MLL1 SAM-Screener™ Assay Kit

Item No. 600580

www.caymanchem.com
Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	384 wells Quantity/Size	1,920 wells Quantity/Size	Storage
600491	SAM-Binding Site Assay Buffer (10X)	1 vial/2 ml	1 vial/10 ml	-20°C
600582	MLL1 (human recombinant) Assay Enzyme	1 vial/550 µl	5 vial/550 µl	-80°C
600493	SAM-Binding Site Probe*	1 vial	5 vials	-20°C
600494	SAM-Binding Site Positive Control	1 vial/100 µg	5 vials/100 µg	-20°C
400093	384-Well Solid Plate (low volume; black)	1 plate	5 plates	RT
400023	Foil Plate Covers	1 cover	5 covers	RT

**United States Patent 9,120,820*

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's MLL1 SAM-Screener™ Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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 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. A plate reader capable of fluorescence polarization measurements using sulforhodamine 101 as the fluorophore (see page 13 for details)
2. Adjustable pipettes and a multichannel pipettor

INTRODUCTION

Background

The alteration of chromatin structure through the posttranslational modification of histones provides a key regulatory step for all processes that act upon DNA. Among the numerous covalent modifications, histone lysine methylation has a central role in the epigenetic regulation of the genome.[1] With the exception of Dot1, all histone lysine methyltransferases contain a conserved domain (SET) that utilizes S-adenosyl-L-methionine (SAM or AdoMet) as a co-factor to catalyze the methylation of the epsilon amino group of lysine.[1] Mixed-lineage leukemia (MLL1) is a member of the trithorax group (trxG)/Set1-like family of gene activators that contains histone methyltransferase activity specific for lysine 4 of histone H3.[2] This methylation plays an important role in gene activation at various developmentally regulated loci, such as the Hox gene loci.[2] Of clinical importance, MLL1 gene rearrangements are linked to a variety of leukemias that have poor prognosis.[3] Human recombinant MLL1 in this assay kit encompasses residues 3,762 – 3,969 and contains the conserved SET methyltransferase domain.

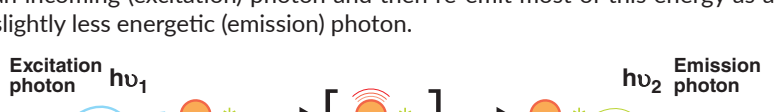
About This Assay

This fluorescence polarization assay is based upon a proprietary small molecule fluorescent probe that binds to the SAM binding pocket in MLL1. Binding of the small molecule probe to MLL1 induces an increase in fluorescence polarization. Binding of the probe can be competed with the endogenous cofactor SAM or by the inhibitor sinefungin, but is unaffected by the histone H3 peptide substrate. The MLL1 SAM-Screener™ Assay is robust (Z' >0.6) and exhibits a greater than 100 mP shift over a range of 0-500 nM MLL1. The assay is suitable for high-throughput screening in the provided 384-well plate or can be scaled to higher density plate formats (*e.g.*, 1,536-well) if desired. The assay is stable at room temperature for several hours and in the presence of less than 2% DMSO.

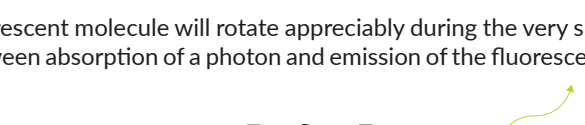
Introduction to FP

Fluorescence polarization (FP) assays are homogeneous, single-step assays ideally suited for high-throughput screening (HTS) of large numbers of samples. All FP assays employ a large molecular species, or binding partner (BP) in conjunction with a small, low molecular weight fluorescent analyte (FA).

Fluorescence is by definition the ability of a molecule to absorb the energy of an incoming (excitation) photon and then re-emit most of this energy as a new, slightly less energetic (emission) photon.



A small fluorescent molecule will rotate appreciably during the very small interval of time between absorption of a photon and emission of the fluorescence photon.



If the excitation light is polarized, rotation of a small molecule freely tumbling in solution will result in complete randomization of the plane of the emitted light. Thus, small fluorescent molecules depolarize an excitation pulse of polarized light (see Figure 1, below).

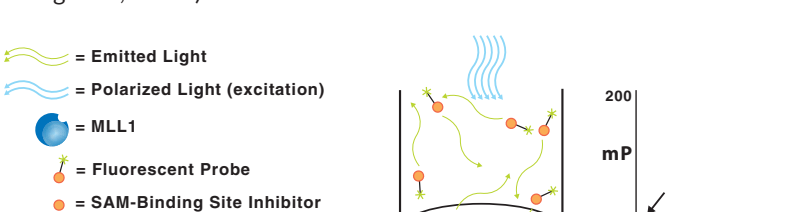


Figure 1. Small fluorescent molecules rotate rapidly and depolarize the excitation light

Large fluorescent molecules (MW >100,000) rotate much more slowly in solution than the small molecules discussed above. These molecules will therefore emit light that retains some of the polarization of the polarized excitation light. This polarization is determined by measuring the parallel and perpendicular fluorescence emitted from an assay sample. The polarization is quantified as milli-polarization units (mP) and can be calculated with the equation below.

$$\mathbf{FP} = 1{,}000 \times \frac{(P - G \times S)}{(P + G \times S)}$$

Where FP: Fluorescence polarization, mP

P: Parallel fluorescence intensity

S: Perpendicular fluorescence intensity

G: An instrument specific correlation factor

When a small fluorescent molecule becomes tightly bound to a molecule of substantially larger mass, as in the binding of the SAM-Binding Site Probe to the MLL1 enzyme, the rotational speed of the small molecule is abruptly reduced to that of the entire complex (see Figure 2, below).

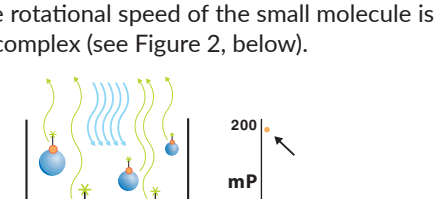


Figure 2. Large fluorescent molecules rotate slowly and emit light that retains some of the polarization of the excitation light

Therefore, when the SAM-Binding Site Probe is bound to MLL1, the probe displays a dramatic increase in FP as compared to the free probe. Cayman's MLL1 SAM-Screener™ Assay is designed to characterize molecules that bind to the SAM binding site of MLL1. Small molecules such as the positive control compound sinefungin compete directly with the SAM-Binding Site Probe for SAM binding on the MLL1 enzyme.

Displacement of the SAM-Binding Site Probe by a small molecule will cause a detectable loss of FP in the assay in a concentration-dependent manner (see Figure 3, below). Plotting mP *versus* competitor molecule allows for the calculation of IC_{50} values.

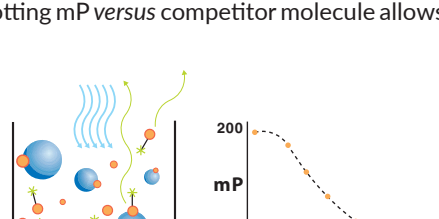


Figure 3. Loss of fluorescent polarization due to displacement of the fluorescent SAM-Binding Site Probe by the competing small molecule

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for FP. UltraPure water may be purchased from Cayman Chemical (Item No. 400000).

Buffer Preparation

2 ml vial SAM-Binding Site Assay Buffer (10X) (384-well kit; Item No. 600491): Add 18 ml of UltraPure water to the vial. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.* Store the diluted buffer at 4°C; it will be stable for approximately one month.

OR

10 ml vial SAM-Binding Site Assay Buffer (10X) (1,920-well kit; Item No. 600491): Dilute to a total volume of 100 ml with UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.* Store the diluted buffer at 4°C; it will be stable for approximately one month.

Sample Preparation

Samples may be prepared in organic solvents such as DMSO, DMF or short chain alcohols (*e.g.*, MeOH, EtOH), as long as the final concentration of organic solvents in the assay is <2%.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

MLL1 (human recombinant) Assay Enzyme

On ice, thaw one tube of MLL1 (human recombinant) Assay Enzyme (Item No. 600582) per 384-well plate and briefly centrifuge the tube before opening. For each 384-well plate, dilute 500 µl of MLL1 to a final volume of 4 ml in 1X SAM-Binding Site Assay Buffer. Mix gently (do not vortex) and keep on ice. Long-term storage of the diluted enzyme is not recommended.

SAM-Binding Site Probe

For each 384-well plate, add 2 ml of 1X SAM-Binding Site Assay Buffer into a vial containing the SAM-Binding Site Probe (Item No. 600493) and vortex briefly. The probe is a dried film on the bottom of the container and may not be visible to the human eye. Keep the solution in the dark to prevent photobleaching. Unused solutions may be stored at -20°C for approximately two weeks.

SAM-Binding Site Positive Control

For each 384-well plate, add 250 µl of 1X SAM-Binding Site Assay Buffer into a tube containing the SAM-Binding Site Positive Control (Item No. 600494) and vortex briefly. Unused solutions may be stored at -20°C for approximately two weeks.

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Do not expose the pipette tip to the reagent(s) already in the well.
- Avoid introducing bubbles into the wells.

Follow the steps below to accurately measure mP in the assay. Allow all reagents except for the MLL1 enzyme to equilibrate to room temperature prior to performing the assay. Keep the MLL1 enzyme on ice until just prior to use. NOTE: Volumes indicated below are for a 384-well plate format with a 20 µl final assay volume. The customer may scale as needed for higher or lower density plate formats.

1. Inhibitor Samples

   Dilute inhibitor samples in 1X SAM-Binding Site Assay Buffer to a concentration that is 4X the desired final concentration (*e.g.*, if 1 µM is desired, prepare a 4 µM solution). This solution may contain up to 8% of an organic solvent (*e.g.*, DMSO). Add 5 µl of this dilution to the desired wells. For best results, perform the assay in duplicate.

   It is recommended that inhibitor compounds be tested in a concentration-response format with at least eight independent concentrations that span approximately a 1,000-fold range around the expected IC_{50} value of the inhibitor.

2. Positive and Negative Control Samples

   For positive (inhibitor control) control wells, add 5 µl of the reconstituted SAM-Binding Site Positive Control to the desired wells.

   For negative (no inhibition) control wells, add 5 µl of 1X SAM-Binding Site Assay Buffer to the desired wells. If inhibitor samples from Step 1 contain organic solvent, add an equivalent amount of the solvent into the assay in this step.

3. MLL1 Assay Enzyme

   Add 10 µl of the diluted MLL1 Assay Enzyme to every well of the 384-well plate.

4. Pre-incubation (optional)

   If desired, incubate the control and sample wells for 15 minutes at room temperature to allow pre-equilibration of the inhibitor and control compounds with the MLL1 enzyme.

5. SAM-Binding Site Probe

   Add 5 µl of the reconstituted SAM-Binding Site Probe to every well.

6. Incubation of the Plate

   Seal the plate with the provided adhesive aluminum seal and incubate at room temperature for 30 minutes. For automation purposes, the plate does not have to be sealed, but it must remain in the dark to prevent photobleaching.

7. Reading the Plate

   Read the plate(s) with excitation and emission wavelengths of 575 nm and 620 nm, respectively. The plate reader used at Cayman Chemical also employs a dichroic filter at 595 nm. Some instruments may not utilize this type of filter. The measurements are taken with the G-factor set to 0.87, however this is instrument specific.

Well Type	SAM-Binding Site Positive Control (µl)	1X Assay Buffer (µl)	Test Sample (µl)	MLL1 (µl)	SAM-Binding Site Probe (µl)
Positive Control	5	-	-	10	5
Negative Control	-	5*	-	10	5
Experimental Samples	-	-	5	10	5

Table 1. Pipetting summary
*If an organic solvent is used at concentrations >2% in the test samples, include it in the negative control wells at the same concentration as the sample wells to control for solvent effects.

Effects of Solvents

Samples may be prepared in organic solvents such as DMSO, DMF, or short chain alcohols (*e.g.*, MeOH, EtOH), as long as the final concentration of organic solvents in the assay is <2%. If conditions require different solvents or higher concentrations, additional assays may be required to assess solvent interference. Simply prepare a 2-fold serial dilution of the solvent in the 1X SAM-Binding Site Assay Buffer. Add reagents to the plate as described above and measure the polarization in the presence of the undiluted solvent and for each of the dilutions. As a control, measure polarization using only 1X SAM-Binding Site Assay Buffer with the SAM-Binding Site Probe.

ANALYSIS

Calculations

The sinefungin positive control wells represent the basal level of polarization for the SAM-Binding Site Probe. Average this value from all replicate wells and subtract it from the test inhibitor and negative control wells to generate baseline-corrected FP values.

A plot of baseline corrected mP versus inhibitor concentration on semi-log axes results in a sigmoidal dose-response curve typical of competitive binding assays. This data can be fit to a 4-parameter logistic equation.

Performance Characteristics

Z'-Factor:

Z'-factor is a term used to describe the robustness of an assay,[4] which is calculated using the equation below.

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
µ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z´-factor is 1.0. A robust assay has a Z´-factor >0.5. The Z´-factor for Cayman's MLL1 SAM-Screener™ Assay was determined to be 0.71.

Sample Data

The data shown below and on page 17, are examples of the data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

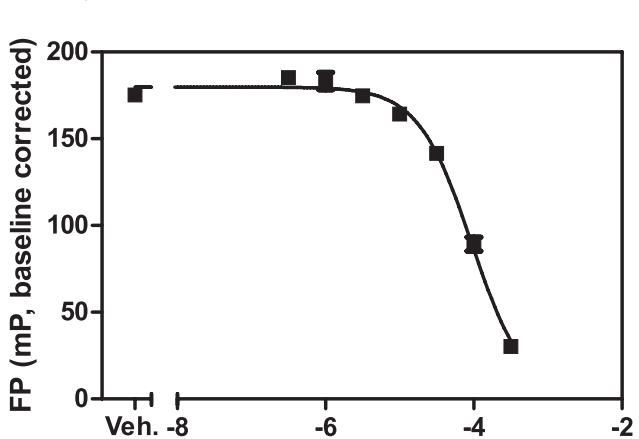


Figure 4. Typical inhibition curves for the displacement of the SAM-Binding Site Probe from MLL1 by sinefungin. Data are shown as the mean±SEM.

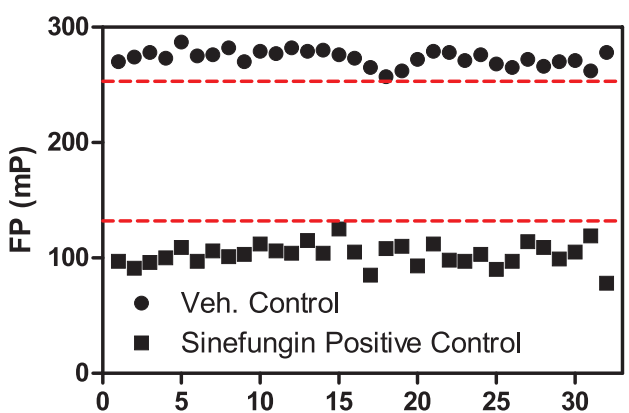


Figure 5. Typical Z' data for the MLL1 SAM-Screener™ Assay. Data are shown from 64 replicate wells of both positive and negative controls were prepared as described in the kit booklet. The calculated Z' from this experiment was 0.71. The red lines correspond to three standard deviations from the mean for each control value.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Bubble in the well B. Poor pipetting/ technique	A. Centrifuge the plate briefly
Little increase in mP in negative control wells	A. Fluorescent molecules in sample matrix B. MLL1 enzyme handled improperly	A. Test sample matrix for interference before running samples in the assay B. Keep the protein frozen at -80°C until ready to use; thaw protein and keep on ice until adding to assay

References

1. Bannister, A.J. and Kouzarides, T. Regulation of chromatin by histone modifications. *Cell Res.* **21**, 381-395 (2011).
2. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* **10**, 1107-17 (2002).
3. Dou, Y., and Hess, J. L. Mechanisms of transcriptional regulation by MLL and its disruption in acute leukemia. *Int J Hematol* **87**, 10-8 (2008).
4. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4(2)**, 67-73 (1999).

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.