Application Note

Detection of HMT G9a with the Transcreener® EPIGEN Methyltransferase Assay

This Application note will serve as a guide for using the Transcreener® EPIGEN MethyltransferaseAssay to detect the initial velocity enzyme activity of HMT G9a with an assay window suitable for inhibitor screening and dose response measurements. It should be used as an adjunct to the Transcreener EPIGEN MT Assay.

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Histone Methyltransferase G9a

G9a catalyzes the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to the amino function of L-lysine residues (mono-, di- and trimethylation), espesially of lysine-9, of histone H3[2]. G9a is a SET-domain type HMT which in complex with the highly homologous G9a like proteins (GLP) is the major source of mono- and di-methylated H3K9 in euchromatin. G9a is overexpressed in a variety of cancers and knockdown of G9a/GLP in the MCF7 breast cancer line increases apoptosis[2]. Human recombinant G9a (residues 786-1210; Genbank Accession # NM_006709) expressed as an N-terminal GST fusion protein in E.coli was obtained from Reaction Biology Corp (Malvern, PA).

Transcreener® EPIGEN Methyltransferase Assay

The Transcreener® EPIGEN Methyltransferase Assay is a universal biochemical HTS assay for enzymes that produce S-adenosylhomocysteine (SAH), including all enzymes within the histone (HMTs) and DNA (DNMTs) methyltransferase families. It combines the extensively validated Transcreener AMP²/GMP² Assay, which relies on fluorescent immunodetection of AMP, with coupling enzymes that convert SAH to AMP. Enzyme activity is signaled by a decrease in fluorescence polarization as the bound tracer is displaced from the AMP²/GMP² Antibody. The assay used a simple mix-and-read format with two liquid addition steps. Methyltransferase (MT) enzyme reactions are first quenched with Stop Buffer and then the SAH Detection Mixture containing coupling enzymes, AMP²/GMP² antibody, and tracer is added. The assay provides excellent signal at low substrate conversion, with an assay window greater than 100 millipolarization units (mP) and Z' ≥ 0.7 under normal reaction conditions.

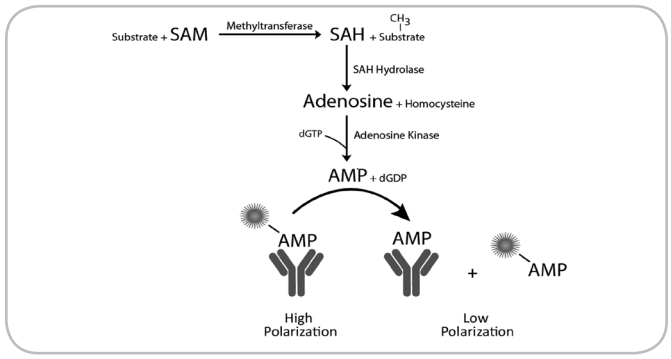

Figure 1. Transcreener Epigen methyltransferase assay principle: SAH produced in a methyltransferase reaction is converted to AMP in two sequential enzymatic steps. AMP is detected using a competitive fluorescence polarization immunoassay.

Summary of protocol

Step 1) Determine the optimal AMP²/GMP² antibody concentration for the G9a enzyme reaction.

Step 2) Perform a G9a titration to determine the amount required to produce a good assay window.

Step 3) Run dose response curves.

Materials and Methods

Instrument: Microplate Reader capable of measuring Fluorescence Polarization.

Microplates: Corning® 384 Well Low Volume Black Round Bottom PS NBS™ Microplate (Product #3676).

Note: Non binding or Low binding black plates are necessary for a good assay window.

Reagent	Catalog #
Transcreener® EPIGEN MT Assay	3017-1K
G9a (Reaction Biology)	HMT-11-102
H3 peptide (1-25) (Anaspec)	61703

HMT Enzyme Buffer: 50 mM Tris-HCl (pH 8.5), 4 mM DTT, 5 mM $MgCl_2$.

Step 1) Determine the optimal AMP²/GMP² antibody concentration for the G9a enzyme reaction.

Note:The optimal antibody concentration is primarily dependent on the SAM (2 µM) and the peptide (10 µM) concentration and to a lesser degree on other enzyme specific components such as metals and salts.

1) Titrate the AMP²/GMP² Antibody using a two fold dilution in a volume of 10 µL in the HMT Buffer containing 2 µM SAM and 10 µM H3 peptide.

2) Add 10 µL of Detection Mix comprising AMP²/GMP² Tracer, Cofactor, Detection buffer, Coupling enzyme 1 and Coupling enzyme 2. The final concentrations of the components in 20 µL reactions were 4 nM tracer, 0.125X cofactor, 0.125X detection buffer, 2 µg/mL coupling enzyme 1 and 1 µg/mL coupling enzyme 2.

3) Mix the plate on a plate shaker, cover with a plate seal, and incubate at RT for an hour.

4) The plates were read at EXC 630 nm and EMS 670 nm to measure fluorescence polarization.

5)Plot polarization vs. log [Antibody] and determine the concentration that produces 85% of the maximal polarization change - the EC85; 5 µg/mL in this case. In general for Transcreener assays, using the EC85 antibody concentration will allow robust detection of enzyme initial velocity (less than 20% conversion of susbtrate to product).

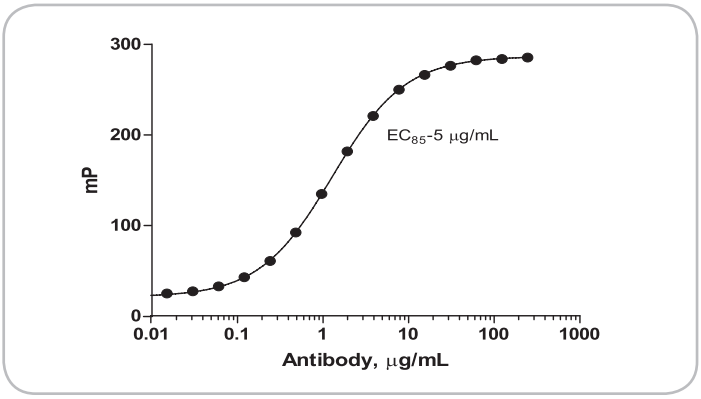

Figure 2. AMP²/GMP² antibody titration in the presence of 2 µM SAM and 10 µM H3 (1-25) peptide. An optimal concentration of 5 µg/mL was determined based on this titration.

Step 2) Perform a G9a titration to determine the optimal enzyme concentration to use.

1)The final reaction volume is 15 µL; reactions are initiated by adding 7.5 µL of substrate to 7.5 µL of titrated enzyme. Titrate G9a using two-fold dilutions by adding 7.5 µL aliquots to wells containing 7.5 µL enzyme buffer starting at 50 ng/µL and ending at 0 ng/µL (the maximum enzyme concentration will be 25 ng/µL after addition of substrate. We suggest running duplicates, with separate controls lacking the H3 peptide and SAM.

2) Initiate the reaction by adding 7.5 µL of substrate - Mix for final concentrations of 2 µM SAM and 10 µM H3 (1-25) peptide in the 15 µL reactions per the instructions in the Transcreener EPIGEN MT Assay Technical Manual. Prepare other controls by adding just SAM or H3 peptide to the titrated enzyme reactions.

3) Incubate the enzyme reaction for two hours at 30°C.

4) Meanwhile prepare a 2 µM SAM/SAH standard curve with 10 µM H3 peptide in the buffer at different percent conversions. Add 15 µL of the standards to the same plate and let them incubate at 30°C along with the enzyme reaction.

Note:For detailed instr.uctions on how to run a standard curve please refer to the EPIGEN Methyltransferase technical manual.

5) At the end of two hours, add 2.5 µL of Stop Buffer A to all the wells followed by 2.5 µL of detection mix. The detection mix comprises of AMP²/GMP² tracer, AMP²/GMP² antibody, cofactor, detection buffer, coupling enzyme 1 and coupling enzyme 2. The final concentrations of the components in 20 µL reactions were 4 nM tracer, 5 µg/mL of AMP² antibody, 0.125X cofactor, 0.125X detection buffer, 2 µg/mL coupling enzyme 1 and 1 µg/mL coupling enzyme 2.

6) Mix the plate well and after 90 min of incubation read the plate in an instrument that measures fluorescent polarization at 633 nm EXC and 670 nm EMS.

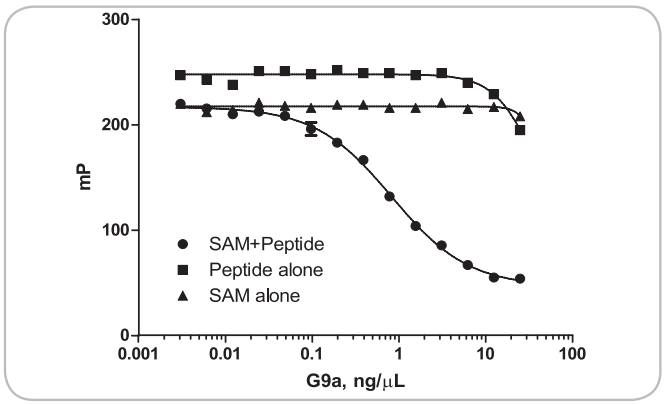

Figure 3. G9a enzyme titration in the presence of 2 µM SAM and 10 µM H3 (1-25) peptide . A concentration (EC_{80}) of 3 ng/µL was determined optimal based on this titration.

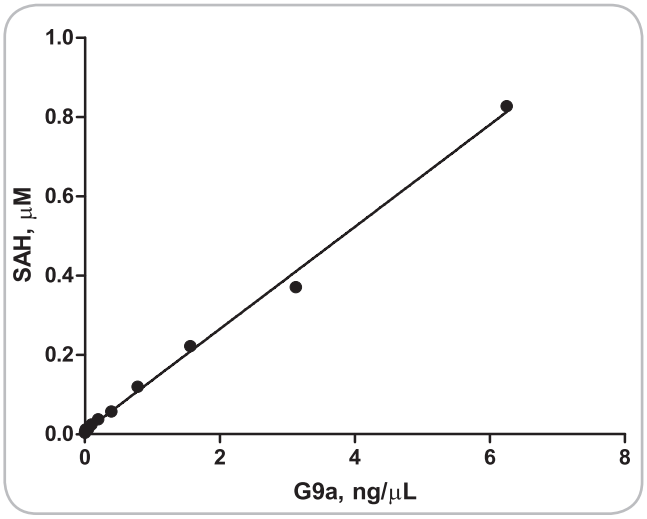

Figure 4. Linear relationship between G9a concentration and SAH formation. The polarization values were converted into SAH (product formed) using a standard curve set up under similar conditions.

Step 3) Run dose response curve with sinefungin.

1) Titrate sinefungin using two- fold dilution by adding 7.5 µL aliquots to wells containing 7.5 µL enzyme buffer starting at 5000 µM and ending at 0 ng/µL (the maximum inhibitor concentration will be 2500 µM after addition of enzyme and substrate. We suggest running duplicates, with separate controls lacking the H3 peptide and SAM.

2) Add 5 µL of 9 ng/µL of G9a at EC_{80} concentration, such that the final concentration of enzyme in the 15 µL reaction is at 3 ng/µL.

3) To one row of wells add 2.5 µL of substrate for final concentrations of 2 µM SAM and 10 µM peptide. Separate controls lacking SAM and H3 peptide are recommended. Incubate the plate at 30°C for two hours.

4) Add 2.5 µL of Stop Buffer A followed by 2.5 µL of detection mix.

5) Mix the plate well, incubate for an hour and read the plate in an instrument that measures fluorescent polarization at 633 nm EXC and 670 nm EMS.

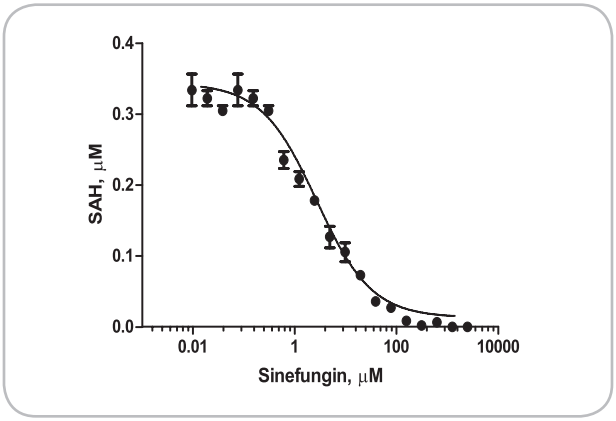

Figure 4. Sinefungin dose response curve. The polarization values were converted into product formed using a standard curve. The IC_{50} value was determined to be 3 µM.

Conclusions

This application note demonstrates a streamlined approach to develop a Transcreener EPIGEN Methyl transferase Assay for G9a. These steps can be followed to easily adapt the Transcreener EPIGEN Assay for doing high throughput screening with G9a.

References & Notes

1. Klink TA, Staeben M, Twesten K, Kopp AL, Kumar M, Schall Dunn R, Pinchard CA, Kleman-Leyer KM, Klumpp M, Lowery RG: Development and Validation of a Generic Fluorescent Methyltransferase Activity Assay Based on the Transcreener AMP/GMP Assay. J Biomol Screen 2012 Jan;17(1):59-70.

2. Makoto Tachibana, Kenji Sugimoto, Tatsunobu Fukushima and Yoichi Shinkai: SET Domain-containing Protein, G9a, Is a Novel Lysine-preferring Mammalian Histone Methyltransferase with Hyperactivity and Specific Selectivity to Lysines 9 and 27 of Histone H3. J Biol Chem 2001 Jul;6: 25309-25317.

Additional Information

Ordering Information

Please visit www.bellbrooklabs.com or contact BellBrook Labs for pricing for the Transcreener® Assays. Custom quotes are available for bulk orders.

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Fax Orders:
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