G9a Methyltransferase Inhibitor Screening Assay Kit

Item No. 700500

TABLE OF CONTENTS

Materials Supplied

Item Number	Item	Quantity/Size
700141	MT Assay Buffer	1 vial/20 ml
700142	MT Assay Buffer Additive	1 vial/200 µl
700143	MT Enzyme Mixture	3 vials/300 µl
700151	MT Fluorometric Mixture	3 vials
700146	MT Assay S-Adenosylmethionine	3 vials/200 µl
700501	G9a (human recombinant) Assay Reagent	2 vials/70 µl
700153	MT DMSO	1 vial/1 ml
700502	G9a Acceptor Peptide	2 vials/600 µl
700145	MT AdoHcy Positive Control	1 vial/200 µl
400091	Half Volume 96-Well Solid Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -80°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the capacity to measure fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.
2. Adjustable pipettes and a repeat pipettor.
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable.

INTRODUCTION

Background

Methylation of key biological molecules and proteins plays important roles in numerous biological systems, including signal transduction, biosynthesis, protein repair, gene silencing, and chromatin regulation.[1] The S-adenosylmethionine (SAM) dependent methyltransferases use SAM, the second most commonly used enzymatic cofactor after ATP. SAM, also known as AdoMet, acts as a donor of a methyl group that is required for the modification of proteins and DNA.[2] Aberrant levels of SAM have been linked to many abnormalities, including Alzheimer's Disease, depression, Parkinson's Disease, multiple sclerosis, liver failure, and cancer.[1,2]

Methylation of lysines can promote transcriptional activation or repression and is critical in regulating histone function.[3] G9a (EHMT2) is a SET domain-containing mammalian histone methyltransferase. G9a can mono- or dimethylate lysine 9 and lysine 27 on histone H3 (H3), contributing to early embryogenesis, genomic imprinting, and lymphocyte development.[4-7] G9a is expressed in aggressive lung cancer cells and its elevated expression correlates with poor prognosis.[8] Recent evidence has shown that G9a is responsible for transcriptional repression of HIV-1 by promoting repressive dimethylation of H3K9 and for the maintenance of viral latency.[9] G9a and GLP (G9a-like protein; EHMT1) specifically methylate the tumor suppressor p53 at lysine 373 which correlates with inactive p53.[10] G9a is overexpressed in various cancers and is a potential inhibitory target for cancer treatment.

About This Assay

Cayman's G9a Methyltransferase Inhibitor Screening Assay provides a convenient method for screening human G9a inhibitors. Figure 1, on page 6, outlines the general scheme of the assay.[11] The transfer of the methyl group from SAM to the acceptor peptide by G9a generates S-adenosylhomocysteine, which is rapidly converted to S-ribosylhomocysteine and adenine by adenosylhomocysteine nucleosidase. This rapid conversion prevents the buildup of adenosylhomocysteine and its feedback inhibition on the methylation reaction. Finally, the adenine is converted to hypoxanthine, by adenine deaminase, which in turn is converted to urate and hydrogen peroxide. The reaction between hydrogen peroxide and 10-acetyl-3,7,-dihydroxyphenoxazine (ADHP) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

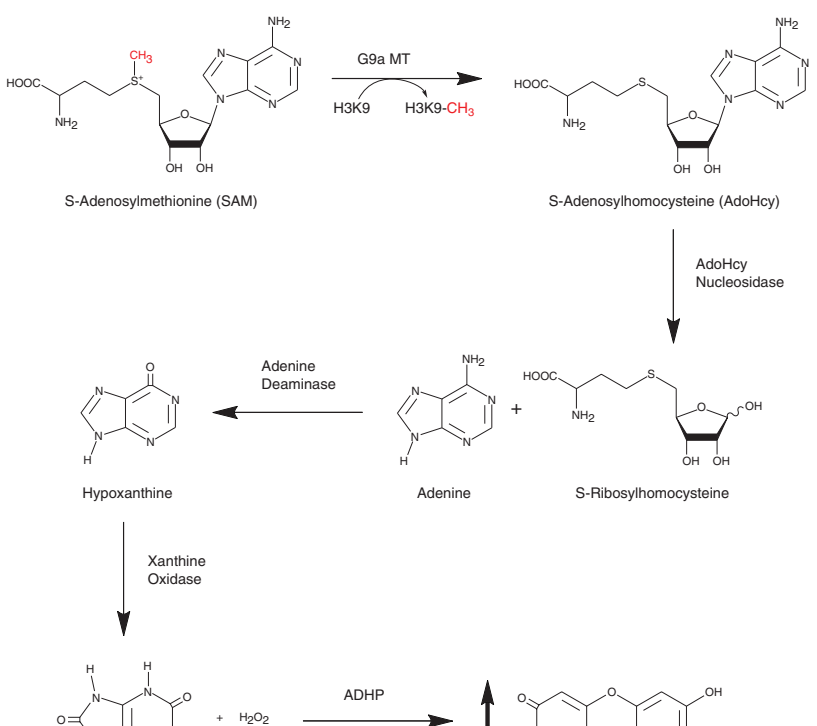

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. MT Assay Buffer - (Item No. 700141) and MT Assay Buffer Additive (Item No. 700142)

Thaw the Assay Buffer and Assay Buffer Additive at room temperature. Add the entire volume of the Additive into the Assay Buffer and mix thoroughly. Mark the Additive box on the Assay Buffer vial. Store the Assay Buffer at room temperature; do not freeze after the addition of Additive.

2. MT Enzyme Mixture - (Item No. 700143)

Each vial contains 300 µl of enzyme mixture. Thaw on ice only the number of vials you will be using for your experiment. We do not recommend repeated freeze/thaw cycles of the enzyme mixture. The enzyme mixture is ready to use to prepare the Master Mixture.

3. MT Fluorometric Mixture - (Item No. 700151)

The vials contain a clear lyophilized powder of ADHP. Immediately prior to making the Master mixture, add 100 µl of MT DMSO (Item No. 700153) to the vial and vortex. Then add 400 µl of Assay Buffer containing additive and vortex. Prepare additional vials as needed. The reconstituted mixture is stable for 45 minutes. After 45 minutes, increased background fluorescence will occur.

4. MT Assay S-Adenosylmethionine - (Item No. 700146)

Each vial contains 200 µl of SAM. Thaw on ice only the number of vials you will be using for your experiment. We do not recommend repeated freeze/thaw cycles. SAM is ready to use to prepare the Master Mixture.

5. G9a (human recombinant) Assay Reagent - (Item No. 700501)

Each vial contains 70 µl of human recombinant G9a methyltransferase (N-terminal GST-tagged G9a amino acids 785-1210). Thaw the enzyme on ice. Prior to assaying, add 490 µl of Assay Buffer to the vial. This is enough enzyme for assaying 56 wells. Dilute the additional vial if assaying the entire plate. The diluted enzyme is stable for four hours on ice.

6. Methyltransferase DMSO - (Item No. 700153)

The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

7. G9a Acceptor Peptide - (Item No. 700502)

Each vial contains 600 µl of 1.25 mM Histone H3 peptide (amino acids 1-21). The peptide is ready to use in the assay. *NOTE: The final concentration of peptide in the assay as described below is 100 µM. This concentration may be reduced with Assay Buffer at the user's discretion. The K_m for the peptide is 21 µM.*

8. AdoHcy Positive Control - (Item No. 700145)

The vial contains 200 µl of a 1 mM solution of S-adenosylhomocysteine (AdoHcy). AdoHcy can be used to assay for interference (see page 14).

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% Initial Activity wells and three wells designated as Background wells. A typical layout of samples and inhibitors to be measured in triplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells
A - 100% Initial Activity Wells
1-30 - Inhibitor Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 125 µl in all the wells.
- All reagents except the enzyme must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Performing the Assay

1. In a suitable tube, prepare the Master Mixture according to the table below:

Reagent	36 wells	72 wells	100 wells
Assay Buffer + Additive	2.9 ml	5.8 ml	8.7 ml
MT Enzyme Mixture	1 vial/300 µl	2 vials/600 µl	3 vials/900 µl
MT Fluorometric Mixture	200 µl	400 µl	600 µl
MT SAM	1 vial/200 µl	2 vials/400 µl	3 vials/600 µl

Table 1. Master Mixture preparation

2. **100% Initial Activity Wells** - add 100 µl of Master Mixture, 10 µl of G9a Acceptor Peptide, and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
3. **Background Wells** - add 100 µl of Master Mixture, 10 µl of G9a Acceptor Peptide, and 5 µl of solvent (same solvent used to dissolve the inhibitor) to three wells.
4. **Inhibitor Wells** - add 100 µl of Master Mixture, 10 µl of G9a Acceptor Peptide, and 5 µl of inhibitor* to three wells.

	Master Mixture	Acceptor Peptide	Solvent	Inhibitor
100% Initial Activity	100 µl	10 µl	5 µl	-
Background	100 µl	10 µl	5 µl	-
Inhibitor	100 µl	10 µl	-	5 µl

Table 2. Pipetting Summary

5. Initiate the reactions by adding 10 µl of G9a to the 100% Initial Activity and Inhibitor wells. Add 10 µl of Assay Buffer to the Background wells.
6. Cover the plate with the plate cover and incubate for twenty minutes at 37°C.
7. Remove the plate cover and read at an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

*Inhibitors can be dissolved in Assay Buffer, methanol, DMSO, or ethanol and should be added to the assay in a final volume of 5 µl. In the event that an appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made. For determination of IC_{50} values, use multiple concentrations of inhibitor to cover a large range.

ANALYSIS

Calculations

1. Determine the average fluorescence of the background, 100% initial activity (IA), and inhibitor wells.
2. Subtract the average fluorescence of the background wells from the average fluorescence of the 100% initial activity and inhibitor wells.
3. Determine the percent inhibition or percent Initial Activity for each inhibitor using one of the following equations.

$$\% \text{ Inhibition} = \left[\frac{\text{IA - Inhibitor}}{\text{IA}} \right] \times 100$$

$$\% \text{ Initial Activity} = \frac{\text{Inhibitor}}{\text{IA}} \times 100$$

4. Graph the percent inhibition or percent initial activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). The inhibition of human recombinant G9a methyltransferase by the specific G9a histone methyltransferase inhibitor, UNC0224, is shown in Figure 3 (see page 13) as an example.[12]

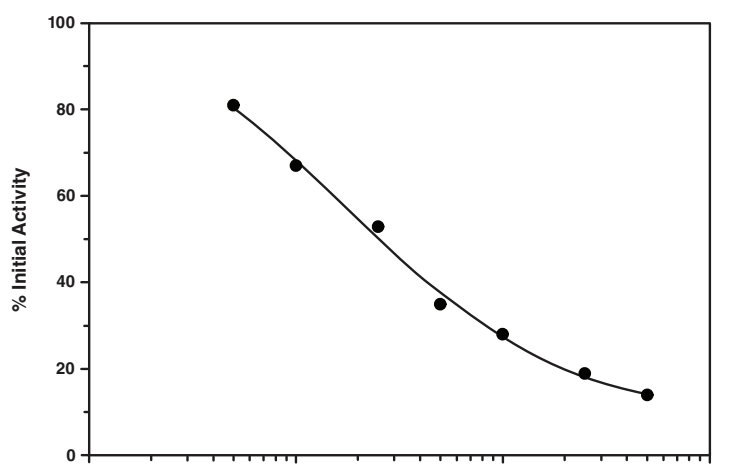

Figure 3. Inhibition of human recombinant G9a methyltransferase by UNC0224 (IC_{50} = 25.2 nM using the final concentration of 30 µM acceptor peptide)

Performance Characteristics

Precision:

When a series of eight G9a measurements were assayed on the same day, the intra-assay coefficient of variation was 4.1%. When a series of eight G9a measurements were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.7%.

Interferences

It is possible that a compound tested for G9a inhibition will interfere with the downstream enzymes in the assay. Potential interference can be tested by assaying the compound in question with the AdoHcy Positive Control. A procedure is outlined below.

Testing for Interference

1. Thaw the AdoHcy Positive Control (Item No. 700145) on ice. Dilute 10 µl of AdoHcy with 190 µl of Assay Buffer containing Additive.
2. **AdoHcy wells** - add 100 µl of Master Mixture and 5 µl of solvent (the same solvent used to dissolve the compound) to three wells.
3. **Compound wells** - add 100 µl of Master Mixture, 5 µl of compound to three wells.
4. Initiate the reactions by adding 10 µl of diluted AdoHcy to the AdoHcy wells and the compound wells.
5. Cover the plate with the plate cover and incubate for 10 minutes at 37°C.
6. Remove the plate cover and read the plate at an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the Percent Interference

1. Determine the average fluorescence of the AdoHcy and the compound wells.
2. Determine the percent interference for the compound. To do this, subtract each compound fluorescence value from the AdoHcy fluorescence value. Divide the result by the AdoHcy fluorescence value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be considered as not affecting coupled enzymes in the assay.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the inhibitor wells	A. Enzyme or acceptor was not added to the well(s). B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all of the components to the wells B. Reduce the concentration of the inhibitor and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition was seen with inhibitor	A. The inhibitor concentration is not high enough B. The inhibitor is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

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3. Kurash, J.K., Lei, H., Shen, Q., *et al.* Methylation of p53 by Set7/9 mediates p53 acetylation and activity *in vivo*. *Mol. Cell* **29**, 392-400 (2008).
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12. Liu, F., Chen, X., Allali-Hassani, A., *et al.* Discovery of a 2,4-diamino-7-aminoalkoxyquinazoline as a potent and selective inhibitor of histone lysine methyltransferase G9a. *J. Med. Chem.* (2009).

Related Products

HAT Inhibitor Screening Assay Kit - Item No. 10006515
HDAC Activity Assay Kit - Item No. 10011563
HDAC1 Inhibitor Screening Assay Kit - Item No. 10011564
JMJD2A Inhibitor Screening Assay Kit - Item No. 700360
JMJD2D Inhibitor Screening Assay Kit - Item No. 700370
LSD1 Inhibitor Screening Assay Kit - Item No. 700120
Methyltransferase Colorimetric Assay Kit - Item No. 700140
Methyltransferase Fluorometric Assay Kit - Item No. 700150
SET7/9 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700270
SET8 Methyltransferase Inhibitor Screening Assay Kit - Item No. 700350
SIRT1 Direct Fluorescent Screening Assay Kit - Item No. 10010401
SIRT1 FRET-Based Screening Assay Kit - Item No. 10010991
SIRT2 Direct Fluorescent Screening Assay Kit - Item No. 700280
SIRT3 Direct Fluorescent Screening Assay Kit - Item No. 10011566
SIRT6 Direct Fluorescent Screening Assay Kit - Item No. 700290

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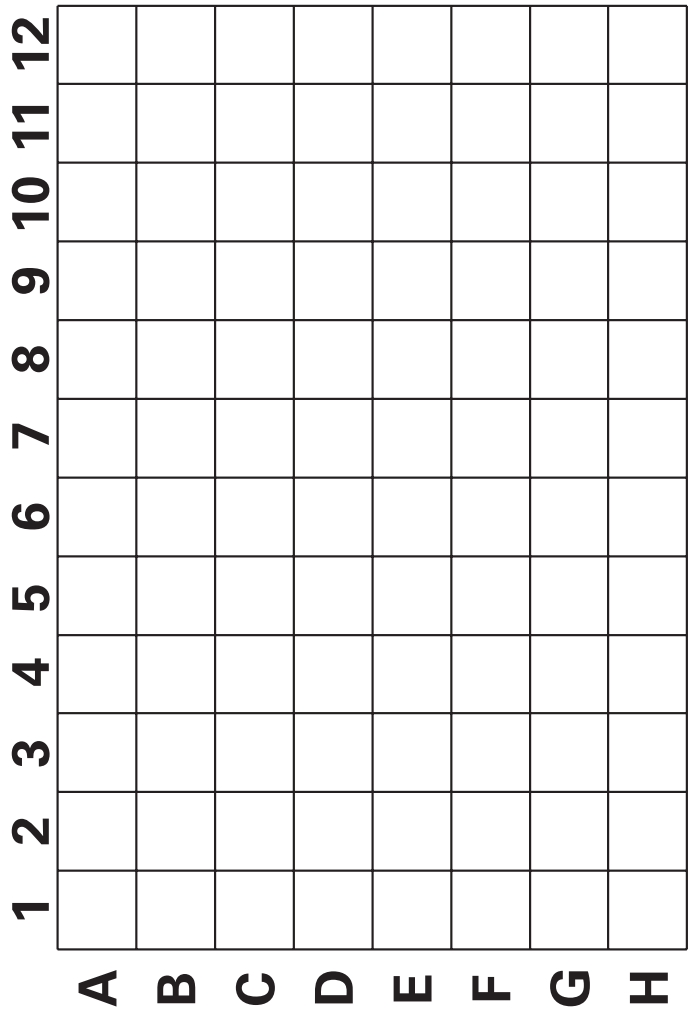
Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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