

SAMfluoro: SAM Methyltransferase Assay

A Fluorescent, Continuous Enzyme Assay

INTRODUCTION

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. Aberrant levels of SAM have been linked to many abnormalities, including Alzheimer's, depression, Parkinson's, multiple sclerosis, liver failure and cancer (2).

The fluorescent SAMfluoro: SAM Methyltransferase Assay is a continuous enzyme coupled assay that can continuously monitor SAM-dependent methyltransferases (3) without the use of radioactive labels or endpoint measurements.

Figure 1 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 the included 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 (H₂O₂). The rate of production of hydrogen peroxide is measured with 10-acetyl-3,7,-dihydroxyphenoxazine (ADHP), which produces the highly fluorescent compound resorufin. Resorufin production can easily be measured with an excitation wavelength of 530-540nm and an emission wavelength of 585-595nm. The kit is supplied with enough reagents for 100 microwell assays.

The assay is supplied with AdoHcy as a positive control. The assay can be adapted to be used with any purified SAM dependent methyltransferase or a purified enzyme that produces 5-adenosylhomocysteine or 5'-methylthioadenosine, due to the specificity of AdoHcy nucleosidase.

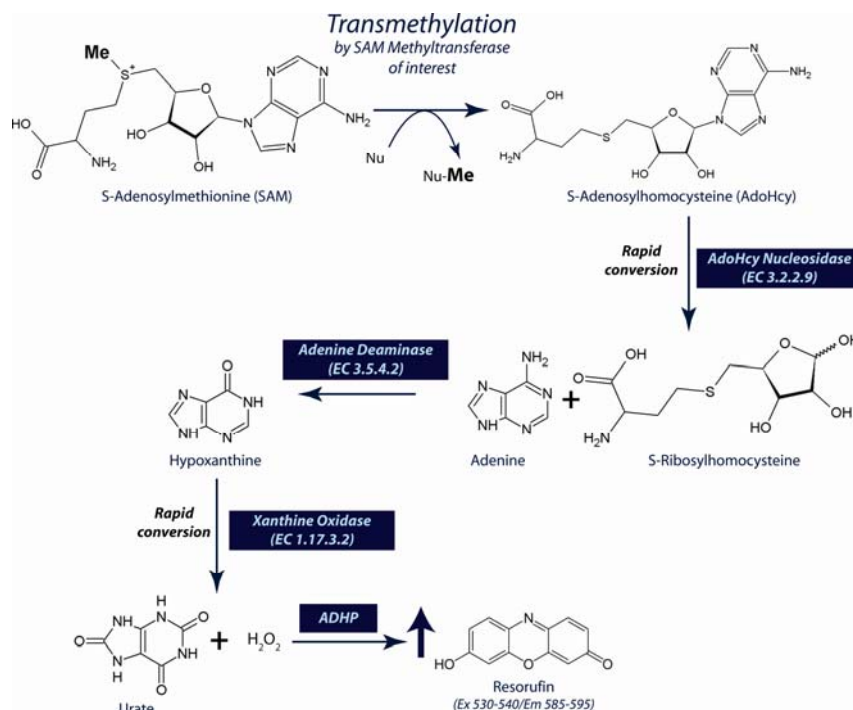


Figure 1: SAMfluoro: SAM Methyltransferase Assay Scheme

Patent Pending, available under a licensing agreement with Washington State University, Pullman WA



ITEM(S) SUPPLIED	Cat # 786-431
SAM Methyltransferase Assay Buffer	20ml
SAM Methyltransferase Assay Buffer Additive	0.2ml
SAM <i>fluoro</i> Enzyme Mix	5 x 300µl
SAM <i>fluoro</i> Fluorometric Mix	3 vials
Positive Control: Adenosylhomocysteine [1mM]	0.2ml
S-Adenosylmethionine	5 x 200µl
Resorufin Standard	100µl
DMSO	1ml
Resorufin Buffer	50ml

STORAGE CONDITIONS

The kit is shipped on **dry ice**. Upon arrival, store the kit at **-70°C**. The kit components are stable for up to 1 year, when stored and used as recommended.

ITEMS NEEDED BUT NOT SUPPLIED

- Purified SAM Methyltransferase to be tested
- Appropriate methyltransferase acceptor substrate
- Fluorometer to measure fluorescence using an excitation of 530-540nm and an emission wavelength of 585-595nm
- 96-well plate suitable for fluorescence measurements

PREPARATION BEFORE USE

- Thaw SAM Methyltransferase Assay Buffer and SAM Methyltransferase Assay Buffer Additive solution at room temperature. Add the entire volume of SAM Methyltransferase Assay Buffer Additive into the SAM Methyltransferase Assay Buffer and mix it thoroughly. Store SAM Methyltransferase Assay Buffer at room temperature, do not freeze after addition of Additive.
- SAM*fluoro* Fluorometric Mix is a clear lyophilized powder of ADHP (10-acetyl-3,7,-dihydroxyphenoxazine). Immediately prior to preparing the Master Mix (Protocol; step 2), add 100µl DMSO to the vial and vortex. Next, add 400µl assay buffer with additive and vortex. Prepare additional vials as needed. The reconstituted mixture is stable for 30 minutes. After this time increased background fluorescence will occur.
- Positive Control: Adenosylhomocysteine: The vial contains 200µl of a 1mM solution of adenosylhomocysteine (AdoHcy). Thaw the vial on ice. Prior to use, dilute 10µl with 90µl of assay buffer with additive.
- Thaw all other kit components **on ice**. Each assay requires:
 - 15µl/ well of SAM*fluoro* Enzyme Mix, supplied in 300µl vials. Thaw on ice only the number of vials you require for your assay. We do not recommend repeated freeze/thawing of the SAM*fluoro* Enzyme Mix.
 - 10µl/well S-Adenosylmethionine (SAM), supplied in 200µl vials. Thaw on ice only the number of vials you require for your assay. We do not recommend repeated freeze/thawing of the S-Adenosylmethionine.
 - 10µl/well SAM*fluoro* Fluorometric Mix, see above for preparation.
- Sample Preparation:** Prepare your test sample, containing the purified SAM dependent methyltransferase to be assayed, according to your own standard protocol. Avoid the use of reducing agents and metal chelators as these have an inhibitory effect on the reaction. If these reagents are present, we recommend using our Tube-O-DIALYZER™ micro devices to dialyze the sample against 0.1M Tris-HCl, pH8.0.
- Prepare the specific substrate for the methyltransferase to be assayed using the SAM Methyltransferase Assay Buffer or the buffer of your own choice. Avoid the use of reducing agents and metal chelators as these have an inhibitory effect on

the reaction.

- G. **Standard Preparation:** Dilute 20 μ l resorufin standard with 1.98ml resorufin buffer to yield a stock solution of 100 μ M. Label 8 clean glass test tubes with A-H and prepare the standards as indicated below. The diluted standards are stable for 4 hours at room temperature.

Tube	Resorufin Stock (μ l)	Resorufin Buffer (μ l)	Final Concentration (μ M)
A	0	1000	0
B	5	995	0.5
C	10	990	1
D	50	950	5
E	100	900	10
F	200	800	20
G	250	750	25
H	500	500	50

PROTOCOL

NOTE: The positive control supplied is a control for the SAM Methyltransferase Assay. Some acceptor substrates, inhibitors, activators or buffer components may interfere with the assay. We highly recommend testing the compatibility of these reagents with the SAM Methyltransferase Assay using the positive control, by substituting in suspected non compatible reagent into the positive control reactions. It is necessary to titrate each enzyme/ substrate system in the assay to determine optimal conditions.

NOTE: The deaminase in the SAM methyltransferase assay requires the presence of divalent metal ions, present in the SAM Methyltransferase Assay Buffer. The presence of chelators, such as EDTA, will deplete divalent metal ions and inhibit the deaminase enzyme. If EDTA is required then supplement additional manganese ions into the reaction.

NOTE: Reducing agents, including DTT, β -mercaptoethanol and TCEP, have an inhibitory effect on the assay. If present, we recommend dialysis against 0.1M Tris-HCl, pH8.0.

1. **Standard Assay:** Add 190 μ l Resorufin Buffer and 10 μ l of each standard per well of a fluorescent compatible plate. Perform in duplicate. Read the plate after 5 minutes using an excitation wavelength of 530-540nm and an emission wavelength of 585-595nm. Reading the samples prior to the actual assay allows the appropriate GAIN to be established for detecting the entire range of standards. This GAIN must also be used for the SAM Methyltransferase assays.
2. Equilibrate the SAM Methyltransferase Assay Buffer + Additive to 37°C.
3. Aliquot a total volume of 10 μ l of your SAM methyltransferase samples to at least two wells of a 96 well plate. Use the SAM Methyltransferase Assay Buffer or 0.1M Tris, pH8.0 as a diluent. We recommend performing the reactions and controls in at least duplicate.

For the background control, aliquot 10 μ l SAM Methyltransferase Assay Buffer into each background control well.

For the positive control, add 10 μ l Positive Control and 10 μ l SAM Methyltransferase Assay Buffer to each positive control well. We recommend performing the reactions in duplicate.

4. Add the appropriate acceptor substrate to the sample and background control wells in a total volume of 10 μ l to the wells, using SAM Methyltransferase Assay Buffer or 0.1M Tris, pH8.0 as a diluent.

NOTE: If assaying inhibitors or activators, adjust the acceptor substrate concentration so that the substrate and activators or inhibitors are added in a final volume of 10 μ l.

5. Immediately prior to use and in a suitable tube, prepare the SAM Methyltransferase Assay Master Mix according to the

table below:

	1 well	10 wells	50 wells	100 wells
SAM Methyltransferase Assay Buffer + Additive	145µl	1.45ml	7.25ml	14.5ml
SAM _{fluoro} Enzyme Mix	15µl	150µl	750µl	1500µl
SAM _{fluoro} Fluorometric Mix	10µl	100µl	500µl	1000µl
S-Adenosylmethionine	10µl	100µl	500µl	1000µl

6. To initiate the reaction, rapidly add 180µl pre-warmed SAM Methyltransferase Master Mix to the wells. Immediately, read the plate at 37°C every minute for 30 minutes using an excitation wavelength of 530-540nm and an emission wavelength of 585-595nm.

ANALYSIS

- A. **Plot the standard curve:** Calculate the average fluorescence of the standards. Subtract the fluorescence value of standard A from itself and all other standards. Plot these values against the final concentrations (see Preparation Before Use, Step F). See Figure 2 for a standard curve.
- B. **Calculate Methyltransferase Activity:** Calculate the average fluorescence of each sample and plot fluorescence against time.
- C. Calculate the change in fluorescence (ΔRU) per minute, by:
- Obtain the slope (rate) of the linear portion of the curve. See figure 3 for an example using humane lysine specific histone Methyltransferase, SET7/9).
 - Or, calculate the change in fluorescence between two points on the linear portion of the curve using the following equation:

$$\Delta\text{RU}/\text{min} = \frac{(\text{RU at Time 2}) - (\text{RU at Time 1})}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

- D. Calculate the rate of ΔRU/min for the background control wells and subtract this rate from the sample well rate.
- E. Calculate Methyltransferase Activity: Use the following equation to calculate the Methyltransferase activity:

$$\text{Methyltransferase Activity (nmol/min/ml)} = \frac{\Delta\text{RU}/\text{min}}{\text{Slope of Resorufin standard curve (RU/}\mu\text{M)}} \times \text{Sample Dilution}$$

One unit of Methyltransferase activity is defined as the amount of enzyme that will cause the formation of 1nmol of fluorophore per minute at 37°C.

- F. If activators or inhibitors were assayed, determine the percent activation/ inhibition for each sample as follows:

$$\% \text{ Inhibition} = \frac{(\text{Activity untreated sample}) - (\text{Activity Inhibitor Treated})}{\text{Activity untreated sample}} \times 100$$

$$\% \text{ Activation} = \frac{(\text{Activity Activator Treated}) - (\text{Activity untreated sample})}{\text{Activity untreated sample}} \times 100$$

ASSAY RANGE

The detection range of the assay is from 0-50µM of Resorufin.

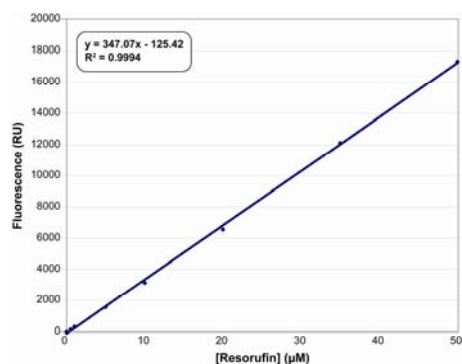


Figure 2: Resorufin Standard Curve.

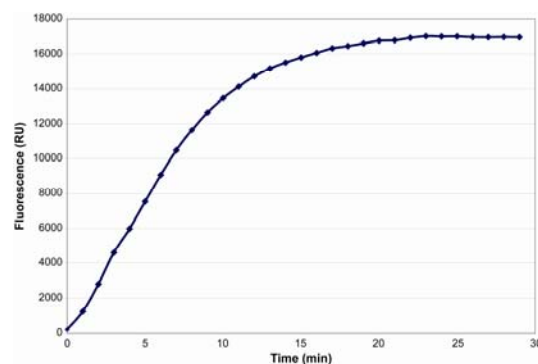


Figure 3: Human lysine specific histone Methyltransferase SET7/9 assayed with 20µM TAF-10 as the acceptor substrate.

TROUBLESHOOTING

Issue	Possible Cause	Recommended Solution
Erratic values; dispersion duplicates/ triplicates	A. Poor pipetting technique B. Air Bubbles in well	A. Use a repeating pipettor and do not eject solution too vigorously B. Gently tap plate to dislodge air bubbles
No fluorescence detected above background control	A. Sample too dilute or acceptor substrate not added B. Acceptor substrate interferes with assay	A. Re-assay using a lower dilution and ensure acceptor substrate is added B. Add the acceptor substrate in with the positive control to test compatibility
Fluorometer exhibited “MAX” values for the samples	A. GAIN setting too high	A. Recalibrate GAIN with the resorufin standards
No inhibition or activation was seen with added compounds	A. The compound concentration was too low B. Compound is not an inhibitor or activator	A. Increase compound concentration and re-assay

REFERENCES

1. Cheng, X. and Blumenthal, R.M. (1999) *S-Adenosylmethionine Dependent Methyltransferases: Structures and Functions*, World Scientific, Singapore.
2. Schubert, H.L. et al. (2003) *Trends Biochem. Sci* **28**: 329-335.
3. Dorgan, K.M. et al. (2006) *Anal. Biochem.* **350**:249-255.

RELATED PRODUCTS

1. **SAM265: SAM Methyltransferase Assay (Cat # 786-425)**: A UV version of the SAM Methyltransferase assay.
2. **SAM510: SAM Methyltransferase Assay (Cat. # 786-430)**: A colorimetric version of the SAM Methyltransferase assay.
3. **Tube-O-DIALYZER™ (Micro) (Cat. # 786-610-786-614)**: Micro dialysis device for the removal of chelators and reducing agents from Methyltransferase preparations. Suitable for 20-200µl volumes and available with 1K, 4K, 8K, 15K and 50K MWCO.

NOTE: For other related products, visit our web site at www.GBiosciences.com or contact us.