Bioluminescent Succinate Detection Enables Inhibitor Screening of 2-Oxoglutarate Dioxygenase Target Class of Enzymes

Hicham Zegzouti, Juliano Alves, Gediminas Vidugiris, and Said Goueli

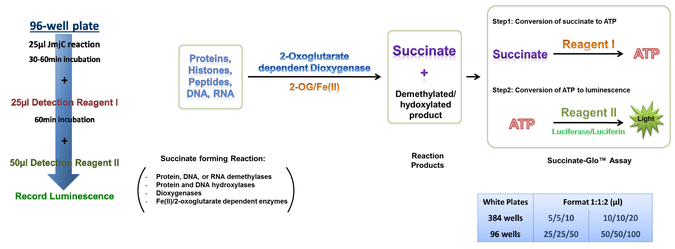
Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711

1. Introduction

The covalent modifications of histone proteins, DNA and RNA by Fe(II)/2-oxoglutarate-dependent dioxygenases are key to the modulation of biological processes such as epigenetics, hypoxic signaling and DNA/RNA repair. Of these, JumonjiC domain-containing histone lysine demethylases (JMJCs), the ten-eleven-translocation (TET) DNA dioxygenases, the ALKB DNA/RNA hydroxylases and the prolyl hydroxylases EGLN1-3 have generated increased interest as potential drug targets for the treatment of a number of pathological conditions, including cancer. Therefore, there is strong need for biochemical assays to monitor the activity of this class of enzymes, study their modes of regulation, and to search for selective and potent inhibitors without relying on cumbersome technologies such as radiometry or antibody-based methods. Since succinate is a common product to all Fe(II)/2-oxoglutarate-dependent dioxygenases, we determined the substrate specificities, the apparent kinetic constants for several JMJCs and members of the dioxygenase superfamily, as well as inhibition profiles of reported inhibitors using a novel bioluminescent and homogenous succinate detection assay. Our results demonstrate that a universal succinate detection is a useful strategy for the characterization of multiple Fe(II)/2-oxoglutarate-dependent dioxygenases with distinct substrate requirements, enabling the investigation of a large number of enzymes and their modulators that cannot be evaluated in a miniaturized or high-throughput manner with currently available methods.

2. Succinate Detection Assay Principle

Principle: Succinate is Converted into ATP that is Detected via Bioluminescence

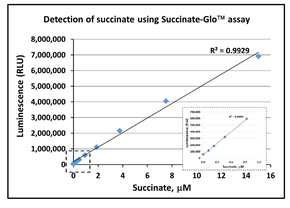


White Plates	Format 1:1:2 (µl)	
384 wells	5/5/10	10/10/20
96 wells	25/25/50	50/50/100

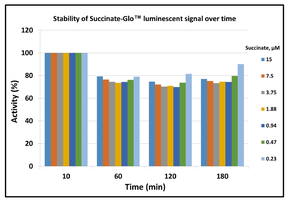
- Two Step Detection: After the demethylase reaction, the Succinate Detection Reagents are added in 1:1:2 ratio
- Luminescence signal is proportional to the succinate produced and to the demethylase activity
- Simple “Add and Read”: No radioisotopes. No product separation. No antibodies

3. High Sensitivity, Linearity and Signal Stability

Linearity



Signal Stability



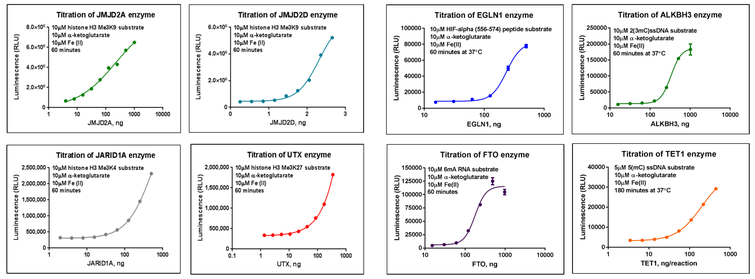
Sensitivity

Signal to Background ratios at different succinate concentrations									
Succinate, µM	15	7.5	3.75	1.88	0.9375	0.47	0.234	0.12	0
S/B values	118	69	37	19	10	5	3	2	1

- Succinate detection is linear up to 15µM and it has a high dynamic range
- It can detect as low as 200nM with a signal/background (SB) of 2
- The signal is stable for up to 3 hours with ~80% remaining signal

4. Universal Activity Assay for Diverse Fe(II)/2-Oxoglutarate Dependent Dioxygenases

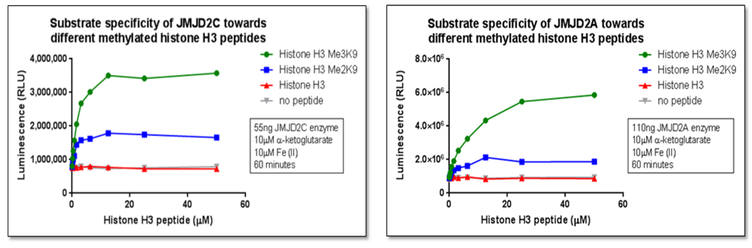
JMJC Histone Lysine Demethylases | **Protein, DNA and RNA Hydoxylases/Dioxygenases**



Succinate detection is suitable for measuring the activity of:

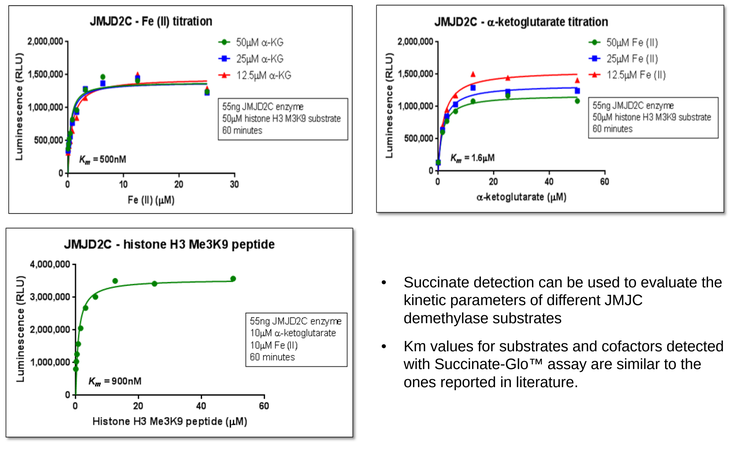
- JMJC demethylases regardless of substrate methylation state and position
- 2-oxoglutarate dependent dioxygenases and hydroxylases

5. JMJC Demethylase Substrate Specificity Studies



Succinate detection can be used to study JMJC demethylase specificity towards diverse methylated substrates

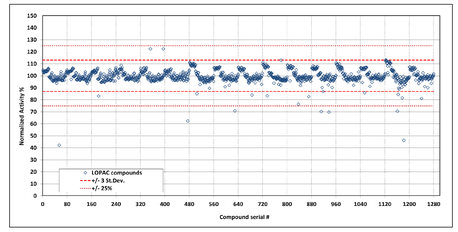
6. Evaluating Kinetic Parameters Using Succinate Detection



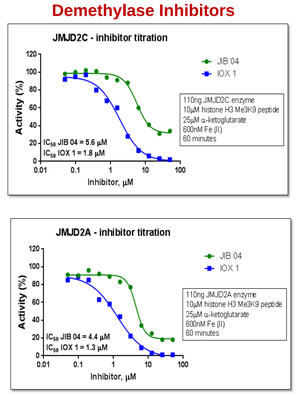
- Succinate detection can be used to evaluate the kinetic parameters of different JMJC demethylase substrates
- Km values for substrates and cofactors detected with Succinate-Glo™ assay are similar to the ones reported in literature.

7. Enzyme Inhibition Studies

LOPAC Screening on Succinate Detection Reagents



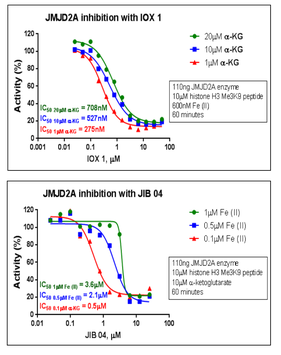
Demethylase Inhibitors



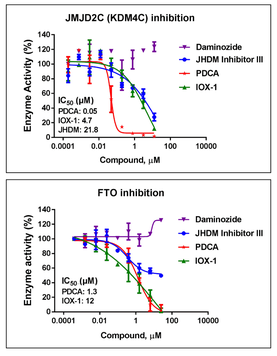
- Small molecule inhibitors do not interfere with succinate assay detection
- JMJC activity detected with succinate assay is inhibited with selective inhibitors

8. Inhibitor’s Mode of Action Studies and Profiling

Mode of action study of JMJC Demethylase inhibitors



Profiling of different 2-Oxoglutarate inhibitors



Bioluminescent succinate assay can be used to:

- Evaluate competitive inhibitors toward different substrates.
- Create selectivity profiles of different inhibitors

9. Conclusions

Bioluminescent succinate detection proved here to be a useful strategy for the characterization of diverse Fe(II)/2-oxoglutarate-dependent dioxygenases. The Succinate-Glo assay has the following advantages over currently available technologies:

Universality:

- This assay can be used with the majority of JMJC demethylases and 2-oxoglutarate dioxygenases regardless of substrate chemical structure, methylation state or position

Versatility:

- Easy to use assay. 2-step addition and read
- Suitable for studying substrate specificity, kinetic parameters and mode of action of inhibitors

HTS friendly:

- Sensitive in low volume format and signal is stable for batch processing
- Resistant to chemical interference and suitable for inhibitor studies

Corresponding author: hicham.zegzouti@promega.com