SAMfluoro™: SAM Methyltransferase Assay

G-Biosciences

Abstract

A Fluorescent, Continuous Enzyme Assay.

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Guidelines

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-adenosyl-L-methionine (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.

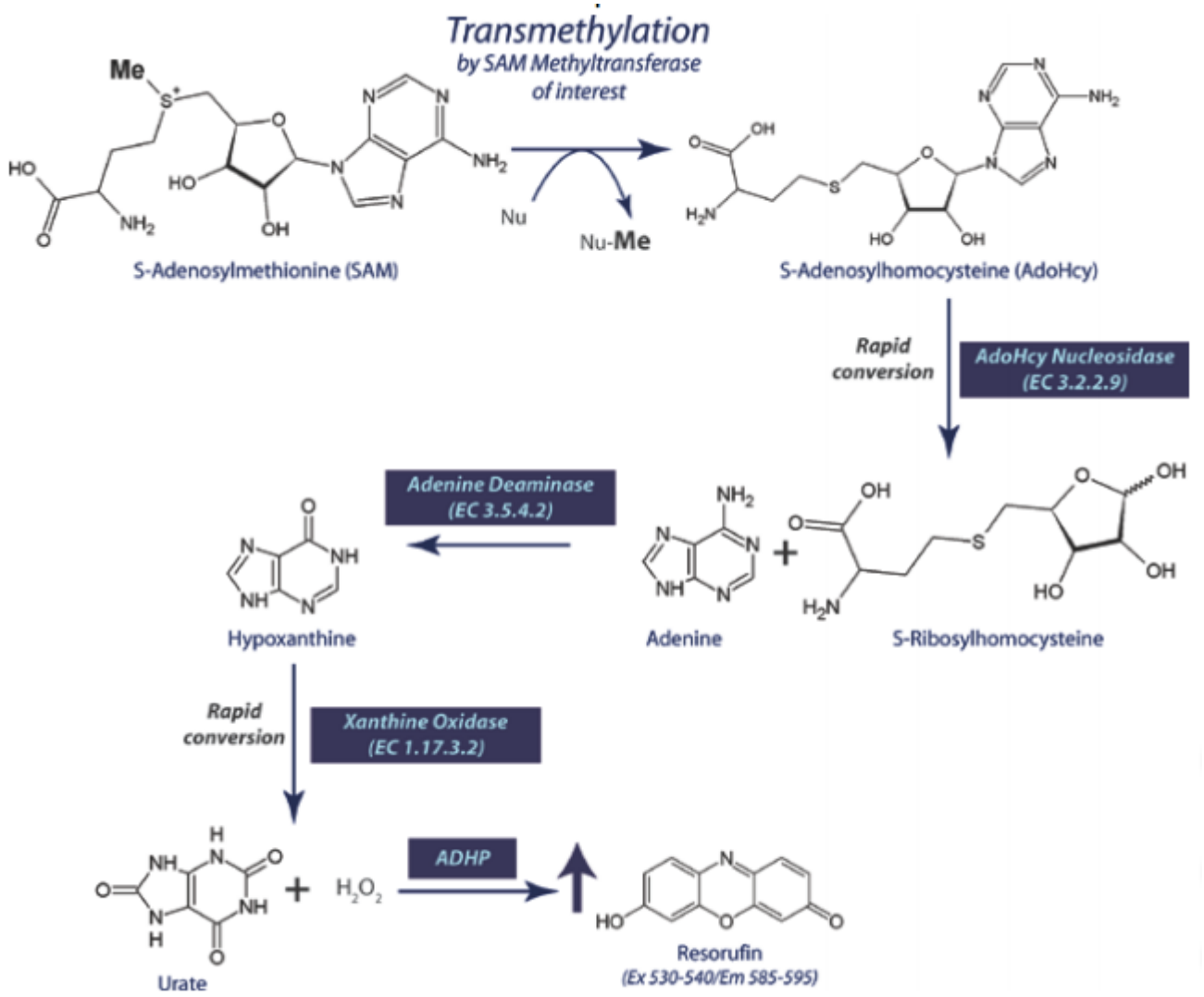

Figure 1: SAMfluoro: SAM Methyltransferase Assay Scheme

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 Sribosylhomocysteine 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.

ITEM(S) SUPPLIED (Cat. # 786-431)

Description	Size
SAM Methyltransferase Assay Buffer	20ml
SAM Methyltransferase Assay Buffer Additive	0.2ml
SAM Enzyme Mix	3 x 300µl
SAMfluoro Fluorometric Mix	3 vials
Positive Control: Adenosylhomocysteine [1mM]	0.2ml
S-Adenosylmethionine	3 vials
Resorufin Standard	100µl
DMSO	1ml
HCl Assay Reagent [20mM]	1ml
Resorufin Buffer [10X]	5ml

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.

ADDITIONAL ITEMS REQUIRED

• Purified S-adenosyl-L-methionine dependent methyltransferase

• Appropriate methyltransferase acceptor substrate

• Fluorometer to measure fluorescence using an excitation of 530-540nm and an emission wavelength of 585-595nm

• Adjustable pipettes and repeat pipettor

• Ultra pure water; G-Biosciences Proteomic Grade Water (Cat. # 786-229) is recommended.

• 96-well plate suitable for half volume (115µl) fluorescence measurements (Corning, Cat. # 3694)

IMPORTANT INFORMATION

• The final volume of the assay is 115µl in all the wells

• All reagents, except the enzymes, must be equilibrated to room temperature before use

• We recommend assaying samples in triplicate.

• Assay is performed at 37°C

• Monitor fluorescence with 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: i. 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). ii. Or, calculate the change in fluorescence between two points on the linear portion of the curve using the following equation:

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

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:

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

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:

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

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

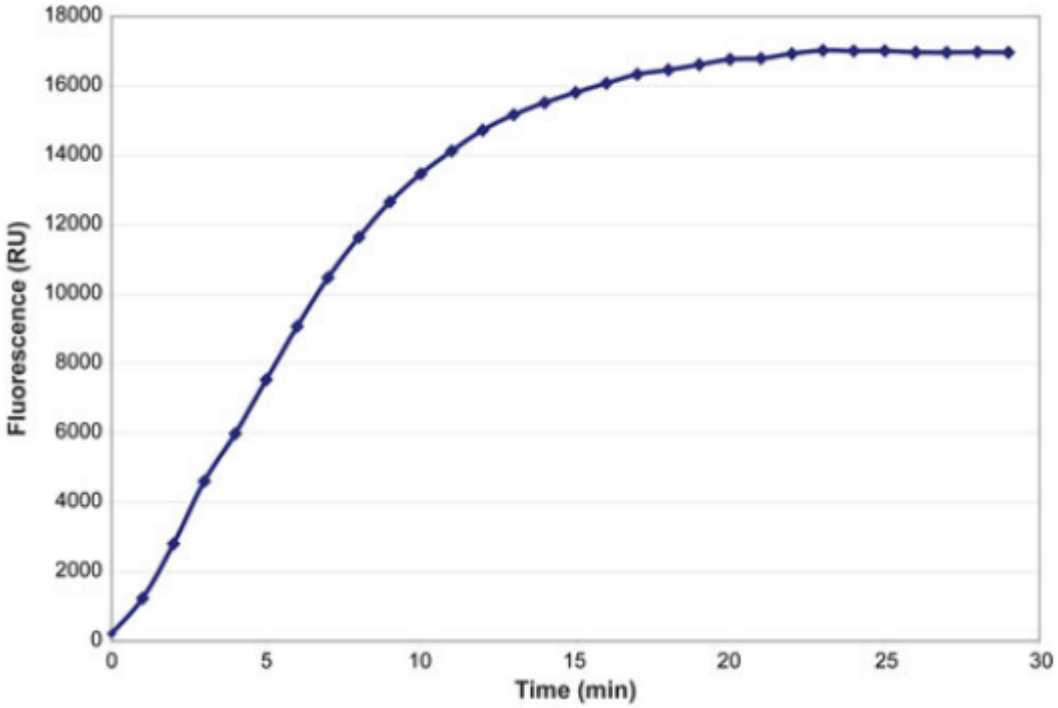

Figure 3: Human lysine specific histone Methyltransferase SET7/9 assayed with 20µM TAF-10 as the acceptor substrate.

ASSAY RANGE

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

TROUBLESHOOTING

Issue	Possible Cause	Recommended Solution
Erratic values; dispersion duplicates/ triplicates	A. Poor pipetting technique	A. Use a repeating pipettor and do not eject solution too vigorously
	B. Air Bubbles in well	B. Gently tap plate to dislodge air bubbles
No fluorescence detected above background control	A. Sample too dilute or acceptor substrate not added	A. Re-assay using a lower dilution and ensure acceptor substrate is added
	B. Acceptor substrate interferes with assay	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
High Background	SAMfluoro Fluorometric Mix has begun to degrade	Make fresh immediately prior to addition to the reaction

PROTOCOL NOTES

- 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.
- 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.
- 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

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.

Materials

SAM-fluoro: SAM Methyltransferase Assay 786-431 by G-Biosciences

Protocol

PREPARATION BEFORE USE

Step 1.

Thaw SAM Methyltransferase Assay Buffer and SAM Methyltransferase Assay Buffer Additive solution at room temperature.

PREPARATION BEFORE USE

Step 2.

Add the entire volume of SAM Methyltransferase Assay Buffer Additive into the SAM Methyltransferase Assay Buffer and mix it thoroughly.

PREPARATION BEFORE USE

Step 3.

Store SAM Methyltransferase Assay Buffer at room temperature, do not freeze after addition of Additive.

PREPARATION BEFORE USE

Step 4.

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 SAM Methyltransferase Assay Buffer with Additive.

PREPARATION BEFORE USE

Step 5.

SAMfluoro Enzyme Mix, supplied in 300µl vials. Each vial is suitable for 36 assays. Thaw on ice only the number of vials you require for your assay.

NOTES

Colin Heath 08 Jun 2016

We do not recommend repeated freeze/thawing of the SAMfluoro Enzyme Mix.

PREPARATION BEFORE USE

Step 6.

S-Adenosylmethionine (SAM), supplied lyophilized. Reconstitute the contents ofthe vial with 100µl HCl Assay Reagent [20mM] to yield 6.9mM SAM.

NOTES

Colin Heath 08 Jun 2016

Each vial is suitable for 36 assays.

We do not recommend repeated freeze/thawing of the SAdenosylmethioninesolution.

PREPARATION BEFORE USE

Step 7.

SAMfluoro Fluorometric Mix. Immediately prior to making the Master Mix, add 100µl DMSO to the vial and vortex. Add 400µl proteomic grade or ultra pure water and vortex.

NOTES

Colin Heath 08 Jun 2016

200µl SAMfluoro Fluorometric is suitable for 36 assays.

The reconstituted mixture is stable for 60 minutes. After this time increased background fluorescence will occur.

PREPARATION BEFORE USE

Step 8.

Resorufin Buffer: Add 4ml Resorufin Buffer [10X] to 36ml ultra pure water to generate 1X Resorufin Buffer.

PREPARATION BEFORE USE

Step 9.

Sample Preparation: Prepare your test sample, containing the purified SAM dependent methyltransferase to be assayed, according to your own standard protocol.

NOTES

Colin Heath 08 Jun 2016

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.

PREPARATION BEFORE USE

Step 10.

Prepare the specific substrate for the methyltransferase to be assayed using the SAM Methyltransferase Assay Buffer or the buffer of your own choice.

NOTES

Colin Heath 08 Jun 2016

Avoid the use of reducing agents and metal chelators as these have an inhibitory effect on the

reaction.

Step 11.

Standard Assay: Dilute 25µl resorufin standard with 475µl 1X Resorufin Buffer.

Step 12.

Standard Assay: Mix 500µl of this diluted standard with 4.5ml 1X Resorufin to yield a stock solution of 10µM.

Step 13.

Standard Assay: 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	10µM Resorufin Stock (µl)	1X Resorufin Buffer (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	1000	0	10

NOTES

Colin Heath 06 Sep 2016

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.

Colin Heath 06 Sep 2016

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.

Colin Heath 06 Sep 2016

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.

Step 14.

Standard Assay: Add 115µl of each standard per well of a fluorescent compatible plate. Perform in duplicate.

Step 15.

Standard Assay: Blank the plate against 1X Resorufin Buffer.

DURATION
00:05:00

Step 16.

Read the plate after 5 minutes using an excitation wavelength of 530-540nm and an emission wavelength of 585-595nm.

NOTES
Colin Heath 08 Jun 2016

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.

Step 17.

Equilibrate the SAM Methyltransferase Assay Buffer + Additive to 37 °C.

NOTES
Colin Heath 08 Jun 2016

NOTE: The SAM Buffer + Additive must be at 37°C prior to performing the assay. Failure to prewarm will result in artifactual results.

Step 18.

Aliquot a total volume of 5µl 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.

a. **For the background control**, aliquot 5µl SAM Methyltransferase Assay Buffer into each background control well. We recommend performing the reactions in duplicate.

b. **For the positive control**, add 5µl Positive Control and 10µl SAM Methyltransferase Assay Buffer to each positive control well. We recommend performing the reactions in duplicate.

Step 19.

Add 10µl the appropriate acceptor substrate to the sample and background control wells, using SAM Methyltransferase Assay Buffer or 0.1M Tris, pH8.0 as a diluent.

NOTES
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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 10µl.

Step 20.

Immediately prior to use and in a suitable tube, prepare the SAM Methyltransferase Assay Master Mix according to the table below:

Reagent	36 wells	72 wells	100 wells
SAM Methyltransferase Assay Buffer + Additive	3ml	6ml	9ml
SAMfluoro Enzyme Mix	1 vial/ 300µl	2 vial/ 600µl	3 vial/ 900µl
SAMfluoro Fluorometric Mix	200µl	400µl	600µl
S-Adenosylmethionine	1 vial/ 100µl	2 vials/ 200µl	3 vials/ 300µl

Step 21.

Immediately initiate the reaction by adding 100µl SAM Methyltransferase Master Mix to the wells.

Step 22.

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.

DURATION
00:30:00

NOTES
Colin Heath 06 Sep 2016

Perform the analysis as described in the Guidelines.