Methyltransferase Fluorometric Assay Kit

Item No. 700150

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GENERAL INFORMATION

Materials Supplied

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

Item Number	Item	Quantity/Size	Storage
700141	MT Assay Buffer	2 vials/20 ml	-20°C
700142	MT Assay Buffer Additive	2 vials/200 µl	-20°C
700143	MT Enzyme Mixture	3 vials/250 µl	-80°C
700002	ADHP Assay Reagent	3 vials	-20°C
700145	MT Assay AdoHcy Positive Control	1 vial/200 µl	-80°C
700146	MT Assay S-Adenosylmethionine	3 vials	-80°C
700023	Resorufin Standard	1 vial/500 µl	-20°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
700012	HCl Assay Reagent (20 mM)	1 vial/1 ml	-20°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.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3640

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 if stored 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. Purified S-adenosyl-L-methionine dependent methyltransferase
2. Appropriate methyltransferase acceptor substrate
3. A plate reader with the ability to measure fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm
4. Adjustable pipettes and a multichannel or repeating pipette
5. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Methylation of key biological molecules and proteins play 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]

About This Assay

Cayman's Methyltransferase Fluorometric Assay Kit is a continuous enzyme-coupled assay that can monitor SAM-dependent methyltransferases.[3] Figure 1, on page 7, outlines the general scheme of the assay. The removal of the methyl group from SAM generates S-adenosylhomocysteine (AdoHcy), which is rapidly converted to S-ribosylhomocysteine and adenine by AdoHcy nucleosidase. This rapid conversion prevents the buildup of AdoHcy 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 ADHP (10-acetyl-3,7,-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The assay is supplied with AdoHcy as a positive control. The assay can be used with any purified SAM-dependent methyltransferase.

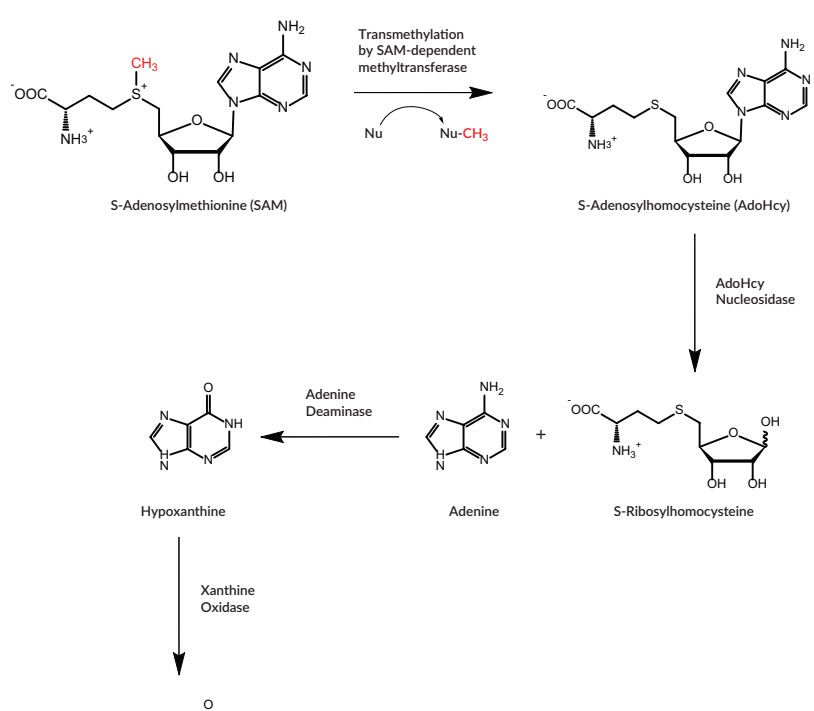


Figure 1. Assay Scheme

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 MT enzyme mixture. Thaw only the number of vials you will be using for your experiment on ice. 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)**

   These vials contain a lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). Immediately prior to making the Master Mixture, 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 AdoHcy Positive Control - (Item No. 700145)**

   This vial contains 200 µl of a 1 mM solution of adenosylhomocysteine (AdoHcy). Thaw the vial on ice. Prior to use, mix 10 µl with 90 µl of MT Assay Buffer containing Additive.

5. **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.

6. **Resorufin Standard - (Item No. 700023)**

   This vial contains 500 µl of 2 mM resorufin in DMSO. It is ready to use to prepare the standard curve.

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

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

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

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

Sample Preparation

This assay is suitable for use with all purified SAM-dependent methyltransferases. It is necessary to titrate each enzyme/substrate system in the assay to determine optimal conditions. An example of human lysine specific histone methyltransferase, SET7/9, assayed with 20 µM of the acceptor substrate TAF-10, is shown in Figure 3 on page 18.[4] Avoid the use of reducing agents (including DTT, β-mercaptoethanol, and TCEP) and metal chelators, such as EDTA and EGTA, as these have an inhibitory effect on the reaction. If these reagents are present, dialysis against 0.1 M Tris-HCl, pH 8.0, is recommended.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a resorufin standard curve in duplicate must be assayed with the samples. We suggest that each sample be assayed at least in duplicate. Two wells should be designated as background wells and two wells should be designated as the positive control. A typical layout of samples to be measured in duplicate is shown in Figure 2 below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	PC	PC	5	5	13	13	21	21	29	29
B	B	B	S	S	6	6	14	14	22	22	30	30
C	C	C	SB	SB	7	7	15	15	23	23	31	31
D	D	D	IA	IA	8	8	16	16	24	24	32	32
E	E	E	1	1	9	9	17	17	25	25	33	33
F	F	F	2	2	10	10	18	18	26	26	34	34
G	G	G	3	3	11	11	19	19	27	27	35	35
H	H	H	4	4	12	12	20	20	28	28	36	36

A-H = Resorufin Standards
PC = AdoHcy Positive Control
S = Sample
SB = Sample Background
IA = Sample + Inhibitor/Activator
1-36 = Other Samples

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps to 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 115 µl in all the wells.
- All reagents except the enzymes 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 fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Mix 100 µl of the Resorufin Standard (Item No. 700023) with 1.9 ml of MT Assay Buffer containing Additive. Mix 500 µl of this diluted Resorufin Standard with 4.5 ml of MT Assay Buffer containing Additive to yield a stock concentration of 10 µM. Label eight clean glass test tubes A-H. Add the amount of Resorufin Stock (10 µM) and MT Assay Buffer containing Additive to each tube as described in Table 1. The diluted Standards are stable for four hours at room temperature.

Tube	10 µM Resorufin Stock (µl)	MT Assay Buffer containing Additive (µl)	Final Concentration (µM)
A	125	875	1.25
B	250	750	2.5
C	375	625	3.75
D	500	500	5
E	625	375	6.25
F	750	250	7.5
G	875	125	8.75
H	1,000	0	10

Table 1. Preparation of Resorufin standards

Performing the Assay

1. **Standard Wells** - add 115 µl of Standard (tubes A-H) per well to the designated wells on the plate (see **Sample plate format**, Figure 2, page 11).
2. Read the plate after five minutes using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. Reading the standards prior to measuring sample activity allows an appropriate *gain* to be established for detecting the entire range of the standards. This *gain* must also be used when assaying the samples.
3. In a suitable tube, prepare the Master Mixture according to the table below:

Reagent	35 wells	70 wells	105 wells
Assay Buffer + Additive	3 ml	6 ml	9 ml
MT Enzyme Mixture	250 µl	500 µl	750 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 2. Master Mixture Preparation

4. **AdoHcy Positive Control Wells** - add 10 µl of MT Assay Buffer and 5 µl of Positive Control to the designated wells on the plate (see Sample plate format, Figure 2, page 11).
5. **Sample Wells** - add 5 µl of sample to at least two wells. To obtain reproducible results, the amount of methyltransferase added to the wells should fall within the range of the assay. When necessary, samples should be diluted with MT Assay Buffer or 0.1 M Tris-HCl, pH 8.0, to bring the enzymatic activity to this level.
6. **Sample Background Wells** - add 5 µl of MT Assay Buffer to two wells.
7. Add 10 µl of the appropriate acceptor substrate to only the sample and sample background wells.
8. Initiate the reactions by quickly adding 100 µl of Master Mixture to the positive control, sample, and sample background wells.
9. Read the plate in a plate reader at 37°C every minute for 30 minutes using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Well	Assay Buffer	Positive Control	Sample	Acceptor	Master Mix
Positive Control	10 µl	5 µl	-	-	100 µl
Sample	-	-	5 µl	10 µl	100 µl
Background	5 µl	-	-	10 µl	100 µl

Table 3. Pipetting summary

NOTE: If assaying inhibitors or activators, adjust the enzyme and acceptor substrate concentration so that all three components are added to the assay in a final volume of 15 µl (i.e., 5 µl methyltransferase, 5 µl inhibitor/activator, and 5 µl acceptor substrate). Keep the methyltransferase volume at 5 µl.

ANALYSIS

Calculations

Plot the Standard Curve

1. Determine the average fluorescence of the standards. Plot the fluorescence values of each standard as a function of the final concentration of resorufin from Table 1.

Determine Methyltransferase Activity

1. Determine the average fluorescence of each sample and plot fluorescence as a function of time.
2. Determine the change in fluorescence (RFU) per minute:
   a. Obtain the slope (rate) of the linear portion of the curve. An example of human lysine specific histone methyltransferase, SET7/9 assayed with 20 µM TAF10 is shown in Figure 3, on page 18.

   OR

   b. Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation:

$$\text{RFU/min} = \frac{\text{RFU(Time 2)} - \text{RFU(Time 1)}}{\text{Time 2(min)} - \text{Time 1(min)}}$$

3. Determine the rate of RFU/min for the sample background and subtract this rate from that of the sample.
4. Calculate the methyltransferase activity using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of fluorophore per minute at 37°C.

$$\text{Methyltransferase Activity (nmol/min/ml)} = \frac{\text{RFU/min}}{\text{Slope from Resorufin standard curve (RFU/µM)}} \times \text{Sample Dilution}$$

5. If inhibitors or activators were assayed, determine the percent inhibition/activation for each sample by subtracting the activity of each inhibitor/activator sample from the activity of its corresponding non-treated sample. Divide the result by the activity of the non-treated sample, and multiply by 100 to give the percent inhibition/activation.

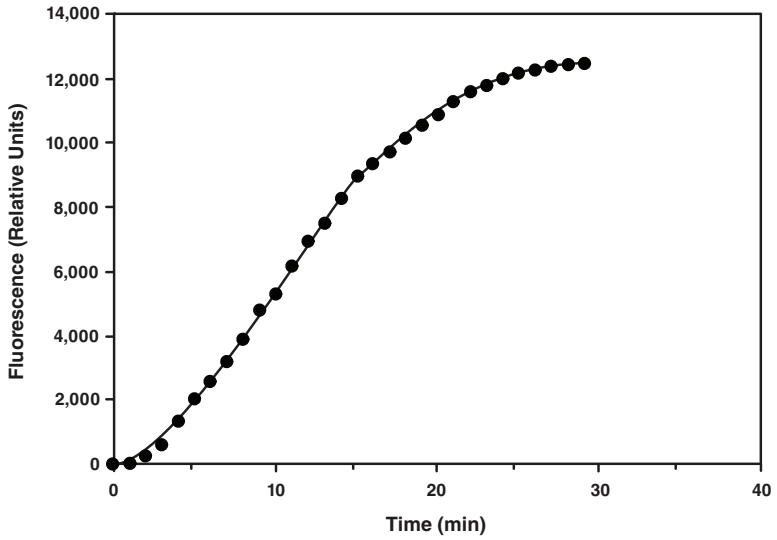


Figure 3. Human lysine specific histone methyltransferase, SET7/9 assayed with 20 µM TAF-10 as the acceptor substrate.

Performance Characteristics

Assay Range:

Under the standardized conditions of the assay, the dynamic range of the kit is 0-10 µM of Resorufin.

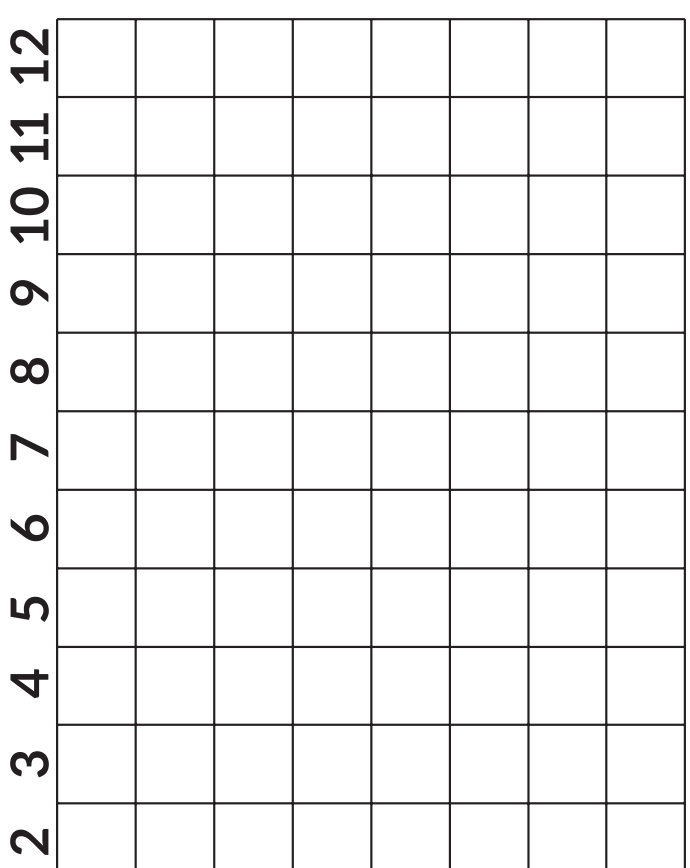
RESOURCES

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 sample wells	Sample was too dilute or acceptor substrate was not added	Re-assay using a more concentrated sample and make sure the appropriate acceptor substrate is added
The fluorometer exhibited 'MAX" values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition/activation was seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor/activator of the enzyme	Increase the compound concentration and re-assay

References

1. Loenen WAM. *Biochem Soc Trans* **34(2)**, 330-333 (2006).
2. Chiang, P.K., Gordon, R.K., Tal, J., *et al. FASEB J.* **10**, 471-480 (1996).
3. Dorgan, K.M., Wooderchak, W.L., Wynn, D.P., *et al. Anal. Biochem.* **350**, 249-255 (2006).
4. Couture, J.-F., Collazo, E., Hauk, G., *et al. Nature Structural and Molecular Biology* **13(2)**, 140-146 (2006).



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.