibitors or other test compounds are to be included in the reaction, but are not to be preincubated with LSD1, replace some portion of the Assay Buffer with the volume of the test solution added. If the test compound is dissolved in a solvent other than Assay Buffer, also prepare a 2x Substrate solution (vehicle control) in which the same volume of Assay Buffer is replaced with that solvent. See Table 1.)

8. Also shortly before performing the assay, prepare a working dilution of HRP in room temperature Assay Buffer such that each 45 µl contains 2 µl of the 50x HRP stock (step 5). Each assay well will receive 45 µl of this solution. For example, to prepare 1 ml, mix 955.6 µl of Assay Buffer with 44.4 µl of 50x HRP stock.

Performing the Assay:

1. The basic assay procedure consists of mixing 50 µl of "2x Enzymes", already present in the assay well, with 50 µl of "2x Substrates" (from, e.g. a reagent trough or the wells of a "mother plate"), and immediately taking fluorescence readings (e.g. Ex. 530 nm, Em. 590 nm) in kinetic mode (intervals of 30-60 sec.).
2. Table 1 gives examples of the compositions of "2x Enzymes" and "2x Substrates" for various sample types, based on 50 µl total volumes for each. In general, within the "2x Enzymes" and "2x Substrates" groupings, these components can be combined and prepared in bulk, in order to facilitate pipetting. So, for example, a bulk solution containing 43 µl Assay Buffer plus 2 µl 50x HRP per 45 µl, may be prepared and 45 µl distributed to each assay well. It is, however, recommended that the 0.1 µg/µl dilution of LSD1 (step 4, "Preparing Reagents for Assay") be kept on ice until shortly before use and distributed 5 µl per assay well, just prior to mixing in the 50 µl of "2x Substrates"
3. The assay procedure described here assumes that any test compounds will not be preincubated with LSD1. An example of a procedure that includes a preincubation step is described in a later section ("Application Examples", Concentration Dependence of Tranylcypromine Inhibition).
4. Initiate and read the LSD1 reactions as follows:
   a. Aliquot 45 µl of the room temperature HRP working dilution (Assay Buffer:50x HRP, 43:2) to assay wells.
   b. Prepare "2x Substrates" solution(s) at room temperature and place in vessel(s) suitable for pipetting to assay wells (e.g. reagent reservoir or wells of a "mother plate"). Be sure to prepare volumes in excess of 50 µl per well, so that a full 50 µl is available for transfer to all wells.
   c. Add 5 µl of 0.1 µg/µl LSD1 to all appropriate wells and 5 µl of Assay Buffer to any "No LSD1" wells (see Table 1).
   d. Mix 50 µl of appropriate "2x Substrates" solution(s) into the assay wells and immediately transfer plate to the fluorimeter.
   e. Read the fluorescence (Ex. 530-570 nm; Em. 590 nm) in kinetic mode (30-60 sec intervals) for 5-30 min.

TABLE 1. ASSAY MIXTURE EXAMPLES

	2x Enzymes, 50 µl Total			2x Substrates, 50 µl Total			
Sample Type	Assay Buffer	LSD1 (0.1 µg/µl)	HRP (50x)	Assay Buffer	Solvent or Test Cpd. in Solvent	H3K4Me2 (0.5 mM)	CELLestial® Red (100x)
Control	43 µl	5 µl	2 µl	(49-y) µl	0 µl	y µl*	1 µl
Vehicle Control	43 µl	5 µl	2 µl	(49-x-y) µl	x µl	y µl	1 µl
No Peptide	43 µl	5 µl	2 µl	(49-x) µl	x µl	0 µl	1 µl
No LSD1	48 µl	0 µl	2 µl	(49-x-y) µl	x µl	y µl	1 µl
Test Compound	43 µl	5 µl	2 µl	(49-x-y) µl	x µl	y µl	1 µl

*For example, to make the 2x H3K4Me2 concentration 40 µM and the final concentration 20 µM, y = 4.

H_2O_2 STANDARD CURVE PROTOCOL

Dilution of Standards and the Detection Reaction

1. Prepare 500 µl of a "Detection Reagent Mix", by mixing 475 µl Assay Buffer (BML-KI566, room temperature), 5 µl CELLestial® Red Peroxidase Substrate (100x, BML-KI565, room temperature in DMSO) and 20 µl of Horseradish Peroxidase Concentrate (50x, BML-KI564)

2. Dilute 5 µl of H_2O_2 Stock Solution (BML-KI567) with 875 µl of Assay Buffer to prepare 5 mM solution. Dilute 5 µl of the 5 mM solution with 120 µl Assay Buffer to produce a 200 µM solution. Dilute this solution 20-fold (5 µl + 95 µl Assay Buffer) to prepare a 10 µM solution.

3. Designate a column (8 wells) of one of the ½-volume microplates for the standard curve. Pipette 50 µl of Assay Buffer into 7 of the wells (B-H) and 75 µl of the 10 µM H_2O_2 into well A.

4. Perform serial dilutions (1/3) on wells A-G by transferring 25 µl from well A to B, mixing, transferring 25 µl from B to C etc. After mixing well G, remove and discard 25 µl. The H_2O_2 concentrations in wells A-H will now be: 10, 3.3, 1.1, 0.37, 0.12, 0.041 and 0.014 µM.

5. Mix 50 µl of the "Detection Reagent Mix" with the 50 µl in each standard well and incubate 5 min. at room temperature. The final H_2O_2 concentrations in wells A-H will be: 5.0, 1.7, 0.56, 0.19, 0.062, 0.021 and 0.0069 µM.

6. Read samples in a microplate reading fluorimeter capable of excitation at a wavelength in the range 530-570 nm and detection of emitted light ca. 590 nm. Adjustment of the fluorimeter's gain setting may be necessary to obtain a reading in which the fluorescence readings from all wells are on scale. Be sure to use this same gain setting for any subsequent experiments in which the standard curve is to be used to convert fluorescence to molar quantities (see Data Analysis, below).

7. Plots of fluorescence vs. [H_2O_2], and the reverse, are shown in Figures 1A and 1B.

Data Analysis with Standard Curve Slopes

Create one of the two types of plot shown in Figure 2 and obtain a slope, either as Arbitrary Fluorescence Units (AFU)/µM (Fig. 1A) or µM/AFU (Fig. 1B).

Two sample calculations are given below for the conversion of LSD1 rate data in units of AFU/sec to pmol/min/µg of LSD1. The numbers used in the examples come from the initial rate data for 0.5 µg LSD1 shown in Fig. 2B:

Rate at 20 µM H3K4Me2 = 11.5 AFU/sec

Background Rate at 0 µM H3K4Me2 = 0.771 AFU/sec

Net Rate at 20 µM = 11.5 AFU/sec – 0.771 AFU/sec

= 10.7 AFU/sec

EXAMPLE 1.

Standard Curve Slope (Fig. 1A) = 7546 AFU/µM

Rate (pmol/min/µg of LSD1) =

$$\frac{10.7 \text{ AFU/sec} \times 60 \text{ sec/min} \times 100 \text{ µl } (10^{-6} \text{ L})}{0.5 \text{ µg} \times 7546 \text{ AFU/µM } (\text{AFU} \cdot \text{L}/(10^{-6} \text{ mol}))}$$

= 17.0 pmol/min/µg (10^{-12} mol/min/µg)

EXAMPLE 2.

Standard Curve Slope (Fig. 1B) = 1.325×10^{-4} µM/AFU

Rate (pmol/min/µg of LSD1) =

$$\frac{10.7 \text{ AFU/sec} \times 60 \text{ sec/min} \times 100 \text{ µl} \times 1.325 \times 10^{-4} \text{ µM/AFU}}{0.5 \text{ µg}}$$

= 17.0 pmol/min/µg

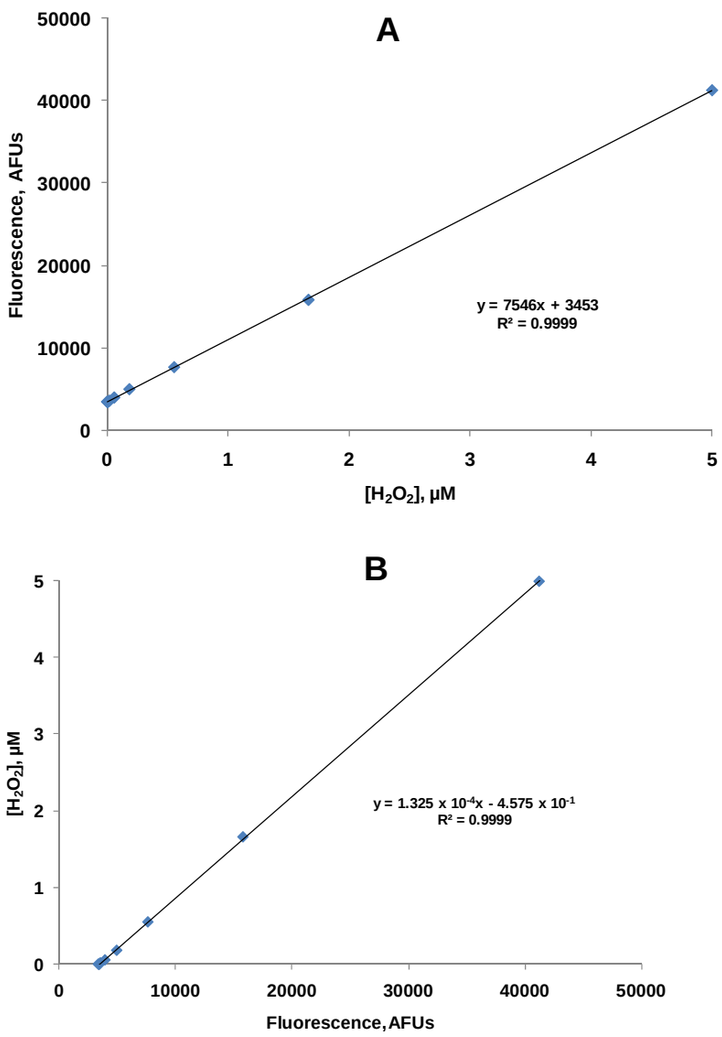

Figure 1. Fluorescence Standard Curves. A hydrogen peroxide dilution series was prepared, as described (50 µl/well; "H_2O_2 Standard Curve Protocol") in the wells of a clear microplate (80-2404) and 50 µl of "Detection Reagent Mix" mixed into each well. After 5 min., room temperature (23°C), fluorescence was measured on a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 530 nm, Em. 590 nm, gain = 60). H_2O_2 concentrations are those in the final 100 µl volume. The same data is plotted as both Fluorescence (AFU) as a function of [H_2O_2] (µM), (A), and the reverse (B).

APPLICATION EXAMPLES

Time Courses

LSD1 demethylation reactions at several concentrations of the H3K4Me2 peptide substrate were set up as described under “Assay Procedures” and the fluorescence over time recorded (Figure 2)

Note that the rate of fluorescence increase diminishes over time (Fig. 2A), but remains linear during approximately the first 300 sec. (5 min.) of the reaction at room temperature (23°C; Fig 2B).

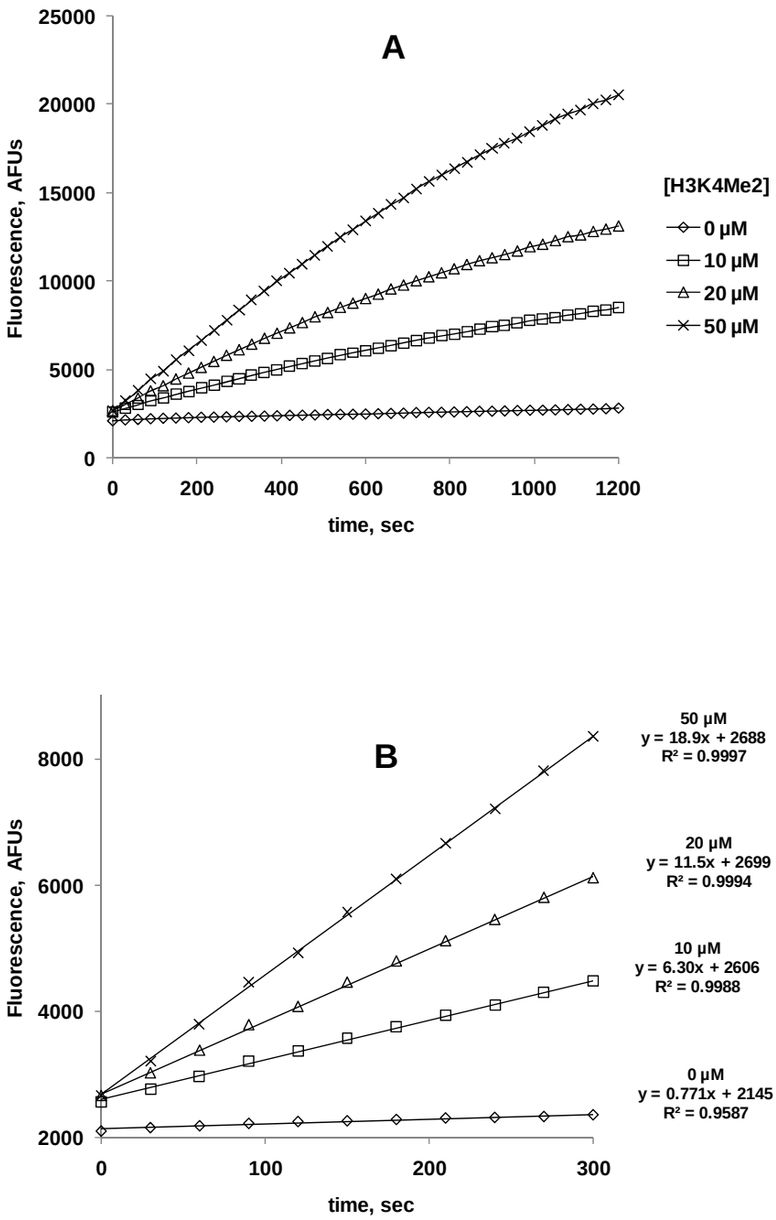

Figure 2. Time Courses of H3K4Me2 Peptide Substrate Demethylation by LSD1. LSD1 enzyme (0.5 µg/well) was incubated with the indicated concentrations of substrate, at room temperature (23°C), as described in “Assay Procedures”. Fluorescence was measured at 30 sec intervals on a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 530 nm, Em. 590 nm, gain = 60). The full 1200 sec time courses are plotted in (A). Data from the first 300 sec are replotted in (B) and shown with a linear least-squares fit to the 0-300 sec plot for each concentration of H3K4Me2 peptide.

Dependence of LSD1 Kinetics on [H3K4Me2]

1. Time courses were performed as described above at [H3K4Me2] = 0, 2, 5, 10, 20, 50, 100 and 200 µM.

2. Initial rates were obtained from linear least-squares fits of 0-300 sec plots as in Figure 2B. The small background rate in the absence of peptide (0 µM) was subtracted to yield the net rates due to H3K4Me2 demethylation. (See Fig. 2B and Data Analysis with Standard Curve Slopes.)

3. The initial, net rates were converted to units of pmol/min./µg by reference to an H_2O_2 Standard Curve (See "H_2O_2 Standard Curve Protocol".)

4. LSD1 demethylation rates were plotted as a function of [H3K4Me2] and the data fitted by non-linear least-squares to the Michaelis-Menten equation (Figure 3). Note that the value obtained for the H3K4Me2 peptide K_m, 120 µM, is substantially higher than one reported in the literature (4.2 µM; F. Forneris *et al. J. Biol. Chem.* 2005 **280** 41360). Presumably this is due to differences between the buffers used in the two assays. The 4.2 µM K_m value was observed at low ionic strength (50 mM HEPES, buffer alone) and addition of 80 mM NaCl was reported to triple the peptide K_m (F. Forneris *et al. J. Biol. Chem.* 2005 **280** 41360).

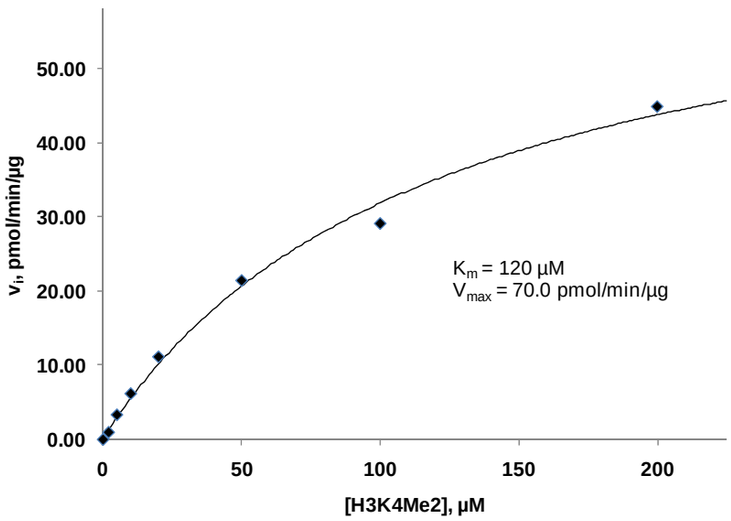

Figure 3. Kinetics of H3K4Me2 Peptide Substrate Demethylation by LSD1. LSD1 enzyme (0.5 µg/well) was incubated with the indicated concentrations of substrate, at room temperature (23°C), as described in "Assay Procedures". Fluorescence was measured at 30 sec intervals on a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 530 nm, Em. 590 nm, gain = 60). Initial rates were obtained from linear least squares fits to the 0-300 sec data as described (Time Courses) and converted to units of pmol/min/µg ("H_2O_2 Standard Curve Protocol"). K_m and V_{max} values were obtained from a direct least-squares fit to the Michaelis-Menten equation, $v = V_{max}[S]/K_m+[S]$ ('Solver' tool, Microsoft, Excel).

Concentration Dependence of Tranylcypromine Inhibition

1. Tranylcypromine is an irreversible, time-dependent inhibitor of LSD1 (see "Background", ref. 12). In order to minimize the kinetic complications of time-dependent inhibition during substrate demethylation, LSD1 was preincubated for 30 min. with varying concentrations of tranylcypromine, diluted 20-fold into the demethylation assay and initial demethylation rates determined from a short time course (141 sec).

2. A 2 mM solution of Tranylcypromine in Assay Buffer was prepared from the 100 mM Tranylcypromine stock solution (Preparing Reagents for Assay, step 3.), A series of six additional dilutions were prepared by successive three-fold dilutions in Assay Buffer.

3. A 0.2 µg/µl dilution of LSD1 was prepared and 5 µl was mixed with 5 µl of each of the seven tranylcypromine dilutions (step 2.) and one 5 µl aliquot of Assay Buffer. The 8 samples were left to incubate at room temperature (23°C) for 30 min.

4. At the end of 30 min, 5 µl (0.5 µg) of each of the eight LSD1 samples was transferred to an assay well to complete the "2x Enzymes" solutions and the demethylation reaction was initiated by mixing with "2x Substrates" (Final [H3K4Me2] = 20 µM; See "Assay Procedures" and Table 1.)

5. Fluorescence was read, as described above (Time Courses), at 47 sec intervals, for 30 min.

6. Initial demethylation rates were determined from linear fits to plots of the first 4 fluorescence readings (0-141 sec.). A dose-response curve was produced by plotting these rates against tranylcypromine concentration and a relative IC_{50} derived from a least squares fit to the 4-parameter Hill-Slope model (Figure 4).

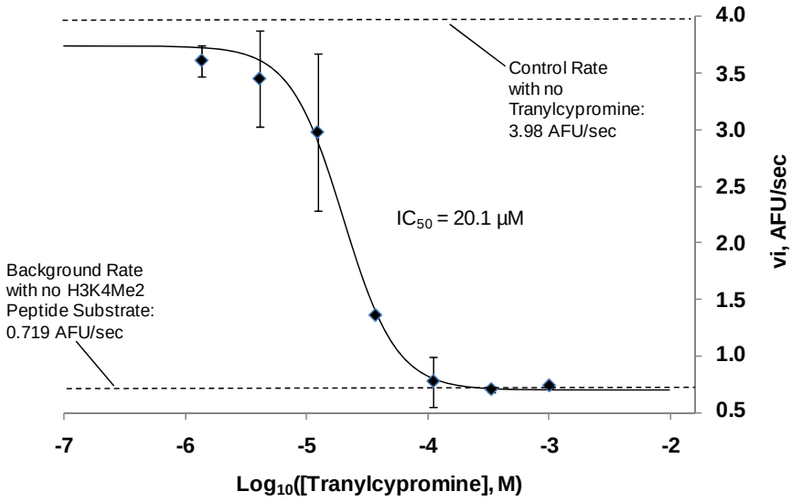

Figure 4. Tranylcypromine Inhibition of LSD1. LSD1 enzyme (0.1 µg/µg) was incubated with the indicated concentrations of tranylcypromine for 30 min. at room temperature (23°C). Samples (0.5 µg, 5 µl) were then transferred to wells for the demethylation assay with 20 µM H3K4Me2 peptide. Fluorescence was measured at 47 sec intervals on a CytoFluor™ II fluorescence plate reader (PerSeptive Biosystems, Ex. 530 nm, Em. 590 nm, gain = 60). Initial rates were obtained from linear least squares fits to the 0-141 sec data as described (Time Courses). The dose-response curve was derived from a least squares fit to the 4-parameter Hill-Slope model, $y = \text{'}bottom + (top - bottom)/(1 + (x/IC_{50})^{slope})$ ('Solver' tool, Microsoft, Excel). The fitted parameter values were: *top* = 3.74 AFU/sec; *bottom* = 0.696 AFU/sec; IC_{50} = 20.1 µM; slope = 1.98.

NOTE: THE APPLICATION EXAMPLES, DESCRIBED HEREIN, ARE INTENDED ONLY AS GUIDELINES. THE OPTIMAL CONCENTRATIONS OF SUBSTRATES AND INHIBITORS, ASSAY VOLUMES, BUFFER COMPOSITION, AND OTHER EXPERIMENTAL CONDITIONS MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PARTICULAR RESULTS, THROUGH THE USE OF THESE PROCEDURES, IS MADE OR IMPLIED.

DETECTION BY ABSORBANCE AT 563 nm

The CELLestial® Red peroxidation product may also be detected by its absorbance at 563 nm. Absorbance standard curves for H_2O_2 concentration ranges of 0-10 µM and 0-50 µM are shown in Figure 5.

Absorbance detection is less sensitive than fluorescence and adjustment to the conditions for LSD1 drug discovery assays may therefore be useful. For example, try choosing an H3K4Me2 Peptide concentration that generates a higher rate than the 20 µM recommended for fluorescence assays, but is still sub-K_m (e.g. 50 µM or 100 µM).

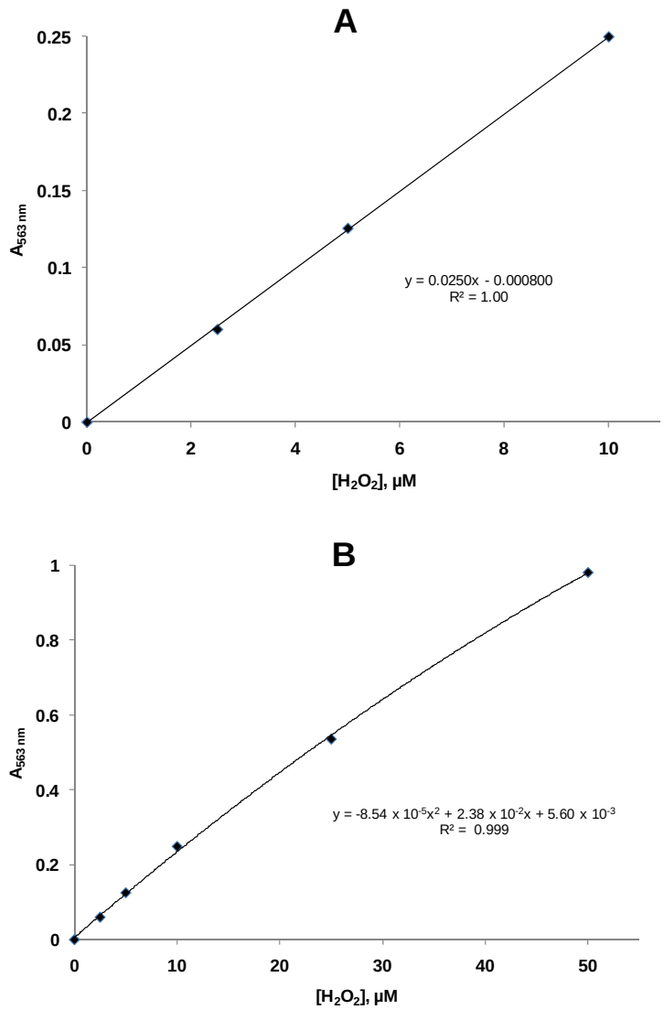

Figure 5. Absorbance Standard Curves. A hydrogen peroxide dilution series was prepared, as described (50 µl/well; "H_2O_2 Standard Curve Protocol", but 0-100 µM) in the wells of a clear microplate (80-2404) and 50 µl of "Detection Reagent Mix" mixed into each well. After 5 min., room temperature (23°C), absorbance at 563 nm was measured on a PowerWave x340 plate reader (Bio-Tek Instruments). H_2O_2 concentrations are those in the final 100 µl volume. Least squares fits to the 0-10 µM data (A) and the 0-50 µM data (B), were linear and 2nd order polynomial, respectively.

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