Measuring JumonjiC Demethylase and Fe(II)/α-KG-Dependent Hydroxylase Activities Using the Succinate-Glo™ Assay and GloMax® Discover System

Promega Corporation

Materials Required

- Succinate-Glo™ Assay (Cat.# V7990, V7991)
- GloMax® Discover System (Cat.# GM3000)
- White, 96-well half-area assay plates (Corning Cat.# 3693)
- Nuclease-Free Water (Cat.# P1195)
- JMJD2C (BPS Biosciences Cat.# 50105)
- [Lys(me3)9]-Histone Hs (1–21) peptide (Anaspec Cat.# 64452)

Caution: We recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents.

Protocols: *GloMax® Discover System Technical Manual* #TM397 and *Succinate-Glo™ Technical Manual* #TM488 are available at: **www.promega.com/protocols/**

Hydroxylation/demethylation reactions catalyzed by Fe(II)/α-Ketoglutarate (KG)-dependent hydroxylases are central to many biological processes, including post-translational modification of histones and non-histone proteins, DNA/RNA repair, metabolism, and oxygen sensing. 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 occurs through the formation of an activated Fe(IV)-oxo intermediate, leading to the formation of succinate as one of the by-products. Members of the hydroxylase/demethylase 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, the fat mass and obesity-associated protein (FTO) for obesity, and the prolyl hydroxylases EGLN1–3 and the asparaginyl hydroxylase for ischemic and inflammatory conditions.

The Succinate-Glo™ Assay is a universal assay for detection of Fe(II)/α-KG-dependent hydroxylase and JumonjiC domain-containing histone lysine demethylase activities. The assay relies on enzymatic conversion of the succinate produced by the Fe(II)/α-KG-dependent hydroxylase reaction into ATP followed by bioluminescent detection of the created ATP. This sensitive assay analyzes the activities of hydroxylases/demethylases with minimal false hits when tested for compound interference using the library of pharmacologically active compounds (LOPAC). Thus the Succinate-Glo™ Assay can be used to identify small molecule modulators of hydroxylases/demethylases during high-throughput screening.

Measuring the luminescence from the Succinate-Glo™ Assay is easy on the GloMax® Discover System because the protocol comes preloaded on the instrument. The extended dynamic range and minimal well-to-well cross talk of the GloMax® Discover System allows you to easily measure signals of varying intensities on the same plate. This Application Note describes the protocol to measure JumonjiC domain-containing histone lysine demethylase activity with the Succinate-Glo™ Assay and GloMax® Discover System.

Succinate-Glo™ Assay Protocol

For detailed instructions and assay notes for various reaction volumes and plate formats, see the *Succinate-Glo™ Assay Technical Manual* #TM488. The following protocol is performed in 384-well plates using a 10µl reaction volume.

1. Prepare a demethylase substrate solution containing the histone peptide substrate, ascorbate and Fe(II)/α-KG at twice the desired final concentration in 1X Demethylase Reaction Buffer. Dispense 5µl into wells of a 384-well plate.
2. Assemble and initiate the demethylase reaction by adding 5µl of the demethylase JMJD2C at twice the desired final concentration in a total reaction volume of 10µl. Include a sample containing only 1X Demethylase Reaction Buffer as a no-enzyme control.
3. Incubate the reaction at room temperature (22–25°C) for the optimal time, generally 60 minutes.

   Note: During the incubation, thaw the Succinate-Glo™ Buffer and Acetoacetyl-CoA, 100X, at room temperature until ready to use in Step 5. Succinate-Glo™ Solution should be thawed on ice only prior to use. Any remaining amount should be returned to –70°C. Calculate the volume of Succinate-Glo™ Buffer, Acetoacetyl-CoA, 100X, and Succinate-Glo™ Solution required for the experiment.
4. Mix the thawed Succinate-Glo™ Buffer and Acetoacetyl-CoA, 100X, by vortexing. Gently mix the thawed Succinate-Glo™ Solution by pipetting. Prepare the required volume of Succinate Detection Reagent I by increasing or decreasing the component volumes provided. For example, make 1ml of Succinate Detection Reagent I by adding 10µl of Acetoacetyl-CoA, 100X, and 10µl of Succinate-Glo™ Solution, to 1,000µl of Succinate-Glo™ Buffer.

   Note: Prepare Succinate Detection Reagent I immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted Succinate Detection Reagent I.
5. Add 10µl of Succinate Detection Reagent I to the completed demethylase reaction, mix briefly and incubate for 60 minutes at room temperature (22–25°C). During the incubation, thaw the ATP Detection Buffer and ATP Detection Substrate (lyophilized) at room temperature until ready to use in Step 7.
6. 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.
7. Add 20µl of Detection Reagent II to reaction wells, and incubate the plate for 10 minutes at room temperature (22–25°C).
8. Measure luminescence on the GloMax® Discover System by selecting the Succinate-Glo™ protocol from the list of preset protocols.

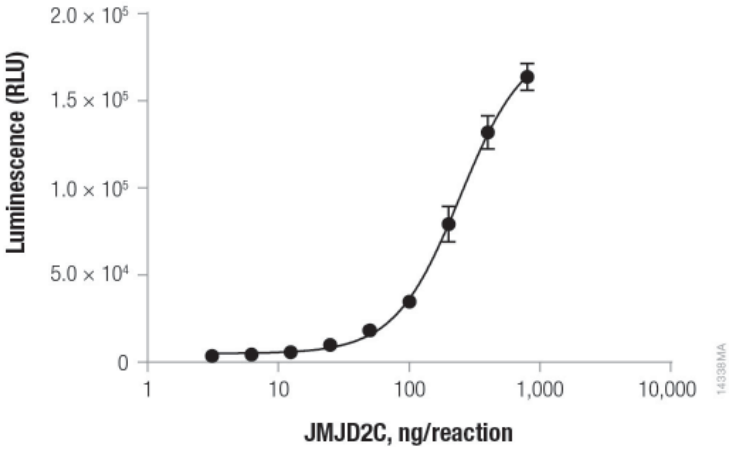

Figure 1. JMJD2C activity. Reactions were assembled with 10µM histone peptide substrate, 100µM ascorbate, 10µM α-ketoglutarate and 10µM Fe(II) in 1X Demethylase Reaction Buffer. The final reaction volume was 10µl. Reactions were incubated for 60 minutes at room temperature. To the completed JMJD2C reactions, 10µl of Succinate Detection Reagent I was added and incubated for 60 minutes at room temperature, followed by addition of 20µl of Succinate Detection Reagent II. Plates were incubated for 10 minutes at room temperature and luminescence was recorded using the GloMax® Discover System.

Conclusion

The GloMax® Discover can detect luminescence generated using the Succinate-Glo™ Assay as shown in Figure 1. An increase in light output was observed when increasing amounts of JMJD2C were included in the assay reaction containing the histone peptide substrate.

The **GloMax® Discover System** offers superior sensitivity and dynamic range and limited well-to-well cross talk. The instrument was developed and optimized with Promega cell and gene reporter assays and may be integrated into low- and medium-throughput automation workflows. The GloMax® Discover System allows flexible use of filters to measure fluorescence intensity, filtered luminescence, BRET, FRET and UV-visible absorbance for a wide variety of laboratory applications. The instrument is operated by an integrated Tablet PC, which provides quick and easy navigation through the control options. Exporting your results is made seamless with a variety of options, including exporting data to your local network.

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