BellBrook Labs

FAR RED TR-FRET

AptaFluor®

SAH Methyltransferase TR-FRET Assay

Technical Manual

Instructions for Part Numbers 3023-1B, 3023-1K, 3023-10K

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AptaFluor SAH Methyltransferase TR-FRET Assay Technical Manual

Contents

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1.0 Introduction

The AptaFluor SAH Methyltransferase TR-FRET Assay is a universal assay for enzymes that convert *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH), with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. The assay can be used for all enzymes within the histone methyltransferase (HMT) and DNA methyltransferase (DNMT) families (**Figure 1**).

The assay is designed specifically for high-throughput screening (HTS), with a simple mix-and-read format and 2 liquid addition steps. It is run in an endpoint mode: methyltransferase (MT) enzyme reactions are quenched with a universal stop reagent (provided) and then the detection reagents are added. The assay offers reagent stability and compatibility with commonly used multimode plate readers.

The AptaFluor SAH Methyltransferase TR-FRET Assay provides the following benefits:

- Simple, mix-and-read format ideal for HTS.
- Robust MT detection under initial velocity conditions (≤20% conversion of SAM to SAH).
- Accommodates SAM concentrations as low as 100 nM and as high as 5 µM.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

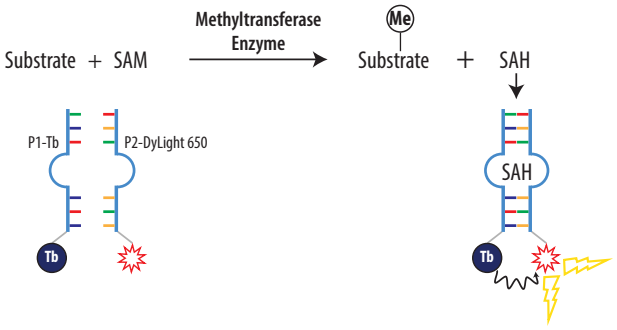

Figure 1. Schematic overview of the AptaFluor SAH Methyltransferase TR-FRET Assay. The detection reagents contain two halves of an SAH-binding RNA aptamer. One half is labeled with a terbium chelate and the other with DyLight® 650. In the presence of SAH, the split aptamer reassembles to generate a TR-FRET signal.

2.0 Product Specifications

Product	Quantity	Part #
AptaFluor SAH Methyltransferase TR-FRET Assay	100 assays*	3023-1B
	1,000 assays*	3023-1K
	10,000 assays*	3023-10K

*The kit contains sufficient reagents for 100, 1,000, or 10,000 wells using concentrations up to 5.0 µM SAM.

Storage

Store reagents at –20°C or –80°C as indicated on individual labels. Reagents are stable for up to 6 months after receipt from BellBrook Labs.

2.1 Materials Provided

Component	Composition	Notes
SAM	500 µM	SAM supplied in the kit should be used for MT enzyme reactions and to create a SAM/SAH standard curve.
SAH	500 µM	SAH is used to create a SAM/SAH standard curve.
Enzyme Stop Reagent, 10X	6% sodium dodecylsulfate (SDS)	Enzyme Stop Reagent is diluted to 1X in SAH Detection Buffer and used to quench the activity of MT enzyme components.
SAH Detection Buffer, 10X	200 mM Tris (pH 8.5), 200 mM $MgCl_2$, 3M NaCl, 0.2 % Brij-35, 10 mM urea	This buffer is used to prepare the SAH Detection Mix and the Enzyme Stop Mix.
P1-Biotin	5 µM	P1-Biotin is a 55-base RNA oligomer with a biotin molecule at the 5' end. It is incubated with Tb-Streptavidin to produce P1-Terbium, the donor piece of the SAH aptamer.
P2-Dylight 650	5 µM	P2-Dylight 650 is an 18-base RNA oligomer with a Dylight 650 molecule at the 3' end. It is the acceptor piece of the SAH aptamer.
Tb-Streptavidin	2 µM	Tb-Streptavidin is used to produce the Tb-labeled (donor) piece of the SAH aptamer.
Conjugation Buffer, 1X	—	This buffer is used for the P1-Biotin/Tb-Streptavidin conjugation reaction.

2.2 Materials Required but Not Provided

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of TR-FRET instruments.

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates, RNA aptamers, and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- ***Methyltransferase Enzyme(s)***—AptaFluor assays are designed for use with purified enzyme preparations. Contaminating enzymes can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds.
- ***Plate Reader***—A microplate reader configured to measure TR-FRET is required for the assay. See Table 1 for information on suitable instruments.
- ***Assay Plates***—It is important to use low-volume assay plates that are entirely white, with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4513) or 96-well plates (Cat. # 3642).
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 0.5 µL into 384-well plates.

Table 1. Instrument filters and settings for commonly used multimode plate readers. A complete list of instruments and instrument-specific application notes can be found at: www.bellbrooklabs.com/technical-resources/instrument-compatibility/

Plate Reader	EXC Filter	EMS Filter	Mirror Module	Other Parameters
Envision (Perkin Elmer)	UV2 320 nm	615 and 665 nm	D400/D630 or D400	Flashes: 100; delay time: 50 µs; window: 200 µs
PHERAstar (BMG Labtech)	337 nm	620 and 665 nm	NA	Integration start/end time: 50 µs; flashes: 50
M1000 (Tecan)	332/320 nm	620 and 665 nm	NA	Integration time: 500 µs; lag time: 60 µs; flashes: 25; flash frequency: 100 Hz
SpectraMax M5/M5e (Molecular Devices)	320 nm	620 and 665 nm	NA	Integration time: 500 µs; delay time: 50 µs; PMT: auto; readings per well: 100

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the TR-FRET instrument and verify that it is compatible with the assay being performed (see **Table 1** in **Section 2.2**).

4.0 Protocol

The AptaFluor™ SAH Methyltransferase TR-FRET Assay protocol consists of 4 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 10 µL enzyme reaction and 20 µL final volume at the time that the plates are read. Increase the volumes to 25 µL enzyme and 50 µL final volume if using 96-well half-volume plates. The use of different plate densities or reaction volumes will require changes in reagent quantities.

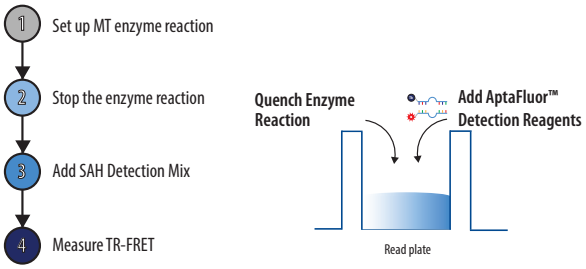

Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Using the optimal instrument settings for TR-FRET is essential to the success of the AptaFluor SAH Methyltransferase Assay. Refer to **Table 1** in **Section 2.2** for filter sets and settings for common multimode plate readers. If you are using a different instrument, verify that it can measure TR-FRET.

4.2 Prepare Reagents

For each step, prepare the reagents for the assay by adding the components in the order and quantity listed.

4.2.1 P1-Terbium

Component	100 Assays (96-Well Format)*	1,000 Assays (384-Well Format)*	10,000 Assays (384-Well Format)*
P1-Biotin	5 µL	20 µL	200 µL
Tb-Streptavidin	4.5 µL	17.8 µL	178 µL
Conjugation Buffer	3.8 µL	15.2 µL	152 µL
Total	**13.3 µL**	**53 µL**	**530 µL**

*96-well assays are performed in a 50 µL final volume; 384-well assays in a 20 µL final volume.

Caution: Do not change the volumes of reagents used in this step. Doing so can produce suboptimal results.

Incubate the P1-Terbium mixture at room temperature (20–25°C) for at least 15 minutes to allow formation of the P1-Biotin/Tb-Streptavidin complex. Store it on ice or at 4°C until required (**Section 4.2.3**).

4.2.2 Enzyme Stop Mix

Component	100 Assays*	1,000 Assays*	10,000 Assays*
Enzyme Stop Reagent, 10X	125 µL	500 µL	5,000 µL
SAH Detection Buffer, 10X	125 µL	500 µL	5,000 µL
Nuclease-free water	1,000 µL	4,000 µL	40,000 µL
Total	**1,250 µL**	**5,000 µL**	**50,000 µL**

*96-well assays are performed in a 50 µL final volume; 384-well assays in a 20 µL final volume.

Store the Enzyme Stop Mix at room temperature (20–25°C) until required (**Section 4.3**, step 2).

4.2.3 SAH Detection Mix

Component	100 Assays*	1,000 Assays*	10,000 Assays*
P1-Terbium	13.3 µL	53 µL	530 µL
P2-Dylight 650	10 µL	40 µL	400 µL
SAH Detection Buffer, 10X	125 µL	500 µL	5,000 µL
Nuclease-free water	1,101.7 µL	4,407 µL	44,070 µL
Total	**1,250 µL**	**5,000 µL**	**50,000 µL**

*96-well assays are performed in a 50 µL final volume; 384-well assays in a 20 µL final volume.

Store the SAH Detection Mix at room temperature for up to 2 hours, or on ice or 4°C for longer periods. Equilibrate it to room temperature (20–25°C) prior to use (**Section 4.3**, step 3).

Caution: Make sure that the enzyme reactions and the SAH Detection Mix are equilibrated to room temperature prior to use.

4.3 Run the Assay

1. Add 10 µL (384-well plate) or 25 µL (96-well plate) of the MT enzyme reaction to each well of a low-volume, white nonbinding plate (e.g., Corning Cat. # 4513). Incubate at the temperature and for the time specific to the enzyme.
2. Add 5 µL (384-well plate) or 12.5 µL (96-well plate) of Enzyme Stop Mix to each well. Incubate at room temperature (20–25°C) for 10 minutes in an orbital shaker. (Refer to **Section 6** for setting up enzyme reactions and standard curves.)
3. Add 5 µL (384-well plate) or 12.5 µL (96-well plate) of SAH Detection Mix to each well. Mix well in an orbital plate shaker. All wells should now contain 20 µL (384-well plate) or 50 µL (96-well plate) of reaction mixture. Cover the microplate with a plate seal, and incubate the plate for 3 hours at room temperature (20–25°C).
4. Remove the plate seal and measure TR-FRET.

5.0 General Considerations

Temperature

The assay is optimal when incubated at room temperature (20–25°C). For consistent results, equilibrate the assay plates and reagents to room temperature before adding the detection reagents. MT enzyme reactions may be run at whatever temperature is optimal for the target, but they should be equilibrated to room temperature after addition of the Enzyme Stop Mix.

Solvents and Other Chemicals

Various chemicals and buffer components were tested for tolerance in the AptaFluor assay. DMSO at 3% or higher in the enzyme reactions interferes with the assay, causing the assay window to drop. EDTA at 2 mM or higher in enzyme reactions also reduces the assay window. Heavy metals, like manganese and zinc, may react with terbium and affect assay performance.

Assay Sensitivity

The lower limit of detection (LLD) is defined as the minimum amount of SAH that generates a Z′ >0. The lower limit of SAH detection in the methyltransferase reaction is 2.5 nM.

Enzyme Substrates

The assay signal is independent of labeled or modified substrates and is based on the generic detection of SAH. Therefore peptides, native histones, nucleosomes, or oligonucleotides can all be used as acceptors of methyl groups.

Enzyme Inhibition and Assay Interference

The Enzyme Stop Mix contains 0.6% SDS, which should cause at least partial denaturation of most enzymes. All of the histone and DNA methyltransferases that we have tested to date have been completely inhibited by the addition of Enzyme Stop Mix.

Dimethylsulfoxide (DMSO) concentrations >3% interfere with the detection reagents and should be avoided. EDTA concentrations ≥2 mM in the MT reaction buffer may interfere with the assay, resulting in a narrower assay window.

Continuous Mode

The assay may be performed in real time by eliminating Enzyme Stop Mix and including the SAH Detection Mix components in the MT reaction. This modification works only when using peptide substrates in the enzyme reaction. Continuous mode should only be used for relative activity comparisons, because the extended signal equilibration time (3 hours) precludes accurate quantitation of SAH. Also, some substrates (e.g., histones) require the Enzyme Stop Mix to stabilize the reaction and prevent interference from the detection reagents.

6.0 Appendix

6.1 Methyltransferase Enzyme Reaction Conditions

The AptaFluor assay was designed for use with buffers and additives commonly used for MT enzymes. Run your enzymatic reaction with optimal buffer and additives at the requisite temperature. The acceptor substrate (e.g., histone or histone-derived peptide) should be present at a concentration similar to or higher than the SAM concentration, to avoid nonlinear kinetics resulting from substrate depletion.

6.2 Determining Optimal Enzyme Concentration

You will need to perform a pilot experiment to determine the optimal enzyme concentration to yield initial velocity conditions (substrate consumption ≤20%) and produce a sufficient assay window. The optimal enzyme concentration should be determined by serial titration, using the same conditions that will be used for screening or profiling. We recommend an enzyme concentration that produces 50–80% of the maximal change in FRET (EC_{50} to EC_{80}; see **Figure 3**).

$$EC_{80} = (80 \div (100 - 80))^{(1 \div \text{hillslope})} \times EC_{50}$$

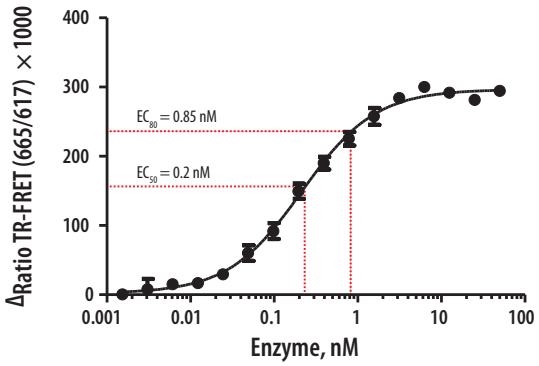

Figure 3. Enzyme titration curve. Based on this curve, the optimal enzyme concentration would be 0.85 nM.

Note: SAM concentration depends upon the K_m of SAM for the enzymes.

1. Prepare serial twofold dilutions of MT enzyme in the optimal buffer for the target, in a volume of 7.5 µL at 1.33X (384-well plate) or 12.5 µL at 2X (96-well plate).
2. Initiate the reactions by adding 2.5 µL at 4X (384-well plate) or 12.5 µL at 2X (96-well plate) of SAM and acceptor substrate. Also set up appropriate controls that lack either SAM or acceptor substrate at this time. Mix the plate, and incubate for the desired amount of time. Make sure that the plate is sealed.

Caution: Make sure that the enzyme reactions and the SAH Detection Mix are equilibrated to room temperature prior to use.

3. Follow the general assay protocol described in **Section 4.3** for preparing all the reagent components.
4. Add 5 µL (384-well plate) or 12.5 µL (96-well plate) of Enzyme Stop Mix to each well. Incubate at room temperature for 10 minutes in an orbital shaker.
5. Add 5 µL (384-well plate) or 12.5 µL (96-well plate) of SAH Detection Mix to each well. Mix well in an orbital plate shaker. All wells should now contain 20 µL (384-well plate) or 50 µL (96-well plate) of reaction mixture. Cover the microplate with a plate seal, and incubate the plate for 3 hours at room temperature (20–25°C).
6. Remove the plate seal and measure TR-FRET.
7. Plot the ratio of 665/617 nm on the Y axis vs. the enzyme concentration on the X axis. Use 4-parameter fit to determine the EC_{80} value as shown in **Figure 3**.

6.3 Determining IC_{50} Values Using the AptaFluor SAH Methyltransferase TR-FRET Assay

The following protocol is an example; the actual volumes used can be adjusted as needed. The volumes provided are intended for a 384-well plate. To perform the assay in a 96-well plate, increase the volumes five-fold.

1. Prepare the assay reagents as described in **Section 4.2**.
2. Prepare two-fold serial dilutions of test compounds in the optimal buffer for the target, for a final concentration of 2X and a final volume of 5 µL per well.
3. Add 2.5 µL of the MT enzyme at 4X to the wells containing the test compounds. Include appropriate controls that lack enzyme. Mix well in an orbital plate shaker for at least 15 minutes at room temperature.
4. Initiate the reaction by adding SAM and acceptor substrate at 4X, in a 2.5 µL volume. All wells should now contain a total volume of 10 µL. Mix well in an orbital plate shaker at the optimal temperature and for the optimal time for the target.
5. Follow the general assay protocol described in **Section 4.3**.
6. Plot the ratio of 665/617 nm on the Y axis vs. the enzyme concentration on the X axis. Use four-parameter fit to determine the IC_{50} value.

6.3.1 Experimental Controls

When screening or profiling inhibitors, two types of controls are needed at a minimum:

- Negative (“no enzyme”) control wells to calculate the basal FRET level due to the reaction components. This control defines the lower limit (lowest FRET ratio) of the assay window.
- Positive (“no inhibitor”) control wells lacking any test compounds to determine the full activity of the MT enzyme being screened. This control defines the upper limit of the assay window.

Z’ values are usually calculated from these two types of controls.

6.4. Setting up SAM/SAH Standard Curves

A standard curve (**Figure 4**) is needed to convert FRET ratios to product formation (SAH) for quantitative data analysis. Whether to use a standard curve is a matter of choice. Many users do not use one for screening purposes, because the criteria for a hit are based on statistical analysis of the raw data. However, if you require quantitative enzyme turnover information for Michaelis-Menten kinetic analysis, you will need to plot a standard curve.

The wells for the standard curve should contain all MT reaction components except the MT enzyme. They should also receive the Enzyme Stop Reagent and SAH Detection Mix (see **Section 4.3**). The curve is constructed to mimic an enzyme reaction. Starting at the SAM concentration used for the MT enzyme reactions, the SAM concentration is decreased incrementally and the SAH concentration is increased proportionally, keeping the sum of their concentrations [SAM + SAH] constant.

We recommend using a 12-point curve with concentrations of SAM and SAH as shown in **Table 2**. Allow reactions to incubate for 3 hours prior to FRET measurement.

1. Prepare 100 nM SAM by adding 1 µL of 500 µM SAM to 4,999 µL of the appropriate buffer.
2. Prepare 100 nM of SAH by adding 1 µL of 500 µM SAH to 4,999 µL of the appropriate buffer.
3. Add proportional quantities of 100 nM SAM and SAH as shown in **Table 2** to generate a 12-point standard curve of 100 nM SAM/SAH.

% Conv.	SAH (nM)	SAM (nM)	SAH (µL)	SAM (µL)
100	100	0	500	0
80	80	20	400	100
60	60	40	300	200
40	40	60	200	300
30	30	70	150	350
20	20	80	100	400
10	10	90	50	450
5	5	95	25	475
2.5	2.5	97.5	12.5	487.5
1	1	99	5	495
0.5	0.5	99.5	2.5	497.5
0.1	0.1	99.9	0.5	499.5
0	0	100	0	500

Table 2. Dilutions of SAM/SAH to prepare a 12-point standard curve.

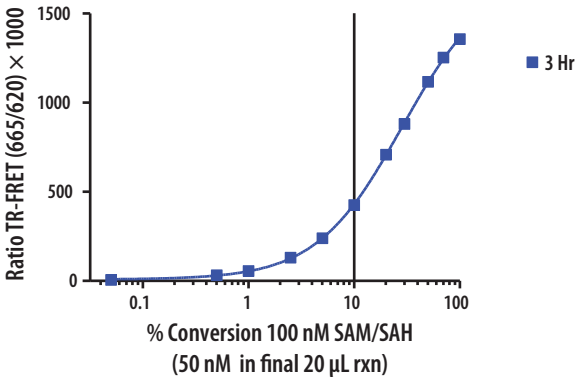

Figure 4. A typical 100 nM SAM/SAH standard curve. Final concentrations were 50 nM in a 20 µL reaction, incubated for 3 hours.

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