SET8 Methyltransferase Inhibitor Screening Assay Kit

Item No. 700350

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
700141	MT Assay Buffer	1 vial/20 ml	-20°C
700142	MT Assay Buffer Additive	1 vial/200 µl	-20°C
700143	MT Enzyme Mixture	3 vials/300 µl	-80°C
700002	ADHP Assay Reagent	3 vials	-20°C
700146	MT Assay S-Adenosylmethionine	3 vials	-80°C
700351	SET8 (human recombinant)	2 vials/150 µl	-80°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
700012	HCl Assay Reagent (20 mM)	1 vial/1 ml	-20°C
700352	SET8 Assay Acceptor Peptide	2 vials/600 µl	-20°C
700145	MT Assay AdoHcy Positive Control	1 vial/200 µl	-80°C
400091	Half-Volume 96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's SET7/9 SAM-Screener™ Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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

Email: techserv@caymanchem.com

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 in the **Materials Supplied** section, on page 3, 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 repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Methylation of key biological molecules plays important roles in numerous systems, including signal transduction, biosynthesis, protein repair, gene silencing, and chromatin regulation.[1] The S-adenosylmethionine (SAM)-dependent methyltransferases use SAM, also known as AdoMet, as a methyl group donor for the modification of both 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]

SET Domain-containing Protein 8 (SET8) is methyltransferase (MT) that selectively mono-methylates histone H4 at lysine residue 20 (H4K20), an vent proven to have an important role in chromatin structure and transcriptional activation.[3,4] SET8 is also a novel regulator of p53, mono-methylating lysine 382 of the tumor suppressor.[5] Its ability to suppress p53 transcriptional activity implies that it may play a significant role in human tumorigenesis.

About This Assay

Cayman's SET8 Methyltransferase Inhibitor Screening Assay provides a convenient method for screening human SET8 inhibitors. Figure 1 outlines the general scheme of the assay.[6] The transfer of the methyl group from SAM by SET8 to the acceptor peptide (H4K20) generates S-adenosylhomocysteine (S-AdoHcy), which is rapidly converted to S-ribosylhomocysteine and adenine by AdoHcy 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 (H_2O_2). The reaction between H_2O_2 and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) produces the highly fluorescent compound resorufin. Resorufin fluorescence is analyzed using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

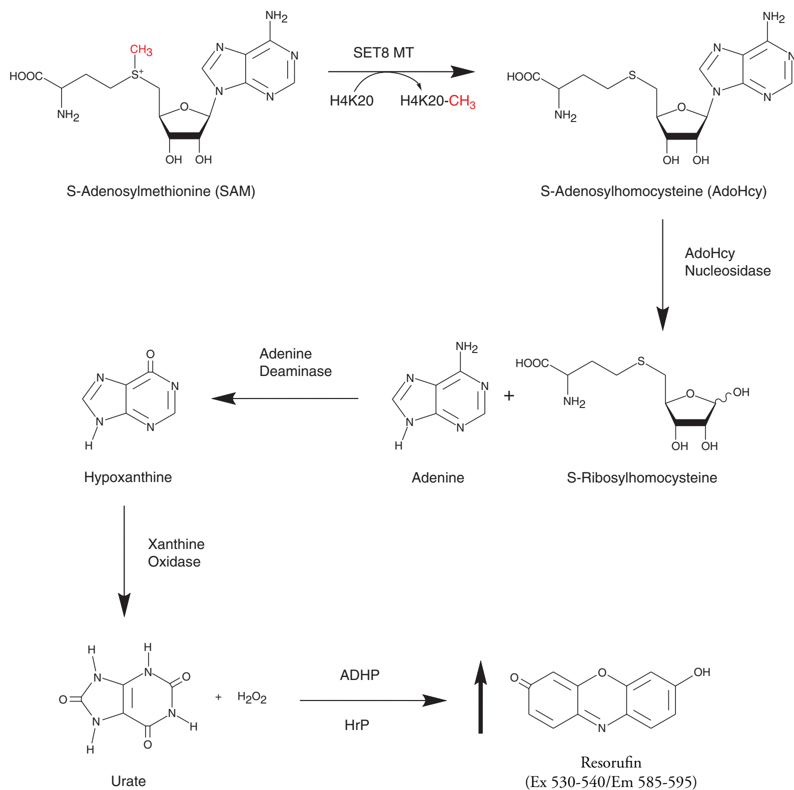

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for FP. UltraPure water may be purchased from Cayman Chemical (Item No. 400000).

Buffer Preparation

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

   Thaw the MT Assay Buffer and MT Assay Buffer Additive at room temperature. Add the entire volume of the Additive into the Assay Buffer and mix thoroughly. 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. **ADHP Assay Reagent - (Item No. 700002)**

   The vials contain a clear lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). Immediately prior to making the Master mixture (see page 12) add 100 µl of DMSO Assay Reagent (Item No. 700001) to the vial and vortex. Then add 400 µl of **HPLC-grade water** and vortex. Prepare additional vials as needed. The reconstituted Mixture is stable for 60 minutes. After 60 minutes, increased background fluorescence will occur.

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

   Each vial contains lyophilized S-adenosylmethionine (SAM). Reconstitute the contents of the vial with 100 µl of 20 mM HCl (Item No. 700012) to yield 6.9 mM SAM. It is ready to use to prepare the Master Mixture. Prepare additional vials as needed.

5. **SET8 (human recombinant) - (Item No. 700351)**

   Each vial contains 150 µl of human recombinant SET8 methyltransferase (N-terminal His-tagged SET8 amino acids 190-352). Thaw the enzyme on ice. Prior to assaying, add 450 µl of Assay Buffer containing additive to the vial. This is enough enzyme for assaying 60 wells. Dilute the additional vial if assaying the entire plate. The diluted Enzyme is stable for four hours on ice.

6. **DMSO Assay Reagent - (Item No. 700001)**

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

7. **SET8 Assay Acceptor Peptide - (Item No. 700352)**

   Each vial contains 600 µl of 3.125 mM human H4K20 peptide (H_2N-AKRHRK-VLRD-NH_2). The peptide is ready to use in the assay. *NOTE: The final concentration of peptide in the assay as described is 250 µM. This concentration may be reduced with Assay Buffer at the user's discretion. The K_m value for the peptide is 120 µM.*

8. **MT Assay AdoHcy Positive Control - (Item No. 700145)**

   The vial contains 200 µl of a 1 mM solution of adenosylhomocysteine (AdoHcy). AdoHcy can be used to assay for interference (see page 15).

9. **HCl Assay Reagent (20 mM) - (Item No. 700012)**

   The vial contains 1 ml of 20 mM hydrochloric acid. The reagent is ready to use as supplied.

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. We suggest that each inhibitor be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 18. A typical layout of samples and inhibitors to be measured in triplicate is shown in Figure 2.

	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

Performing the Assay

Pipetting Hints
• Use different tips to pipette each reagent.
• Do not expose the pipette tip to the reagent(s) already in the well.
• Avoid introducing bubbles into the wells.

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.

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

Reagent	36 wells	72 wells	100 wells
Assay Buffer + Additive	3 ml	6 ml	9 ml
MT Enzyme Mixture	1 vial/300 µl	2 vials/600 µl	3 vials/900 µl
ADHP Assay Reagent	200 µl	400 µl	600 µl
MT SAM	1 vial/100 µl	2 vials/200 µl	3 vials/300 µl

Table 1. Master Mixture Preparation

2. **100% Initial Activity Wells** - add 100 µl of Master Mixture, 10 µl of SET8 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 SET8 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 SET8 Acceptor Peptide, and 5 µl of inhibitor* to three wells.

	Master Mixture	MT 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 SET8 to the 100% Initial Activity and Inhibitor wells and add 10 µl of Assay Buffer to the background wells.
6. Cover the plate with the plate cover and incubate for ten minutes at 37°C.
7. Remove the plate cover and read fluorescence 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.

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

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

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).

Performance Characteristics

Precision:

When a series of 10 SET8 measurements were assayed on the same day, the intra-assay coefficient of variation was 3.9%. When a series of 10 SET8 measurements were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.2%.

RESOURCES

Interferences

It is possible that a compound tested for SET8 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 MT Assay 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 value from the AdoHcy value. Divide the result by the AdoHcy value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the assay.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Bubble in the well(s) B. Poor pipetting/technique	A. Be careful not to splash the contents of the wells A. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence was 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
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. Couture, J.-F., Collazo, E., Brunzelle, J.S., *et al.* Structural and functional analysis of SET8, a histone H4 Lys-20 methyltransferase. *Genes & Development* **19**, 1455-1465 (2005).
4. Yin, Y., Liu, C., Tsai, S.N., *et al.* SET8 recognizes the sequence RHRK20VLRDN within the N terminus of histone H4 and mono-methylates lysine 20. *J. Biol. Chem.* **280(34)**, 30025-30031 (2005).
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6. Dorgan, K.M., Wooderchak, W.L., Wynn, D.P., *et al.* An enzyme-coupled continuous spectrophotometric assay for S-adenosylmethionine-dependent methyltransferases. *Anal. Biochem.* **350**, 249-255 (2006).

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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