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Bioluminescent High-Throughput Succinate Detection Method for Monitoring the Activity of JMJC Histone Demethylases and Fe(II)/2-Oxoglutarate-Dependent Dioxygenases

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1. Introduction

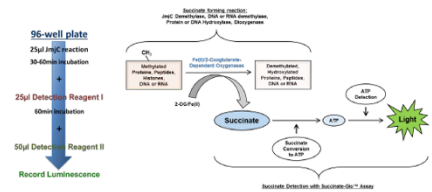
The covalent modification of histone proteins, DNA and RNA by Fe(II)/2-oxoglutarate-dependent dioxygenases are key to the modulation of biological processes such as epigenetics, hypoxic signaling and DNA/RNA repair. Of these, JumonjiC domain-containing histone lysine demethylases (JMJCs), the ten-eleven-translocation (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. Since succinate is a common product to all Fe(II)/2-oxoglutarate-dependent dioxygenases, we developed a novel bioluminescent and homogenous activity detection assay for JmjC histone demethylases and Fe(II)/2-oxoglutarate-dependent dioxygenases based on succinate measurement. The assay was used successfully in the following applications:

- Determine substrate specificities for JmJC enzymes.
- Measure apparent kinetic constants for several JMJCs and members of the dioxygenase superfamily
- Screen a compound library for inhibitors of JmJC demethylase
- Study inhibition mode of action of reported inhibitors.
- Determine selectivity profiles for several compounds against JMJD2A and FTO enzymes.

Our results demonstrate that 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 that cannot be evaluated in a miniaturized or high-throughput manner with the methods currently available.

2. Succinate Detection Assay Principle

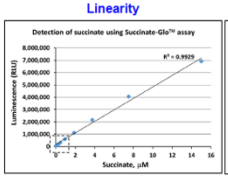
Principle: Succinate is converted into ATP that is detected via a luciferase/luciferin reaction



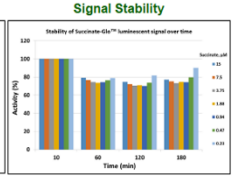
- Two Step Detection: After the demethylase reaction, the Succinate Detection Reagents are added in 1:1:2 ratio
- Luminescence signal is proportional to the succinate produced and to the demethylase activity
- Simple "Add and Read": No radioisotopes. No product separation. No antibodies

3. High Sensitivity, Linearity and Signal Stability

Linearity



Signal Stability



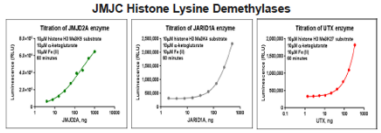
Sensitivity

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

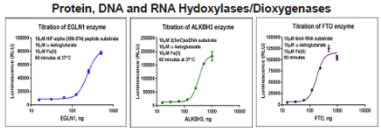
- Succinate detection is linear up to 15µM and it has a high dynamic range
- It can detect as low as 200nM with a signal/background (SB) of 2
- The signal is stable for up to 3 hours with ~80% remaining signal

4. Detection of Different Fe(II)-2-oxoglutarate-Dependent Dioxygenases Activities

JMJC Histone Lysine Demethylases



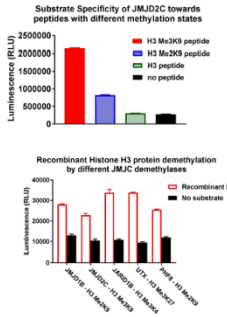
Protein, DNA and RNA Hydoxylases/Dioxygenases



Succinate detection is suitable for measuring the activity of:

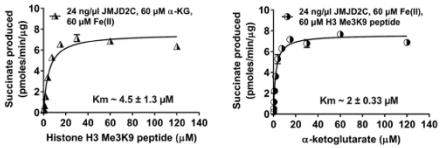
- JMJC demethylases regardless of substrate methylation state and position
- 2-oxoglutarate dependent dioxygenases and hydroxylases

5. JMJC Demethylase Substrate Specificity Studies



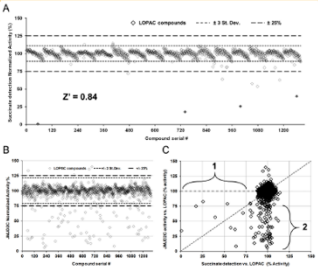
Succinate detection can be used to study JMJC demethylase specificity toward diverse methylated peptide or protein substrates

6. Evaluating Enzyme Kinetic Parameters Using Succinate Detection



Km values for substrates and cofactors detected with Succinate-Glo assay are similar to the ones reported in literature.

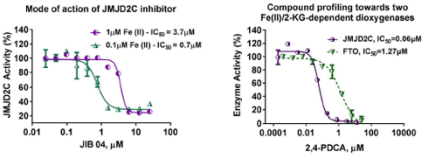
7. JMJC Demethylase Inhibitor Studies



(A) Screening for potential inhibitors of Succinate-Glo reagents performance using LOPAC compound library. (B) Screening for demethylase inhibitors (C) Inhibition correlation graph showing the effect of each compound on the assay reagents at the same time as on the JMJD2C enzyme. All the inhibitors are inhibiting the enzyme (section 2) but not the assay reagents (section 1).

- Small molecule inhibitors do not interfere with succinate assay detection.
- Succinate-Glo assay is robust with high Z' factor.

8. Inhibitor Profiling and Mode of Action Studies



Bioluminescent succinate assay can be used to:

- Study 2-oxoglutarate dependent dioxygenase inhibitors
- Evaluate competitive inhibitors toward different substrates.
- Create selectivity profiles of different inhibitors

9. Conclusions

Universality:

- Bioluminescent succinate assay can be used with the majority of 2-oxoglutarate dependent dioxygenases.
- One assay for diverse JMJC demethylase-substrate combinations, regardless of substrate methylation state and position.

Versatility:

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

HTS friendly:

- Sensitive in low volume format
- Signal is stable for batch processing
- Resistant to chemical interference

Publication: Alves et al,. SLAS Discovery, Online First 2017.
https://doi.org/10.1177/2472555217745657

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