ASSAY PROTOCOL

Bridge-It® S-Adenosyl Methionine (SAM)

Fluorescence Assay

(96-well microplate format)

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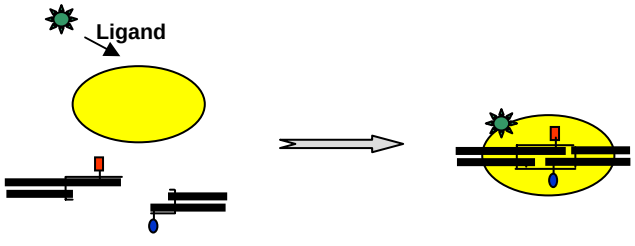
Introduction

**The Bridge-It® S-adenosyl methionine (SAM) Fluorescence Assay Kit is intended solely for laboratory research and development (R&D) purposes. This product has not received government regulatory approval for use in diagnosis or treatment of diseases in humans or animals, monitoring of food products, or any applications other than laboratory R&D and it should not be utilized for such purposes.*

S-adenosyl methionine (also referred to as SAM, SAMe or AdoMet) plays a crucial role in the biological process of methylation in all types of organisms. In the methylation cycle, SAM serves as the donor of the methyl group used in the covalent modification of DNA and proteins. Variability in SAM levels have been linked to the processes of aging, numerous neurological and psychiatric disorders including Alzheimer's disease, depression, HIV-related neurological dysfunction/dementia, multiple sclerosis, Parkinson's disease, spinal cord degeneration, epilepsy, fibromyalgia, migraine headaches, and also chronic liver dysfunction, arteriosclerosis and cancer. Currently, SAM is quantified using the high pressure liquid chromatography (HPLC) method. HPLC is time consuming, costly and, due to the large amount of organic solvent required, not environmentally friendly.

Bridge-It® Fluorescence Assay Platform Design

The common property of all sequence-specific DNA binding proteins is their ability to bind with high affinity and specificity to a DNA duplex containing a unique nucleotide sequence - i.e., the DNA binding site for the protein. Mediomics' novel assay platform design relies on this common characteristic. A DNA duplex containing the sequence-specific DNA binding site for a given target protein is split into two DNA "half-site" duplexes each having a short single-stranded overhand. These single-stranded extensions are short enough so that in the absence of the target protein little spontaneous re-association occurs. When the target protein is present, however, its high affinity for the full-length DNA sequence will drive the re-association of the two half-site DNA duplexes. This re-association can be sensitively detected by incorporating appropriate fluorescence probes into each of the two DNA half-sites. The presence of the DNA binding protein is detected as an increase in the fluorescence signal. As shown schematically below, a simple variation of this basic platform design allows a DNA binding protein (yellow oval) to function as a sensitive biosensor for its specific ligand:



Bridge-It® SAM Fluorescence Assay Principle

Eukaryotic cells contain an estimated 3,000 sequence-specific DNA binding proteins. These important proteins, acting either with or without a specific small molecule co-regulator (ligand), control all aspects of genomic DNA activity including gene expression, DNA replication, and DNA repair. Mediomics is applying its novel fluorescence assay platform to develop *in vitro* assays useful for rapidly and sensitively quantifying the activity of both DNA binding proteins and their small molecule ligands.

The Mediomics Bridge-It® SAM fluorescence assay method is based on a combination of well-established fluorescence measurement techniques and a new assay platform design that utilizes DNA-binding proteins as biosensors for their respective small molecule co-regulators (ligands). The affinity of the DNA sequence-specific MetJ methionine repressor protein for its unique DNA binding site is greatly increased in the presence of its ligand, S-adenosyl methionine. For this assay, the MetJ consensus sequence was split into two approximately equal DNA "half-sites" with one half fragment labeled with fluorescein and the other half fragment labeled with Oyster® 645 fluorophore. The relative amount of SAM present in a test sample will influence the amount of DNA-MetJ protein complex formation in the assay. When this complex forms, it brings the fluorescence labeled-DNA half-sites into close proximity and causes a measurable change (increase) in fluorescence signal emission that can be readily measured using a microplate reader (wavelength settings: absorption 485 nm; emission 665 nm). SAM concentrations in test samples are then determined using a SAM standard curve.

The Bridge-It® SAM fluorescence assay method exhibits highly desirable performance characteristics including a high (>6:1) signal to background (S/B) ratio, a good linear dynamic range (~0.5 µM – 20 µM), and, a detection sensitivity of ~0.5 µM. This detection level (0.5 µM) is useful for quantifying SAM in most test samples of interest including biological fluids, cell culture and fermentation medium, and extracts of tissues and cells. This assay can be modified to become an assay for any enzyme reaction that uses S-adenosyl methionine as either a reactant or a product.

Bridge-It® SAM Fluorescence Assay Reagents and Storage Requirements

The quantity of reagents contained in the assay kit is adequate to perform the indicated number of individual SAM measurements using a black 96-well non-binding surface microplate. Store the Bridge-It® SAM Fluorescence Assay Kit in the freezer at -20ºC until needed for use. **Thaw only the appropriate number of reagent tubes needed for the planned experiment.** Each tube of SAM Assay Solution contains reagent adequate for performing ten (10) SAM measurements using a 96-well black microplate. The SAM assay kit reagents will retain their activity for at least 2 months when stored frozen at -20°C.

Tube Cap Color	Assay Reagent	ml/tube	Storage Conditions
Green	10 mM S-adenosyl methionine standard in 10 mM 2-mercaptoethanol	0.10	Store at -20 ºC. May be thawed up to 5 times
Pink	SAM Assay Solution	1.0	Once thawed, store refrigerated at 4ºC. Stable for at least 1-week. **DO NOT RE-FREEZE.**
Blue	Buffer S*	1.0	Once thawed, store refrigerated at 4ºC**.**

***Note:**
Buffer S may be used for diluting SAM standards and test samples.
96-well non-binding surface black microplates are recommended for this assay and may be purchased from Mediomics, LLC (Cat #163300).

Bridge-It® SAM Fluorescence Assay: General Notes

The following precautionary steps are recommended in order to optimize assay performance and reproducibility:

- Pipette slowly to avoid bubbling and to allow complete recovery and transfer of assay solutions.

- If more than one tube of SAM Assay Solution is required for experimental analysis, mix all tubes together to ensure reagent consistency before use. Thaw only the needed number of tubes.

- After the samples have been transferred into the wells of the 96-well black microplate, gently tap the microplate on the counter top to release any trapped bubbles. Although bubbles generally break with time, large bubbles may be broken with the tip of a small gauge needle (e.g., 27 gauge) being careful to avoid loss of volume through capillary action.

- Fluorescence readings can be affected by temperature. It is recommended for consistency of assay results that all microplates used in a given study be incubated as close to the same temperature as possible (preferably ~25°C).

- To ensure consistency of results, it is recommended that a volume of 18 µl of standard or sample be transferred into the wells of a 96-well black microplate.

- High salt concentration will affect the annealing of DNA in the assay. Thus, use of high salt concentrations should be avoided in preparing the test samples and standards to be measured using the Bridge-It® SAM fluorescence assay.

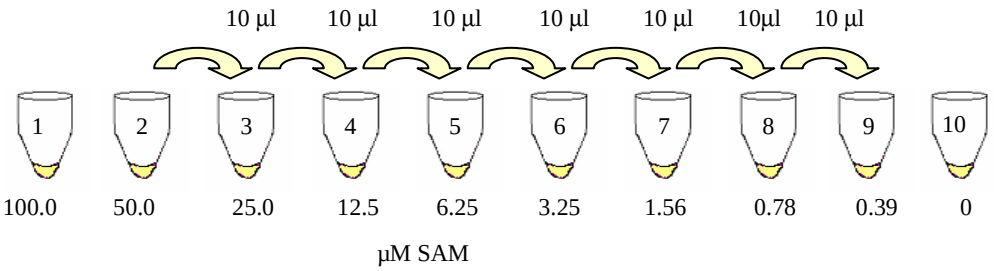
- Certain test samples may exhibit high background fluorescence. In order to take background fluorescence into account, it is recommended that the test sample be diluted in buffer alone (i.e., no assay solution included) and measured against a buffer blank (settings: excitation ~ 485 nm; emission: ~665 nm). Additional information concerning control of background fluorescence is presented elsewhere in this protocol (see data analysis, page 10).

Bridge-It® SAM Assay Standards

If more than one tube of SAM Assay Solution is required for experimental analysis, mix all tubes together to ensure reagent consistency before use. Thaw only the number of SAM Assay Solution tubes needed for the experiment.

It is recommended that the SAM standard curve be prepared in Buffer S or under similar conditions to the test samples. If a buffer other than Buffer S is used, a test to determine the effect of the alternate buffer on assay performance should be conducted prior to using it in the assay. The SAM standards should be prepared as follows:

1. Dilute the 10 mM SAM standard (green cap tube) 1:10 using Buffer S (blue cap tube) for a final concentration of 1 mM SAM. Prepare 10 polypropylene Eppendorf tubes. Add 10 µl of Buffer S to tubes 2-10. Add 10 µl of the 1 mM SAM stock to tubes 1 and 2. Mix the contents of tube 2 by pipetting up and down 5 times. Transfer 10 µl from tube 2 into tube 3. Continue serially diluting the SAM standard through tube 9. After mixing, discard 10 µl from tube 9. All tubes should contain a final volume of 10 µl of SAM standard solution.



Note: 100 µM is the final concentration of tube 1 when a total of 100 µl volume is used. This is equivalent to 10 nmol/well. The SAM standards may be diluted directly in the microtiter plate. Mix by slowly pipetting solution up and down 4-5 times (avoid bubbles).

2. Add 90 µl of SAM Assay Solution to each polypropylene Eppendorf tube and vortex immediately for ~1 second at medium speed.

3. Carefully transfer 90 µl into the appropriate well of a 96-well black microplate. **Transfer 90 µl of Buffer S to a minimum of two wells to measure buffer background (see page 10 for additional information).** The transfer of 90 µl/well rather than 100 µl/well when assayed directly in the microplate will not affect assay sensitivity. It is recommended that the volume used for the standards and test samples be the same.

4. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~ 25°C) for at least 30 minutes.

5. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

Determination of SAM Levels in Various Samples

It is possible to use this assay to determine SAM levels in various types of test samples including biological fluids and extracts of tissues and cells. Liquid samples (including standards) should be diluted in Buffer S. If a different buffer is used, a test to determine the effect of the different buffer on the assay performance should be conducted before using the assay. If the sample has an innate fluorescence, it is recommended a sample blank be measured at excitation ~485 nm; and emission ~665 nm (see page 10).

If more than one tube of SAM Assay Solution is required for experimental analysis, mix all of the SAM Assay Solution tubes together to ensure reagent consistency before use. Thaw only the number of tubes needed for the experiment.

Direct Analysis of a Liquid Sample:

1. Clarify the sample by centrifugation if necessary. Prepare several dilutions of the sample with Buffer S to ensure that the read-out falls within the linear range of the standard curve. Add 10 µl of sample to a polypropylene Eppendorf tube.

2. Add 90 µl of SAM Assay Solution to each polypropylene Eppendorf tube and vortex immediately for ~1 second at medium speed.

3. Carefully transfer 90 µl from each polypropylene Eppendorf tube into the appropriate well of a 96-well black microplate.

4. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~ 25°C) for at least 30 minutes.

5. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

6. **NOTE:** It is possible to use the assay to measure up to 20% v/v of liquid sample to be added directly to the Assay Solution with only a small affect on assay performance. To do this, add 20 µl of the liquid test sample to 80 µl SAM Assay solution. The standard curve including the blank should be prepared as previously described, but an additional 10 µl of Buffer S should be added to the 10 µl of standard in order to bring it to the same 20 µl volume used for the test samples. Add 80 µl of SAM Assay Solution to each well and follow steps 4 and 5 above.

Increase Concentration of SAM in Test Samples

If the SAM level contained in the sample extracts is found to be less than the lower limit of detection for the assay, the SAM in the sample extracts may be concentrated using lyophilization. If the protein content present in such concentrated samples is found to be problematic, ethanol extraction of the sample extract followed by centrifugation and lyophilization of the supernatant (i.e., ethanol containing SAM) is recommended. High salt concentrations affect DNA annealing and should be taken into consideration. It is not recommended that the sample extracts be lyophilized unless they are contained in a volatile solvent such as ethanol. Samples containing very low levels of SAM may be concentrated for use in the assay as follows:

1. Dilute the test samples with cold absolute (100%) ethanol to achieve a final concentration of 70% ethanol. Tightly cap all tubes, vortex and incubate on ice for 15 minutes.

2. Following the 15 minute incubation on ice, centrifuge the sample at ~12,000g for 10 minutes at 4˚C.

3. Carefully transfer the supernatants into a fresh labeled polypropylene Eppendorf tube and lyophilize the supernatant samples (e.g., speed vac dry). Lyophilized samples that are not to be assayed for SAM content immediately should be stored at -20˚C until needed for analysis.

4. It is recommended that tubes containing the 9 assay standards (10 µl per tube) be prepared as described on page 7, lyophilized and stored at -20˚C for assay with the lyophilized test samples.

5. Add 100 µl of SAM Assay Solution to each of the lyophilized standards and test samples. Vortex for ~1 second at medium speed.

6. Transfer 90 µl into the appropriate well of a 96-well black microplate.

7. Cover the microplate with tinfoil to minimize evaporation and exposure to light. Incubate the covered microplate at room temperature (~ 25°C) for at least 30 minutes.

8. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

If the SAM content is too high for measurement using direct dissolution in SAM Assay Solution after ethanol extraction and lyophilization, the samples may be diluted in Buffer S or in water (depending on salt concentration) for direct measurement as described above.

Bridge-It® SAM Fluorescence Assay: Data Analysis

There are several ways of analyzing the fluorescent signal intensity readouts obtained using this assay.

1. Raw fluorescence intensity may be used as a signal proportional to SAM concentration (the x-axis is common log; the y-axis is linear).

2. Relative fluorescence (RF) change may be calculated from the raw fluorescence values. RF values are highly reproducible for the same SAM concentrations and do not depend significantly on the particular microplate reader instrument that is used to read the fluorescence signals.

   RF = Relative Fluorescence
   F_0 = fluorescent intensity of the 0 SAM standard (see page 7)
   F = fluorescence of SAM standard or test sample
   Fb = fluorescence of buffer blank (Buffer S only)
   Fbg = native sample fluorescence

   - Relative fluorescence change (RF) may be calculated from the raw fluorescence values using the formula RF = $(F-F_0)/ F_0$, or alternatively:

   - Buffer and/or standard blanks may be used for the determination of a Buffer or Standard Adjusted Relative Fluorescence (Buffer Adjusted F-Fo/Fo).

   ➢ 90 µl of Buffer S (Fb) is added per each of at least 2 wells per microtitre plate in the experiment. Read for fluorescence at the same time and conditions as the standard curves and samples. The average of the buffer blanks is subtracted from the fluorescence readings (F) of both the standards and samples giving a "buffer adjusted" F-Fo/Fo" or:

   (F-Fb)- (Fo-Fb) / (Fo-Fb).

   ➢ Background fluorescence present in test samples (Fbg) may be accounted for by determining the fluorescence signal of the test sample versus that of Buffer S blank (Fb) (i.e., the fluorescence of test sample in the absence of labeled probes).

   The sample (or sample dilution) should be handled in the same manner as the sample that is actually being assayed. For example, add 10 µl of sample to 90 µl of Buffer S (Fbg), mix, and transfer 90 µl of the solution into the well of a 96-well microplate. Read the fluorescence of the samples compared to the fluorescence readings of the buffer blank (Fb) using a microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm). If Fbg is greater than Fb,

subtract the Fbg to eliminate the background sample fluorescence. The Relative Fluorescence (RF) then becomes

$$RF = [(F-Fbg) – (Fo-Fb)] / Fo-Fb).$$

3. Calculation of the final concentration of SAM in the test sample should incorporate the dilution factor. For example, when 10 µl of the sample is used in the assay and the concentration is found to be 5 µM, the concentration of test sample is 50 µM.

4. A “Sigma Plot” software program was used to analyze and graph the results obtained from the Bridge-It® SAM Fluorescence Assay. Data were converted to RF values and plotted as a sigmoidal, 4-parameter graph (x-axis as common log, y-axis as linear).

Bridge-It® SAM Fluorescence Assay Performance

SAM Standard Curves

The following SAM standard curve was prepared using the Bridge-It® SAM assay. Figure 1 shows the standard curve prepared in Buffer S with the fluorescence signal read after 30 minutes following the addition of SAM Assay Solution into the wells of the 96-well black microplate. The standard errors were from duplicate measurements. Figure 2 shows a portion of the same curve (0.39 to 6.25 µM SAM) at 30 minutes plotted to show the lower range of sensitivity.

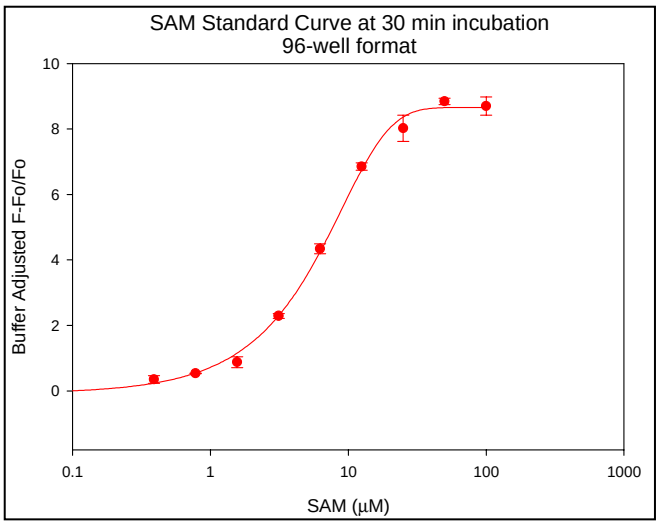


Figure 1

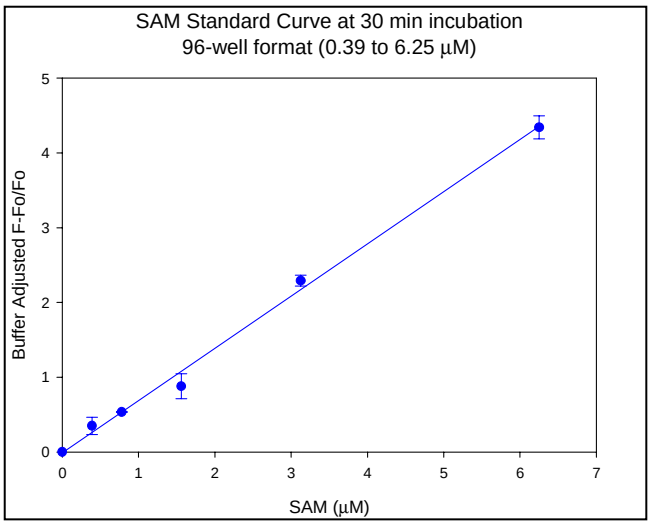


Figure 2

Figure 3 shows a SAM standard curve read at 30 minutes, 1, 2 and 5 hours after the addition of the SAM Assay Solution. Additional information on the data analysis may be found on page 10.

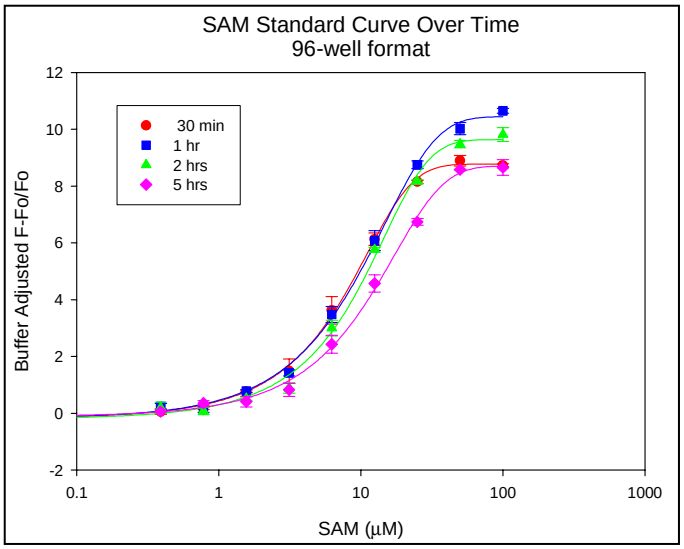


Figure 3

Determination of SAM levels in Biological Samples

Rat Brain Extract:

Rat brain was extracted using the following procedure. Two volumes per gram (v/g) of brain of 50 mM NH_4HCO_3 were used to homogenize the rat brain tissue. After centrifugation at 12,000g for 30 minutes at 4°C, the supernatant brain extract was transferred into another centrifuge tube and diluted with cold absolute (100%) ethanol to a final concentration of 70%. The tube was tightly capped and placed into an ice bath. After 30 minutes of incubation on ice, the ethanol-treated extract was clarified by centrifugation. The supernatant solution was aliquoted into 1 ml samples in polypropylene Eppendorf tubes, lyophilized (speed vac dried) and stored frozen at -20ºC until needed for analysis. An aliquot was later resuspended in 80 µl of cold Buffer S and centrifuged at 12,000 rpm for 15 min at 4ºC. Results of the analysis of the rat brain extract for the presence of SAM are shown on Table 1. The SAM level in the rat brain extract that was prepared and analyzed in our laboratory was determined to be 7.81 nmol SAM per gram of wet weight rat brain tissue.

Yeast Cell Extract:

Following a procedure by Mizunuma et al[5] yeast strain BY4742 was grown in Medium O broth (5% glucose, 1% peptone, 0.5% yeast extract, 0.4% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.75% L-methionine[6]) overnight at 30ºC with gentle agitation. The following day, the culture was diluted to a density of approximately 0.01 OD_{600nm} and again cultured for 48 hours at 30ºC with gentle agitation. The yeast cells were harvested at a density of 15.7 OD_{600nm} by centrifugation at 4ºC. Samples were taken to determine cell dry weight. The yeast cells were washed 2-times with cold double distilled water. After washing, the cell pellet was suspended in a total volume of appropriately $1/50^{th}$ the original culture volume. Using cold 10% perchloric acid, the sample was brought to a final concentration of 0.2% perchloric acid and incubated at room temperature for 1 hour, vortexing occasionally. The sample was centrifuged at 4ºC and the clarified supernatant containing SAM was divided into aliquots and flash frozen on dry ice for storage in a freezer at -20ºC. The following day, a frozen aliquot was thawed and analyzed for SAM using the Bridge-It® SAM fluorescence assay. O Media and media that the yeast was growing in were also analyzed for SAM. The results of this analysis are presented in Table 1. The SAM level in the yeast cells was determined to be 1.40 nmol/gm dry weight. There was no appreciable SAM in either the growth or O Media.

Human Urine:

Samples of urine from two normal adult human subjects were centrifuged and the clarified urine (supernatant) was aliquoted (1 ml/tube) into clean labeled polypropylene Eppendorf tubes and immediately frozen for storage in the freezer at -20ºC until needed for testing. Subsequently the frozen urine samples were thawed, diluted in cold Buffer S, and centrifuged again to remove any precipitate. The clarified supernatant urine was analyzed for SAM content using the Bridge-It® SAM fluorescence assay. Urine dilutions of 1:4 or less were found to have a significant level of background fluorescence. This background fluorescence was taken into account as part of the assay data analysis. The SAM levels

found in two normal human urine specimens using the Bridge-It® SAM fluorescence assay are presented in Table 1.

Human Blood:

Commercially-available, normal, pooled human serum and pooled plasma and also fresh human serum and plasma from a single normal donor were simultaneously analyzed for SAM using the Bridge-It® SAM fluorescence assay. Following dilution in cold Buffer S, the serum and plasma samples were centrifuged to remove any precipitate prior to SAM analysis.

Table 1

SAM Levels Found In Various Types of Test Samples.

Sample	Bridge-It® SAM (µM)
Rat Brain Extract	14.8 ± 2.8
Yeast Cell Extract	900.8 ± 13.3
Human Urine	7.8 ± 0.3
Pooled Human Serum	9.5 ± 2.3
Pooled Human Plasma	8.3 ± 1.7
Fresh Human Serum	7.6 ± 1.6
Fresh Human Plasma	19.2 ± 1.1

Note: The data represents the average value ± standard deviation (n = 2-4)

Specificity of the Bridge-It® SAM Fluorescence Assay

To assess the specificity of the Bridge-It® SAM fluorescence assay, all 20 amino acids were tested up to 100 µM concentration and were shown to have no cross-reactivity with SAM when they were measured using the Bridge-It® SAM fluorescence assay. Further, S-adenosyl homocysteine and homocysteine were tested up to 100 µM and found to have no cross reactivity.

References

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6. Shiozaki, S., Shimzu, S. & Yamada, H. *Agric. Biol. Chem*, **48**, 2293-2300 (1984).

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Product Ordering Information

Bridge-It® SAM Fluorescence Assay Kit:

Product Description[1,2]	No. Measurements	Catalog No.	Price/kit[3]
Bridge-It® SAM Fluorescence Assay Kit	50	1-1-1003A	$164.00
Bridge-It® SAM Fluorescence Assay Kit	96	1-1-1003B	$315.00

96-well black microplate:

Product Description[1]	Catalog No.	Price/microplate[3]
96-well non-binding surface black microplate	163300	$6.25

[1] Bridge-It® is a registered trademark of Mediomics, LLC, St. Louis, Missouri, USA.
[2] Oyster® is a registered trademark of Denovo Biolabels, GmbH, Munster, Germany.
[3] All prices are denominated in U.S. dollars. Shipping and handling cost will be applied. Prices shown may be changed without notice.

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