TECHNICAL MANUAL

Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay

Instructions for Use of Products
V7990 and V7991

3/17
TM488

Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay

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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay[a–e] is a bioluminescent assay for detecting the activity of JumonjiC histone demethylases and several Fe(II)/α-ketoglutarate-dependent dioxygenases that use α-ketoglutarate as substrate and release succinate as a product. Demethylation/hydroxylation reactions catalyzed by demethylases/dioxygenases are central to many biological processes, including post-translational modification of histones (1) and non-histone proteins (2), DNA/RNA repair (3), metabolism (4), and oxygen sensing (5). In particular, JumonjiC domain-containing histone lysine demethylases (JmjCs) play a pivotal role in determining the epigenetic status of the genome by counteracting the activities of histone lysine methyltransferases. These enzymes act as erasers by catalyzing the removal of methyl marks from specific lysine sites in histones, leading to either transcriptional repression or activation of target genes. The hydroxylation reaction catalyzed by these enzymes proceeds via an activated Fe(IV)-oxo intermediate (6).

Members of the demethylase/dioxygenase family have generated increased interest as potential drug targets. Specifically, among the most promising targets are the Jumonji-domain lysine demethylases (KDM) for the treatment of several cancers and inflammation (1,7), the fat mass and obesity associated protein (FTO) for obesity (8), and the prolyl hydroxylases EGLN1–3 and the asparaginyl hydroxylase FIH for ischemic and inflammatory conditions (9).

The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay is a homogenous, double-reagent-addition method to rapidly detect succinate formation in JumonjiC histone demethylase and Fe(II)/α-ketoglutarate-dependent dioxygenase reactions. After the demethylase or dioxygenase reaction, an equal volume of Succinate Detection Reagent I and Succinate Detection Reagent II are added sequentially to convert the succinate product to ATP and generate light in a luciferase reaction. The light generated is detected using a luminometer (Figure 1). Luminescence can be correlated to succinate concentration by using a succinate standard curve. The light output is proportional to the concentration of succinate from low nM to 15µM (Figure 2, Panel A). The assay is highly sensitive (Figure 2, Panel B), a feature that is desirable and essential for measuring the activity of different Fe(II)/α-ketoglutarate-dependent dioxygenases covering the majority of dioxygenase classes (Figure 3). Therefore, the succinate detection assay uses less enzyme in JumonjiC histone demethylase/Fe(II)/α-ketoglutarate-dependent dioxygenase reactions. This assay is easy to use, fast and simple (Figure 4). The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay is performed in a single well of a multiwell plate, and can be used to detect demethylase/dioxygenase activity in as little as a 5µl reaction.

The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable glow-type luminescent signal and improve performance across a wide range of assay conditions. The signal produced by the luciferase reaction initiated by adding the Succinate Detection Reagents I and II is stable for more than 3 hours (Figure 2, Panel C). This extended stability eliminates the need for a luminometer equipped with injectors and allows batch-mode processing of multiple plates. Furthermore, the combination of Ultra-Glo™ Recombinant Luciferase (5) and proprietary formulation of the Succinate Detection Reagents I and II results in luminescence that is much less susceptible to interference from library compounds than other luciferase- or fluorescence-based assays (6). In addition to providing biochemical values comparable to those reported in the literature (10), the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay can be used in screening for specific demethylase/dioxygenase inhibitors and the study of their mode of action (see Section 4.C).

Note: This assay detects only the activity of JumonjiC histone demethylases or Fe(II)/α-ketoglutarate-dependent dioxygenases that use α-ketoglutarate as a substrate and can only be used with purified enzymes. Whole cells and cell extracts cannot be used in this assay, as endogenous ATP or/and succinate interferes with assay performance. However, JumonjiC histone demethylases and Fe(II)/α-ketoglutarate-dependent dioxygenases can be purified from cell extract using immunoprecipitation or affinity tag pull down then used in the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay.

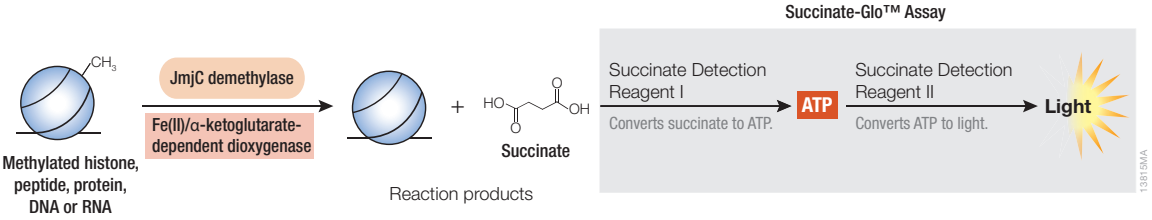
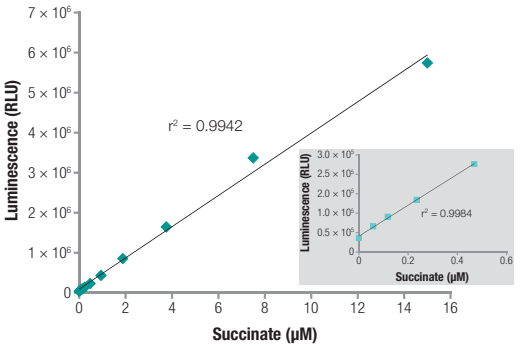


Figure 1. Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay principle. The assay is performed in two steps. After the demethylase/dioxygenase reaction, Succinate Detection Reagent I is added to convert succinate to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated correlates to the amount of succinate produced by the demethylase/dioxygenase, which indicates demethylase/dioxygenase activity.

1. Description (continued)

A.



B.

	Succinate (µM)								
	15	7.5	3.75	1.88	0.94	0.47	0.23	0.12	0.06	0
Signal-to-Background Ratio at 10 minutes	157	92	45	23	12	6	4	2.5	1.8	1

C.

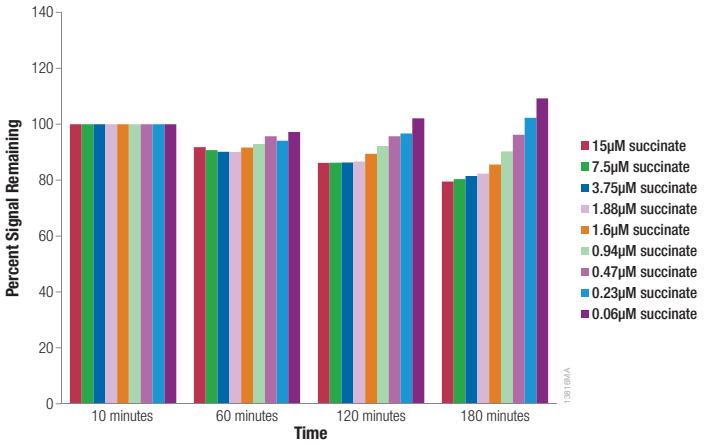


Figure 2. Linearity and sensitivity of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay. Panel A. Succinate standard curve was prepared over the indicated succinate concentration range in 25µl of 1X JmjC Demethylase/Hydroxylase assay buffer in a solid white 96-well plate. (Standard curve preparation is described in Section 3.B.) Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay was performed using 25µl of Succinate Detection Reagent I and 50µl of Succinate Detection Reagent II at room temperature as described in Section 4. Luminescence was recorded using a GloMax® 96 Microplate Luminometer (Cat.# E6501). Values represent the mean of four replicates. Inset graph is an enlargement of data for <0.5µM succinate. **Panel B.** Luminescence was measured 10 minutes after adding the Succinate Detection Reagents, and signal-to-background ratios calculated for each concentration of the succinate standard curve. **Panel C.** To determine signal stability, luminescence was recorded again every hour. The signal-to-background ratio did not change over the time measured (data not shown). RLU = relative light units.

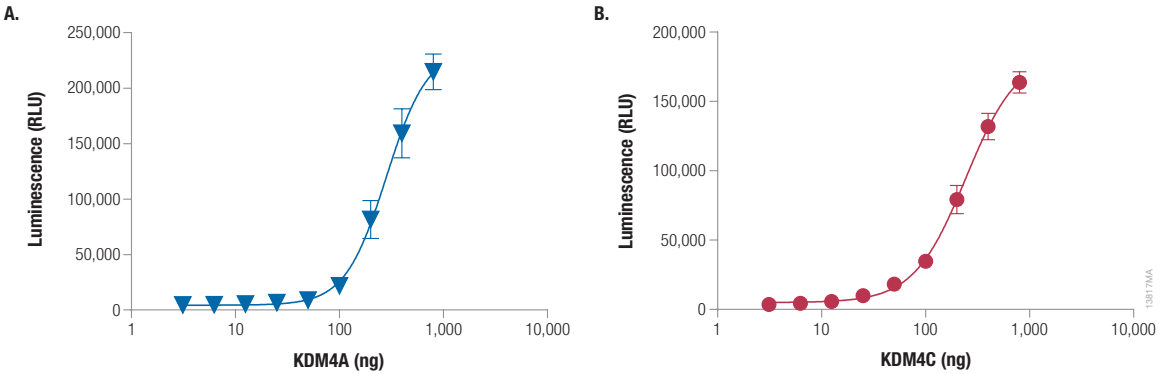


Figure 3. Detection of the activity of KDM4A and KDM4C. Panel A. KMD4A (BPS Bioscience Cat.# 50123) was titrated in 1X JmjC Demethylase/Hydroxylase assay buffer in the presence of 10µM methylated histone H3 peptide substrate (Anaspec Cat.#64452), 100µM ascorbate, 10µM Fe (II) and 10µM α-ketoglutarate. **Panel B.** KDM4C (BPS Bioscience Cat.# 50105) was titrated in 1X JmjC Demethylase/Hydroxylase assay buffer in the presence of 10µM methylated histone H3 peptide substrate (Anaspec Cat.#64452), 100µM Ascorbate, 10µM Fe (II) and 10µM α-ketoglutarate. All enzyme reactions were performed in a total volume of 10µl in a solid white 384-well plate and incubated at 23°C for 60 minutes. The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay was performed as described in Section 4.A. Each point is an average of two experiments, and the error bars represent the standard deviations. Curve fitting was performed using GraphPad Prism® version 6, sigmoidal dose-response (variable slope) software.

1. Description (continued)

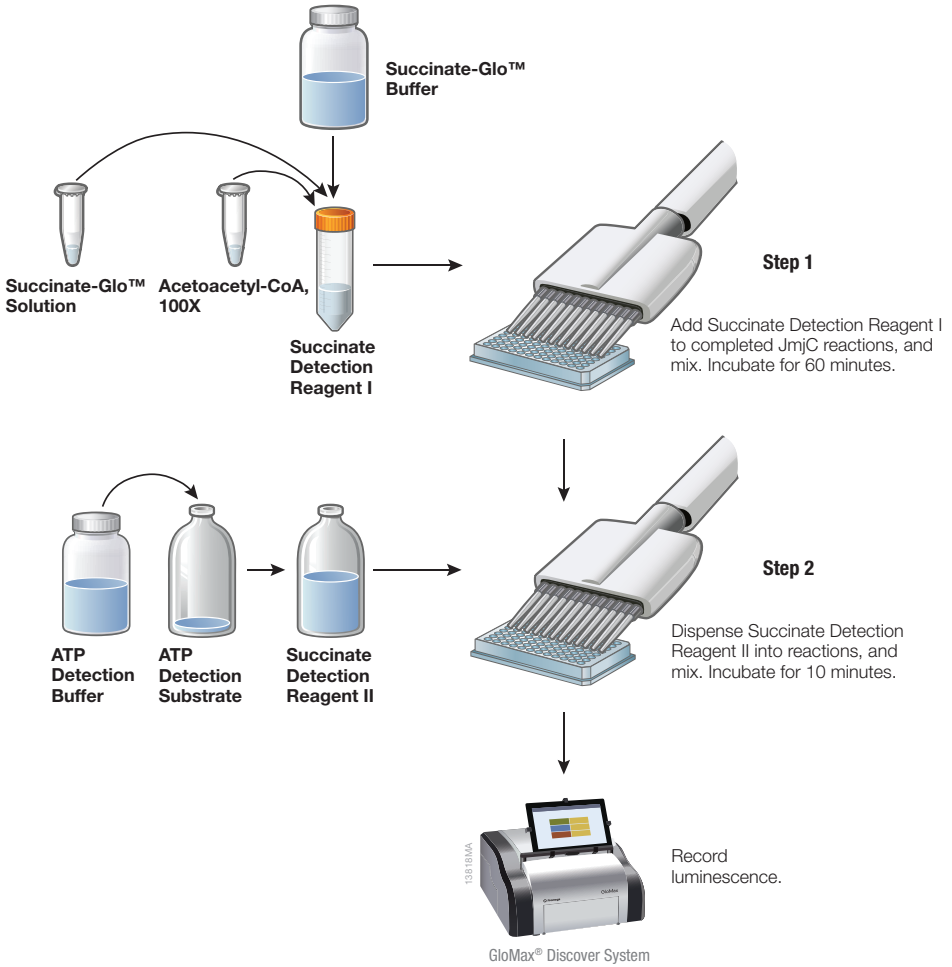


Figure 4. Schematic representation of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay protocol.

Advantages of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay

- **Linear response in the nM to µM range:** Uses low concentrations of α-ketoglutarate, decreasing feedback JumonjiC histone demethylase/Fe(II)/α-ketoglutarate-dependent dioxygenase inhibition issues.
- **High dynamic range:** High signal-to-background ratios at lower concentrations of succinate means using less enzyme during the demethylase/dioxygenase reaction.
- **High sensitivity:** Detect 2–4pmol succinate with twofold difference over background.
- **Reliable, reproducible data:** Routinely obtain Z factor values >0.7 even with low succinate production rates.
- **Universal assay:** Use any demethylase/dioxygenase and demethylase/dioxygenase:substrate combination, including peptide, protein and DNA substrates.
- **Positive correlation:** Assay signal increases linearly with increasing product formation.
- **Luminescence-based succinate detection:** Experience less overall assay interference from chemical compounds.
- **Batch plate processing:** Highly stable luminescent signal with >80% signal remaining after 3 hours.
- **Homogeneous non-radioactive assay:** No safety or waste management issues due to the use of radioisotopes.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay	**1,000 assays**	**V7990**

This system is sufficient for 1,000 assays if performed in 384-well plates using 5:5:10µl ratio corresponding to volumes in microliters of the demethylase/dioxygenase reaction and Succinate Detection Reagents I and II, respectively. The system can also be used in 96-well plates with 25:25:50µl ratio for a total of 200 assays. It includes:

- 50µl Succinate, 10mM
- 50µl Succinate-Glo™ Solution
- 100µl Acetoacetyl-CoA, 100X
- 5ml Succinate-Glo™ Buffer
- 10ml ATP Detection Buffer
- 1 vial ATP Detection Substrate (Lyophilized)

PRODUCT	SIZE	CAT.#
Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay	**10,000 assays**	**V7991**

This system is sufficient for 10,000 assays if performed in 384-well plates using 5:5:10µl ratio corresponding to volumes in microliters of the demethylase/dioxygenase reaction and Succinate Detection Reagents I and II, respectively. The system can also be used in 96-well plates with 25:25:50µl ratio for a total of 2,000 assays. It includes:

- 50µl Succinate, 10mM
- 500µl Succinate-Glo™ Solution
- 1ml Acetoacetyl-CoA, 100X
- 50ml Succinate-Glo™ Buffer
- 100ml ATP Detection Buffer
- 1 vial ATP Detection Substrate (Lyophilized)

Storage Conditions: Store the complete kit at less than –65°C. Alternatively, store the Succinate-Glo™ Solution at less than –65°C and the rest of the components at –30°C to –10°C. Before use, thaw all components completely at room temperature, except the Succinate-Glo™ Solution, which should be thawed on ice only prior to use and any remaining volume should be returned to less than –65°C. Once thawed, all components should be thoroughly mixed before use. For best results, prepare only the amount of Succinate Detection Reagent I needed. If smaller amounts of Succinate Detection Reagent I are used each time, the Succinate-Glo™ Solution should be dispensed into small aliquots and stored at less than –65°C to minimize freeze-thaw cycles.

3. Preparing for the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay

Materials to Be Supplied by the User

- solid white, multiwell plate (**do not** use black plates or clear plates)
- enzyme reaction buffers: used for enzyme dilution and for substrate/compound dilution
- multichannel pipette or automated pipetting station
- ascorbate, Fe (II) and α-ketoglutarate
- substrates [e.g., methylated histone H3 peptide substrates or DNA substrate]
- JmjC demethylase or dioxygenase enzyme
- luminometer capable of reading multiwell plates
- plate shaker

3.A. Preparing the Succinate Detection Reagents

Calculate the required volumes of each reagent needed for your experiment, and increase or decrease the volumes appropriately.

Succinate Detection Reagent I Preparation

1. Equilibrate the Succinate-Glo™ Buffer to room temperature before use.
2. Prepare Succinate Detection Reagent I by adding 10µl of Acetoacetyl-CoA, 100X, and 10µl of Succinate-Glo™ Solution to each 1ml of Succinate-Glo™ Buffer immediately before use.
3. Briefly vortex or mix by gently swirling or by inverting the contents to obtain a homogeneous solution. The Succinate Detection Reagent I is now ready to be added to the assay plate containing succinate.

Notes:

1. Prepare only enough Succinate Detection Reagent I required for the experiment. Store the remaining Succinate-Glo™ Solution at less than −65°C.
2. If Succinate Detection Reagent I will be prepared and incubated at room temperature or on ice for more than 1 hour (e.g. for a high-throughput screening application), we recommend that the Acetoacetyl-CoA, 100X, be added to the JmjC reaction at 1X final concentration as described in Section 4.B. Succinate Detection Reagent I prepared without acetoacetyl-CoA is stable for 3 hours at room temperature, and acetoacetyl-CoA had no effect on JmjC enzymatic reactions.

Succinate Detection Reagent II Preparation

1. Equilibrate the ATP Detection Buffer and ATP Detection Substrate to room temperature before use.
2. Transfer the entire volume (10ml) of ATP Detection Buffer into the amber bottle containing ATP Detection Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the Succinate Detection Reagent II.
3. Mix by gently vortexing, swirling or by inverting the contents to obtain a homogeneous solution. The ATP Detection Substrate should go into solution easily in less than one minute. The Succinate Detection Reagent II is now ready to be used.
4. Use Succinate Detection Reagent II immediately or dispense into aliquots and store at −20°C.

3.B. Generating a Standard Curve for Succinate

To estimate the amount of succinate produced in the demethylase/dioxygenase reaction, we recommend creating a succinate standard curve of 0–15µM succinate.

The succinate standards can be prepared in a separate 96-well or 384-well plate. Once the standards are prepared, transfer the appropriate amount to the same assay plate where the demethylase/dioxygenase reaction is being performed. We recommend assaying each succinate standard concentration in triplicate. Figure 2 shows representative data from a succinate standard curve.

1. Prepare 200µl of 15µM succinate solution in desired 1X demethylase/dioxygenase reaction buffer using the provided 10mM Succinate standard. Then add all 200µl of the 15µM succinate solution to well A1 of a preparative 96-well plate.
2. Add 100µl of 1X demethylase/dioxygenase reaction buffer to wells A2 through A10 of the preparative 96-well plate.

   Note: Depending on the enzymatic reaction requirements, you can use 1X demethylase/dioxygenase buffer containing succinate and other appropriate substrates and co-factors such as 100µM ascorbate, 10µM Fe (II) and 10µM α-ketoglutarate or only succinate.
3. Perform a serial twofold dilution as shown in Figure 5 by transferring 100µl from well A1 to well A2 and pipetting to mix. Transfer 100µl from well A2 to well A3 and pipetting to mix. Repeat for wells A4 through A9. Discard the extra 100µl from well A9. Do not add succinate to the no-succinate control reactions in well A10.

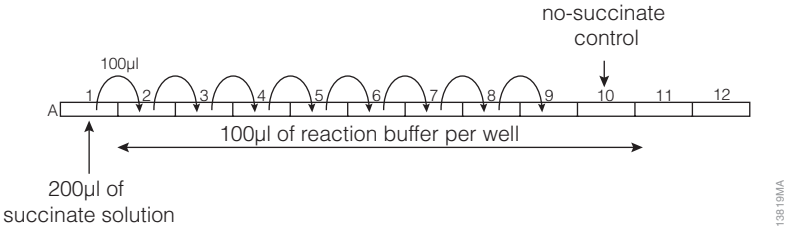


Figure 5. Dilution scheme for creating a succinate standard curve.

4. Transfer the desired volume of each succinate standard from the preparative 96-well plate to the wells reserved for the succinate standard curve on your assay plate.
5. Proceed immediately to the assay protocol (Section 4).

We recommend the following volumes for different assay plate formats:

96-well plate: Transfer 25µl of succinate standards.

384-well plate: Transfer 10µl of succinate standards.

Low-volume 384-well: Transfer 5µl of succinate standards.

The luminescence output of the assay is proportional to the concentration of succinate in the standard curve. Therefore, luminescence can be directly compared to those succinate concentrations generated in a demethylase/dioxygenase reaction sample. However, for accurate comparison, the volume of the succinate standards must be equal to the volume of the demethylase/dioxygenase reaction, and the buffer components must be identical.

4. Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay Protocols

Prior to performing the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay, prepare the reagents and succinate standards as described in Section 3. Calculate the volume of Succinate Detection Reagents required for your experiments, and equilibrate that volume to room temperature before use. Thaw the Succinate-Glo™ Solution on ice prior to use. Store the remaining reagents at −20°C, and any remaining volume of the Succinate-Glo™ Solution should be returned to less than −65°C. The Succinate Detection Reagent I prepared without acetoacetyl-CoA is stable for 3 hours at room temperature with minimal loss of signal and signal-to-background ratios. The Succinate Detection Reagent I with acetoacetyl-CoA should be prepared just prior to use and immediately added to the assay plate.

4.A. Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay Protocol

The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay is performed in two steps after the completed demethylase/dioxygenase reaction as outlined in Figures 1 and 4. For 96-well plates, we recommend a 25µl demethylase/dioxygenase reaction, followed by adding 25µl Succinate Detection Reagent I and 50µl Succinate Detection Reagent II for a total volume of 100µl. For 384-well plates, volumes may be reduced fivefold to a 5µl demethylase/dioxygenase reaction, followed by dispensing 5µl Succinate Detection Reagent I and 10µl Succinate Detection Reagent II for a total volume of 20µl. Other volumes may be used provided the 1:1:2 ratios of demethylase/dioxygenase reaction volume to Succinate Detection Reagents volumes are maintained. The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay protocol for 96-well plates is described below.

1. Perform a 25µl demethylase/dioxygenase reaction using the 1X demethylase/dioxygenase buffer of your choice. (See Section 7 for buffer examples). If the demethylase/dioxygenase reaction was not incubated at room temperature, equilibrate the plate to room temperature before adding the Succinate Detection Reagent I.
2. Prepare the Succinate Detection Reagent I by adding 10µl of Succinate-Glo™ Solution and 10µl of Acetoacetyl-CoA, 100X, to each 1ml of Succinate-Glo™ Buffer.
3. Add 25µl of Succinate Detection Reagent I to each well of the assay plate.

   Note: The Succinate Detection Reagent I terminates the demethylase/dioxygenase reaction; therefore, there is no need to add an inhibitor to terminate the demethylase/dioxygenase reaction (e.g., EDTA, acid, etc.). However, if a demethylase/dioxygenase-termination reagent is added to the enzymatic reaction, do not use a magnesium-chelating agent such as EDTA because the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay requires magnesium.
4. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 60 minutes.
5. Add 50µl of Succinate Detection Reagent II to each well of the assay plate to generate light from the ATP formed in Step 4.
6. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 10 minutes.
7. Measure the luminescence with a plate-reading luminometer.

   Note: Instrument settings depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline. The long signal half-life of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay means plates can be batch processed at room temperature with minimal loss of luminescence signal.

4.B. Succinate Detection Protocol for High-Throughput Screening Applications

Prior to performing the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay, prepare the reagents and succinate standards as described in Section 3, omitting acetoacetyl-CoA in Succinate Detection Reagent I.

Succinate Detection Reagent I prepared with acetoacetyl-CoA and incubated at room temperature or on ice for more than 1 hour can become unstable and generate higher background in the assay. Succinate Detection Reagent I prepared without acetoacetyl-CoA is stable for 3 hours at room temperature. Therefore, we recommend that Acetoacetyl-CoA, 100X, be added to the JmjC reaction at 1X final concentration.

Calculate the volume of Succinate Detection Reagents required for your experiments, and equilibrate the needed volumes to room temperature before use, except the Succinate-Glo™ Solution, which is thawed on ice prior to use. Return the remaining reagents to a –20°C freezer and any remaining Succinate-Glo™ Solution should be stored at less than –65°C. Follow the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay protocol described in Section 4.A, starting at Step 3.

4.C. Optimizing JumonjiC Histone Demethylase/Fe(II)/α-Ketoglutarate-Dependent Dioxygenase Reaction Conditions

Perform the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay after optimizing the amount of demethylase/dioxygenase and substrates in the reaction. If you have determined the optimal amount of demethylase/dioxygenase or its substrates, proceed to Section 4.A.

Note: We recommend optimizing the demethylase/dioxygenase reaction conditions at room temperature to ensure uniform temperature across the plate during the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay.

Preparation of JumonjiC Histone Demethylase/Fe(II)/α-Ketoglutarate-Dependent Dioxygenase Titration Components

This example protocol is written for JumonjiC histone demethylase KDM4C enzyme titration to select an optimal enzyme concentration for use in subsequent experiments such as an inhibitor titration (Figure 3, Panel B). The KDM4C reaction is performed in 96-well plate using 1X Demethylase/Hydroxylase Assay Buffer [50mM HEPES (pH 7.5)], 10µM methylated histone H3 peptide substrate (Anaspec Cat.#64452), 100µM ascorbate, 10µM Fe (II), 10µM α-ketoglutarate and a serial dilution of KDM4C enzyme from 0–800ng/reaction in 10µl volume. A succinate standard curve is performed in the same assay plate to correlate luminescence with the succinate concentrations generated in each KDM4C reaction.

Notes:

1. Different demethylases/dioxygenases have varying specific activities. Therefore, the useful enzyme dilution range may vary greatly and should be determined experimentally prior to inhibitor potency determinations.
2. Different demethylases/dioxygenases may have succinate contamination. Therefore, we recommend that a no-histone H3 peptide substrate mix be tested in parallel to the enzyme titration.

1. **Substrate Mix Preparation:** Prepare 1,000µl of 2X α-Ketoglutarate/Methylated Histone H3 Peptide Substrate Mix (5µl/reaction/well) in a 1.5ml tube as described below, and keep at room temperature until ready to dispense in the assay plate.

Component	Volume
250mM HEPES pH 7.5	200µl
1mM methylated histone H3 peptide substrate	20µl
1mM ascorbic acid	200µl
1mM Fe(II)	20µl
1mM α-ketoglutarate	20µl
DMSO	20µl
ATP-free water	520µl

2. **KDM4C Solution Preparation:** Prepare 100µl KDM4C enzyme solution as described below (5µl/reaction/well). This will give 800ng KDM4C/5µl starting concentration.

Component	Volume
250mM HEPES (pH 7.5)	20µl
KDM4C (2.4µg/µl)	6.7µl
ATP-free water	73.3µl

   a. Prepare 1ml of 1X JmjC Demethylase/Hydroxylase dilution buffer by mixing 200µl of 250mM HEPES (pH 7.5) with 800µl of ATP-free water.

   b. Add KDM4C enzyme solution to well A1 of a 96-well plate.

   c. Add 50µl of 1X JmjC Demethylase/Hydroxylase dilution buffer to wells A2 through A12 of the 96-well plate.

   d. Perform a serial twofold dilution by transferring 50µl from well A1 to well A2, pipetting to mix as described in Table 1. Transfer 50µl from well A2 to well A3, pipetting to mix. Repeat for wells A4–A11. Discard the extra 50µl from well A11. Do not add KDM4C to the no-enzyme control reaction in well A12.

      Note: Do not create bubbles while preparing the dilution series.

4.C. Optimizing JumonjiC Histone Demethylase/Fe(II)/α-Ketoglutarate-Dependent Dioxygenase Reaction Conditions (continued)

Table 1. Performing Serial 1:1 Dilutions of KDM4C.

Well Number	KDM4C (ng)	Starting Volume of Each Well	Volume to Transfer
A1	800	100µl	50µl
A2	400	50µl	50µl
A3	200	50µl	50µl
A4	100	50µl	50µl
A5	50	50µl	50µl
A6	25	50µl	50µl
A7	12.5	50µl	50µl
A8	6.25	50µl	50µl
A9	3.12	50µl	50µl
A10	1.56	50µl	50µl
A11	0.78	50µl	0µl; No transfer
A12	0	50µl	Buffer only

KDM4C Reaction and Succinate Standard Curve Experiment

1. Transfer 10µl of the succinate serial dilution in duplicates into the standard curve-designated wells of the 384-well assay plate.
2. Transfer 5µl of KDM4C dilution samples in duplicates from the wells of the KDM4C titration plate to the wells of the assay plate.
3. Transfer 5µl of the 2X α-Ketoglutarate/Methylated Histone H3 Peptide Substrate Mix to the rows of the KDM4C dilutions.
4. Centrifuge the plate, and mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.
5. Prepare the Succinate Detection Reagent I by adding 10µl of Succinate-Glo™ Solution and 10µl of Acetoacetyl-CoA, 100X, to each 1ml of Succinate-Glo™ Buffer.
6. Add 10µl of Succinate Detection Reagent I to each well of the assay plate.

   Note: The Succinate Detection Reagent I terminates the demethylase/dioxygenase reaction; therefore, there is no need to add an inhibitor to terminate the demethylase/dioxygenase reaction (e.g., EDTA, acid, etc.). However, if a demethylase/dioxygenase-termination reagent is added to the enzymatic reaction, do not use a magnesium-chelating agent such as EDTA because the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay requires magnesium.
7. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 60 minutes.
8. Add 20µl of Succinate Detection Reagent II to each well of the assay plate to generate light from the ATP formed in Step 4.

9. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 10 minutes.
10. Record luminescence using a plate-reading luminometer.

    Note: Instrument settings depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline.

Note: The optimal amount of a demethylase/dioxygenase to use in subsequent experiments including chemical compound screens and IC_{50} determinations is the amount that produces luminescence within the linear range of the demethylase titration curve and generates an adequate signal-to-background ratio.

Because the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay is sensitive, it can detect a small amount of succinate with a good signal-to-background ratio. As a result, a small amount of enzyme that produces low amount of succinate is sufficient for use with the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay. Figure 3 shows that 25ng KDM4C produced 300nM succinate with a threefold signal-to-background ratio.

4.D. Determining IC_{50} Values of Demethylase/Dioxygenase Inhibitors

The following protocol is an example of an inhibitor titration in KDM4C reaction at a final concentration of 10µM methylated histone H3 peptide substrate and 110ng KDM4C (optimal amount with a tenfold signal-to-background ratio produced). Representative inhibitor titration data using KDM4C inhibitor IOX-1 is shown in Figure 6. This protocol is designed for a 384-well plate using a 10µl:10µl:20µl ratio of demethylase/dioxygenase reaction volume to Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay reagents. Other volumes may be used, provided the 1:1:2 ratio of demethylase/dioxygenase reaction to Succinate Detection Reagent I and II volumes are maintained.

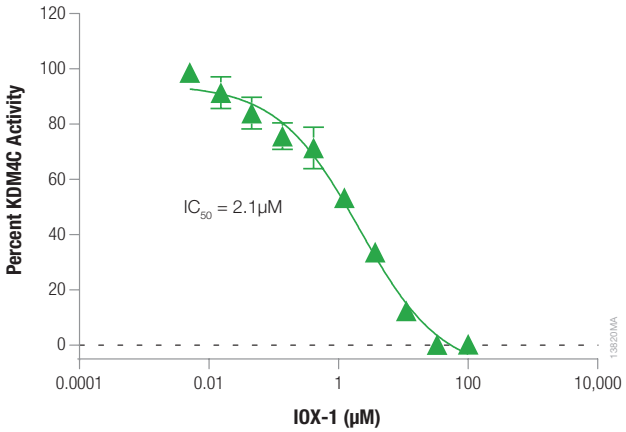


Figure 6. Determining IC_{50} for KDM4C inhibitor. Titration of KDM4C inhibitor IOX-1 (R&D Systems Cat.# 4464) was performed in solid white, half-volume 96-well plate in a total volume of 10µl using 110ng/well KDM4C, 10µM methylated histone H3 peptide substrate (Anaspec Cat.#64452), 100µM ascorbate, 10µM Fe (II), 10µM α-ketoglutarate and the indicated concentrations of inhibitor. KDM4C reactions were incubated for 60 minutes at room temperature (23°C). Curve fitting was performed using GraphPad Prism® version 6, sigmoidal dose-response (variable slope) software.

IC_{50} value for IOX-1 potency determined using the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay shown in Figure 6 compare favorably to the IC_{50} value reported for this compound in the literature (10).

4.D. Determining IC_{50} Values of Demethylase/Dioxygenase Inhibitors (continued)

Preparation of KDM4C Inhibitor IOX-1 Titration Components

1. **IOX-1 Solution Preparation:** Prepare 100µl of 400µM IOX-1 (with 4% DMSO) as described below (final 2.5µl/reaction/well). This gives 100µM IOX-1 (with 1% DMSO) starting concentration in the assay.

Component	Volume
250mM HEPES pH 7.5	10µl
10mM IOX-1 in DMSO	2µl
ATP-free water	38µl

   a. Prepare 1ml of 1X JmjC Demethylase/Hydroxylase dilution buffer (with 4% DMSO) by mixing 200µl of 250mM HEPES (pH 7.5), 40µl of DMSO and 760µl of ATP-free water.

   b. Add IOX-1 solution to well A1 of a 96-well plate.

   c. Add 66.6µl of 1X JmjC Demethylase/Hydroxylase dilution buffer (with 4% DMSO) to wells A2–A12 of the 96-well plate.

   d. Perform a serial threefold dilution by transferring 33.3µl from well A1 to well A2 and pipetting to mix as described in Table 2. Transfer 25µl from well A2 to well A3 and pipet to mix. Repeat for wells A4–A10. Do not add IOX-1 solution to the no-inhibitor control reaction in well A11 and the no-enzyme control reaction in well A12.

Table 2. Performing Serial 1:3 Dilutions of KDM4C Inhibitor IOX-1.

Well Number	Final IOX-1 Concentration (µM)	Starting Volume of Each Well	Volume to Transfer
A1	100	100µl	25µl
A2	33.3	50µl	25µl
A3	11.11	50µl	25µl
A4	1.23	50µl	25µl
A5	0.411	50µl	25µl
A6	0.137	50µl	25µl
A7	0.045	50µl	25µl
A8	0.015	50µl	25µl
A9	0.005	50µl	25µl
A10	0.0016	50µl	0µl; No transfer
A11	No inhibitor	50µl	Buffer only
A12	No enzyme	50µl	Buffer only

2. **KDM4C Solution Preparation:** Prepare 250µl of KDM4C solution (for 50 reactions at 2.5µl/reaction/well with extra volume for pipetting error) in a 1.5ml tube as described below, and keep on ice until ready to dispense in the assay plate. This will give 110ng of KDM4C/reaction.

Component	Volume
250mM HEPES pH 7.5	50 µl
KDM4C (2.4µg/µl)	4.6µl
ATP-free water	195.4µl

3. **Substrate Mix Preparation:** Prepare 600µl of 2X α-Ketoglutarate/Methylated Histone H3 Peptide Substrate Mix (5µl/reaction/well) in a 1.5ml tube as described below, and keep at room temperature until ready to dispense in the assay plate.

Component	Volume
250mM HEPES pH 7.5	120µl
1mM methylated histone H3 peptide substrate	12µl
1mM ascorbic acid	120µl
1mM Fe(II)	12µl
1mM α-ketoglutarate	12µl
ATP-free water	324µl

Inhibitor Titration Experiment

1. Using a multichannel pipette, transfer 2.5µl of IOX-1 samples in duplicate from the inhibitor titration plate to the corresponding wells of the 384-well assay plate (e.g., well A1 from the 96-well titration plate to well A1 and A2 of the 384-well assay plate, well A2 from the 96-well titration plate to well A3 and A4 of the 384-well assay plate, well A3 from the 96-well titration plate to well A5 and A6 of the 384-well assay plate, etc.)
2. Transfer 2.5µl of KDM4C samples in duplicate to wells A1–A22 of the 384-well assay plate.

   Note: Add only 2.5µl of 1X JmjC Demethylase/Hydroxylase dilution buffer to wells A23 and A24 for the no-enzyme control.
3. Mix and incubate at room temperature for 10 minutes.
4. Transfer 5µl of the 2X α-Ketoglutarate/Methylated Histone H3 Peptide Substrate Mix to all the assay wells.
5. Centrifuge the plate. Mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.
6. Prepare the Succinate Detection Reagent I by adding 10µl of Succinate-Glo™ Solution and 10µl of Acetoacetyl-CoA, 100X, to each 1ml of Succinate-Glo™ Buffer.

4.D. Determining IC_{50} Values of Demethylase/Dioxygenase Inhibitors (continued)

Inhibitor Titration Experiment (continued)

7. Add 10µl of Succinate Detection Reagent I to each well of the assay plate.

   Note: The Succinate Detection Reagent I terminates the demethylase/dioxygenase reaction; therefore there is no need to add an inhibitor to terminate the demethylase/dioxygenase reaction (e.g., EDTA, acid, etc.). However, if a demethylase/dioxygenase-termination reagent is added to the enzymatic reaction, do not use a magnesium-chelating agent such as EDTA because the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay requires magnesium.

8. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 60 minutes.

9. Add 20µl of Succinate Detection Reagent II to each well of the assay plate to generate light from the ATP formed in Step 4.

10. Mix assay plate with a plate shaker for 30 seconds, and incubate at room temperature for 10 minutes.

11. Record luminescence.

    Note: Instrument settings depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline.

12. **Calculating Percent Enzyme Activity:** First, subtract the signal of the no-enzyme controls located in wells A23 and A24 from all sample signals. Then use the 0% KDM4C activity (no-enzyme control) and the 100% KDM4C activity (no-inhibitor control) located in wells A21 and A22 to calculate the percent enzyme activity remaining in the presence of the different dilutions of KDM4C inhibitor.

5. General Considerations

Temperature: The intensity and stability of the luminescent signal from the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay depends on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates and reagent to room temperature before adding the Succinate Detection Reagents. Insufficient equilibration may result in a temperature gradient between the wells in the center and at the edge of the plate and therefore, variability across the plate.

Solvents and Other Chemicals: The chemical environment in which the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay is performed will affect the enzymatic rates and thus luminescence intensity. We recommend a pH of 6–9 for the demethylase/dioxygenase buffer. Some vehicles used to resuspend the various test compounds or reagents used in the demethylase/dioxygenase reaction buffer may interfere with the luciferase reaction and thus, affect the light output of the assay. Various chemicals were shown to be compatible with or tolerated by the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay (Table 3). Interference with the assay reaction can be detected by performing a succinate standard curve in the intended buffer compared with a simple buffer (Section 7).

Table 3. Solvents and Chemicals Compatible with the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay.

Chemical	Maximum Concentration Tolerated[1]
NaCl	10mM
DTT	100µM
Tween®-20	0.01%
Triton® X-100	0.01%
DMSO	5%
TCEP	2mM
$MgCl_2$	1mM

[1]Higher concentrations of these chemicals will either decrease or increase the overall luminescence without affecting assay sensitivity. In some instances, higher concentrations might decrease the performance of the assay.

Plates and Instruments: We recommend using standard solid white, multiwell plates suitable for luminescence measurements (e.g., Corning Cat.# 3912, 3693, 3674). Luminescence can be recorded on a variety of plate readers although the relative light units will depend on the instrument. Assay well geometry and small dispensing volumes may affect the efficiency of mixing, thus, poor assay homogeneity in individual wells may result in increased reaction noise or reduced signals or both. A succinate standard curve is useful for liquid handling and instrument optimization.

Testing for Compounds that Interfere with the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay: Compounds that interfere with the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay are rare. We screened 1,280 compounds from the LOPAC chemical library using the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay reagents with 10µM compound; only seven compounds weakly affected the performance of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay. When screening for demethylase/dioxygenase inhibitors, compounds that inhibit only the demethylase/dioxygenase will result in lower luminescence compared to vehicle-only controls and are easily distinguishable from compounds that inhibit other components of the assay. Test compounds that inhibit other components of the assay either alone or together with the demethylase/dioxygenase might increase or decrease the luminescent signal, depending on the level of inhibition of the demethylase/dioxygenase, luciferase or other enzyme components of the assay. To test hits from a demethylase/dioxygenase screen for the possibility of chemical interference with enzymatic conversion of succinate or generation of the luminescent signal, set up mock reactions without demethylase/dioxygenase but with all other assay components present, including a concentration of succinate that mimics the demethylase/dioxygenase reaction results. Add the appropriate concentration of test compound (usually 10µM) or vehicle control (e.g., 1% DMSO) to the mock reactions. A test compound that affects assay performance would alter luminescence by greater than 20% compared to vehicle control reactions without test compounds. Test compounds that inhibit luciferase may result in false hits, albeit rarely. However, the unique combination of Ultra-Glo™ Recombinant Luciferase and proprietary buffer compositions of the Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay will significantly reduce the number of false hits.

6. References

1. Johansson, C. *et al.* (2014) The roles of Jumonji-type oxygenases in human disease. *Epigenomics* **6**, 89–120.
2. Gorres, K. L., and Raines, R. T. (2010) Prolyl 4-hydroxylase. *Crit. Rev. Biochem. Mol. Biol.* **45**, 106–24.
3. Shen, L. *et al.* (2014) Mechanism and function of oxidative reversal of DNA and RNA methylation. *Annu. Rev. Biochem.* **83**, 585–614.
4. Kaelin, W. G., Jr. (2011) Cancer and altered metabolism: potential importance of hypoxia inducible factor and 2-oxoglutarate-dependent dioxygenases. *Cold Spring Harbor Symp. Quant. Biol.* **76**, 335–45.
5. Bishop, T., and Ratcliffe, P. J. (2015) HIF hydroxylase pathways in cardiovascular physiology and medicine. *Circ. Res.* **117**, 65–79.
6. Martinez, S., and Hausinger, R. P. (2015) Catalytic mechanisms of Fe(II)- and 2-oxoglutarate-dependent oxygenases. *J. Biol. Chem.* **290**, 20702–11.
7. Thinnes, C. C. *et al.* (2014) Targeting histone lysine demethylases - progress, challenges, and the future. *Biochim. Biophys. Acta, Gene Regul. Mech.* **1839**, 1416–32.
8. Aik, W. *et al.* (2013) Structural basis for inhibition of the fat mass and obesity associated protein (FTO). *J. Med. Chem.* **56**, 3680–8.
9. Eltzschig, H. K., Bratton, D. L., and Colgan, S. P. (2014) Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases. *Nat. Rev. Drug Discovery* **13**, 852–69.
10. Schiller, R. *et al.* (2014) A cell-permeable ester derivative of the JmjC histone demethylase inhibitor IOX1. *ChemMedChem.* **3**, 566–71.

7. Composition of Buffers and Solutions

1X JmjC demethylase/hydroxylase assay buffer

50mM HEPES (pH 7.5)

1X α-Ketoglutarate/Methylated Histone H3 Peptide Substrate Mix

50mM HEPES (pH 7.5)
10µM methylated histone H3 peptide substrate
100µM ascorbic acid
10µM Fe(II)
10µM α-ketoglutarate

8. Related Products

Product	Size	Cat.#
NanoBRET™ TE Intracellular HDAC Assay*	100 assays	N2080
NanoBRET™ TE Intracellular BET BRD Assay*	100 assays	N2130
HDAC-Glo™ I/II Assay*	10ml	G6420
HDAC-Glo™ Class IIa Assay	10ml	G9560
HDAC-Glo™ 2 Assay	10ml	G9590
MTase-Glo™ Methyltransferase Assay*	400 assays	V7601
SIRT-Glo™ Assay*	10ml	G6450

*Other sizes available.

[a]Patent Pending.

[b]U.S. Pat. Nos. 7,083,911, 7,452,663, and 7,732,128 and other patents.

[c]U.S. Pat. No. 7,700,310 and other patents and patents pending.

[d]U.S. Pat. Nos. 7,741,067, 8,361,739 and 8,603,767 and other patents and patents pending.

[e]U.S. Pat. Nos. 6,602,677, 7,241,584, 8,030,017 and 8,822,170 and other patents and patents pending.