L3MBTL1 MBT Domains TR-FRET Assay Kit

Item No. 601030

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Customer Service 800.364.9897
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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	384 wells Quantity/Size	1,920 wells Quantity/Size	9,600 wells Quantity/Size	Storage
601031	L3MBTL1 MBT Domains Europium Chelate	1 vial/ 420 wells	5 vials/ 420 wells	5 vials/ 2,100 wells	-80°C
601032	L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture	1 vial/ 420 wells	5 vials/ 420 wells	5 vials/ 2,100 wells	-80°C
600508	TR-FRET Assay Buffer 2 (10X)	1 vial/2 ml	1 vial/10 ml	5 vials/10 ml	-20°C
601033	L3MBTL1 Positive Control	1 vial/ 160 nmol	1 vial/ 800 nmol	5 vials/ 800 nmol	-20°C
400093	384-Well Solid Plate (low volume; black)	1 plate	5 plates	25 plates	RT
400023	Foil Plate Covers	1 cover	5 covers	25 covers	RT

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 L3MBTL1 MBT Domains TR-FRET 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:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 time-resolved FRET with an excitation at 340 nm and emission at 620 and 670 nm
2. Adjustable pipettes and a multichannel pipettor
3. DMSO

INTRODUCTION

Background

The post-translational modification (PTM) of chromatin plays a crucial role in the regulation of gene transcription, DNA repair and many other cellular processes. While a broad array of chromatin PTMs have been identified, only a few classes have been extensively studied, including methylation on histone lysine/arginine residues, acetylation on histone lysine residues, and CpG methylation of the DNA.[1-3] Collectively, the combinatorial array of chromatin PTMs forms the foundation of the "Histone Code".[4] This code is interpreted by a large family of protein motifs that have been colloquially dubbed chromatin "reader" domains. These small motifs have evolved to selectively recognize and bind to a particular chromatin PTM. Commonly found in transcription factors and other regulatory proteins, reader domains localize their proteins to chromatin bearing the appropriate PTM(s). Additionally, a given protein will often have multiple reader domains located adjacent to each other, potentially providing additional levels of specificity to the system.

Since the discovery of small molecule bromodomain inhibitors, there has been intense interest in the pharmacological targeting of chromatin reader domains to for oncology, inflammation, and infectious disease indications.[5,6] Unlike many protein-protein interaction domains, chromatin readers appear to be particularly tractable for drug development.[7] Cayman's TR-FRET assays for chromatin readers are designed to assist in the early-stage discovery and development of chromatin reader domain inhibitors.

About This Assay

L3MBTL1 is a member of the polycomb group of transcriptional regulators.[8] Working in conjunction with other polycomb-associated proteins, L3MBTL1 induces chromatin compaction and elicits transcriptional repression.[9,10] L3MBTL1 contains three malignant brain tumor (MBT) domains which selectively recognizes and binds to the histone marks monomethyl H3K4 and dimethyl H4K20.[9] Physiologically, L3MBTL1 functions as a tumor suppressor and plays a key role in embryonic development.[10] This assay is designed to characterize inhibitors of the L3MBTL1 MBT domains.

Cayman's L3MBTL1 MBT Domains TR-FRET Assay Kit is a homogeneous, Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay method amenable to rapid characterization of MBT domain inhibitors in a high-throughput format. The 'donor' fluorophore in this assay consists of a protein containing the three MBT domains of L3MBTL1 (human amino acids 191-530) directly labeled with a europium (Eu^{3+}) chelate. A biotinylated peptide containing target a methylated lysine residue serves as the ligand for the L3MBTL1 MBT domains. Allophycocyanin (APC) labeled avidin binds with high affinity to the peptide substrate *via* the biotin moiety and serves as the 'acceptor' fluorophore in the assay. Inhibition of the MBT domain/peptide interaction displaces L3MBTL1-Eu^{3+} from the APC/avidin resulting in a loss of TR-FRET signal. The L3MBTL1 MBT Domains TR-FRET Assay Kit is robust (Z´ >0.6), and 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 plate) if desired. The assay is stable at room temperature for at least four hours and in the presence of less than 1% DMSO.

Introduction to TR-FRET

TR-FRET is based upon the principles of FRET, but possesses a number of advantages that make it a superior technology for high-throughput screening. When an optically active molecule absorbs a photon it has several options by which it may release that energy: it may release a photon of a longer wavelength (less energy) than the photon it absorbed, it may dissipate the energy as heat, or it can transfer the energy non-radiometrically to a suitable acceptor fluorophore. The latter effect is known as FRET and is a commonly used phenomenon in biological assays. In these assays, a donor flurophore is coupled to one binding partner and an acceptor fluorophore is coupled to the other binding partner. The binding partners are mixed in an assay well and allowed to associate. The donor fluorophore is then excited with a wavelength of light that does not excite the acceptor fluorophore and if the molecules are within approximately 100 Å of each other, the donor fluorophore can non-radiometrically transfer the energy to the acceptor fluorophore, which will then release that photon as light with at a wavelength characteristic of the acceptor fluorophore (see Figure 1 on page 9). For each assay point, the fluorescence intensity of the donor fluorophore and the acceptor fluorophore are measured and the data are generally presented as the ratio of acceptor fluorophore intensity/donor fluorophore intensity. This methodology is particularly sensitive because the FRET efficiency decays as the 6^{th} power of the distance between the two fluorophores. Therefore, unassociated binding partners are unlikely to lie within the distance required for efficient FRET.

TR-FRET is an extension of FRET that utilizes a donor fluorophore with a long fluorescent half-life. These fluorophores are based upon lanthanide (most often Eu^{3+} or Tb^{3+}) chelates that have characteristically large stokes shifts and fluorescent half-lives on the order of milliseconds. The long fluorescent lifetime allows the TR-FRET signal to be sustained for dramatically longer periods of time than standard fluorescence. This is particularly advantageous because it affords the ability to measure the TR-FRET signal after background fluorescence in the assay (*e.g.*, buffer/reagent autofluorescence) has dissipated (see Figure 2 on page 10). The increased signal:noise ratio and the diminished effects of screening compound fluorescence makes TR-FRET assays particularly useful for high-throughput screening applications.

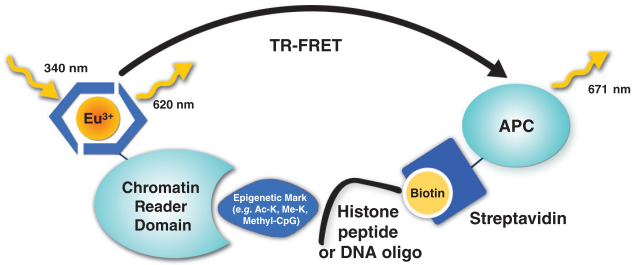

Figure 1. Assay schematic for Chromatin Reader TR-FRET Assay Kits. Upon excitation, the europium chelate can release a photon or transfer its energy to an APC molecule, provided the APC is in close proximity to the europium fluorophore.

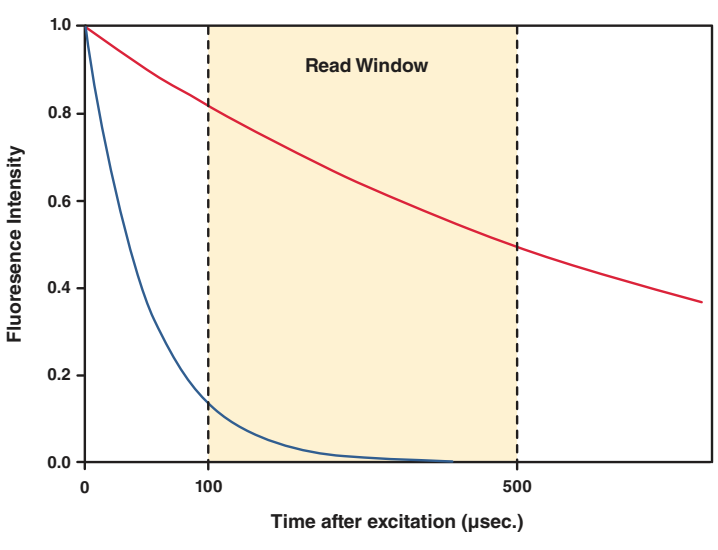

Figure 2. The extended fluorescence lifetimes of Eu^{3+}-based fluorophores allows the samples to be analyzed after background fluorescence has decayed, improving signal to noise and reducing spectral artifacts.

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). UltraPure water may be purchased from Cayman Chemical (Item No. 400000).

Buffer Preparation

2 ml vial TR-FRET Assay Buffer 2 (10X) (384-well kit; Item No. 600508): Add 18 ml of UltraPure water to the vial. For best results, filter completed 1X Assay Buffer with a 0.22 µm filter before use. *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 TR-FRET Assay Buffer 2 (10X) (1,920- or 9,600-well kit; Item No. 600508): For five 384-well plates, dilute 10 ml TR-FRET Assay Buffer to a total volume of 100 ml with UltraPure water. For best results, filter completed 1X Assay Buffer with a 0.22 µm filter before use. *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

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in 1X TR-FRET Assay Buffer 2 at a concentration 4X the desired final assay concentration (*e.g.*, for 1 µM final assay concentration, a 4 µM dilution should be made). This solution may contain up to 4% organic solvents such as DMSO, DMF, or short chain alcohols. The final concentration of organic solvents in the assay will then be <1%. Avoid using high concentrations of metal chelating agents or phosphate buffers.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

L3MBTL1 MBT Domains Europium Chelate (Item No. 601031)

420 well vial L3MBTL1 MBT Domains Europium Chelate (384- or 1,920-well kit):

On ice, thaw one tube of L3MBTL1 MBT Domains Europium Chelate (420 well, Item No. 601031) per 384-well plate and briefly centrifuge tube before opening. Dilute contents to a final volume of 4.2 ml in 1X TR-FRET Assay Buffer 2. Mix gently (do not vortex) and keep on ice. Long-term storage of the diluted protein is not recommended.

OR

2,100 well vial L3MBTL1 MBT Domains Europium Chelate (9,600-well kit):

On ice, thaw one tube of L3MBTL1 MBT Domains Europium Chelate (2,100 well, Item No. 601031) per five 384-well plates and briefly centrifuge tube before opening. Dilute contents to a final volume of 21 ml in 1X TR-FRET Assay Buffer 2. Mix gently (do not vortex) and keep on ice. Long-term storage of the diluted protein is not recommended.

L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture (Item No. 601032)

420 well vial L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture (384- or 1,920-well kit):

For each 384-well plate, add 2.1 ml of 1X TR-FRET Assay Buffer 2 to one vial of the L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture (420 well, Item No. 601032) and gently vortex. Keep the solution in the dark to prevent photobleaching. Long-term storage of the diluted mixture is not recommended.

OR

2,100 well vial L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture (9,600-well kit):

For five 384-well plates, add 3 ml of 1X TR-FRET Assay Buffer 2 to one vial of the L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture (2,100 well, Item No. 601032) and gently vortex. Transfer contents to a new tube and adjust the mixture to a final volume of 10.5 ml in 1X Assay Buffer. Keep the solution in the dark to prevent photobleaching. Long-term storage of the diluted mixture is not recommended. Long-term storage of the diluted mixture is not recommended.

L3MBTL1 MBT Domains Positive Control (Item No. 601033)

160 nmol vial L3MBTL1 MBT Domains Positive Control (384- or 1,920-well kit):

For each 384 well plate, add 200 µl of 1X TR-FRET Assay Buffer 2 to one tube containing the L3MBTL1 MBT Domains Positive Control (160 nmol, Item No. 601033) and vortex gently. Unused solutions may be stored at -20°C for approximately two weeks.

OR

800 nmol vial L3MBTL1 MBT Domains Positive Control (9,600-well kit):

For five 384 well plates, add 1 ml of 1X TR-FRET Assay Buffer 2 to one tube containing the L3MBTL1 MBT Domains Positive Control (800 nmol, Item No. 601033) and vortex gently. 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 the TR-FRET ratio in the assay. Allow all reagents except for the L3MBTL1 MBT Domains Europium Chelate to equilibrate to room temperature prior to performing the assay. Keep the L3MBTL1 MBT Domains Europium Chelate 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 TR-FRET Assay Buffer 2 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 4% 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 L3MBTL1 MBT Domains Positive Control to the desired wells. This will provide a final assay concentration of 200 µM.

For negative (no inhibition) control wells, add 5 µl of 1X TR-FRET Assay Buffer 2 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. L3MBTL1 MBT Domains Europium Chelate

Add 10 µl of the diluted L3MBTL1 MBT Domains Europium Chelate 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 L3MBTL1 MBT Domains Europium Chelate. *Protect from light*.

5. L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture

Add 5 µl of the reconstituted L3MBTL1 MBT Domains Ligand/APC Acceptor Mixture to every well.

6. Incubation of the Plate

Seal the plate with an adhesive aluminum seal and incubate at room temperature for one hour. For automation purposes, the plate does not have to be sealed, but it should remain in the dark to prevent photobleaching.

7. Reading the Plate

Read the plate(s) in time-resolved format by exciting the sample at 340 nm and reading emissions at 620 and 670 nm, using a 100 µs delay and a 500 µs read window. The plate reader used at Cayman Chemical employs a 340/40 nm excitation filter, 620/15 nm and 670/20 nm emission filters. Samples will be stable for analysis for at least five hours if stored at room temperature and protected from light. Data analysis is performed using the TR-FRET ratio (670 nm emission/620 nm emission).

Well Type	L3MBTL1 MBT Domains Positive Control (µl)	1X TR-FRET Assay Buffer 2 (µl)	Test Sample (µl)	L3MBTL1 MBT Domains Europium Chelate (µl)	L3MBTL1 MBT Domains Ligand/ APC Acceptor Mixture (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 >1% 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 <1%. High concentrations of metal chelating agents or phosphate buffers may interfere with the fluorescence of the donor fluorophore and should be avoided. If conditions require different solvents or higher concentrations, additional assays may be required to assess solvent interference.

ANALYSIS

Calculations

A plot of the TR-FRET ratio (670 nm emission/620 nm emission) *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 as shown in Figure 3 to calculate IC_{50} values.

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay,[11] 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 L3MBTL1 MBT Domains TR-FRET Assay Kit was determined to be 0.66.

Sample Data

The data shown here is an example 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.

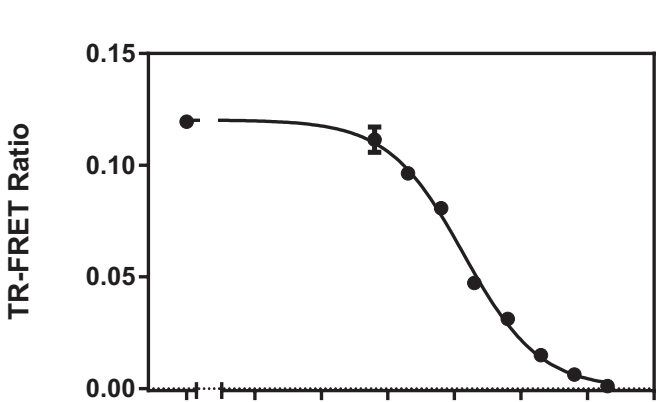

Figure 3. Typical inhibition curves for the displacement of the methylated peptide from L3MBTL1 MBT Domains by the L3MBTL1 MBT Domains Positive Control. "Veh." represents compound vehicle control.

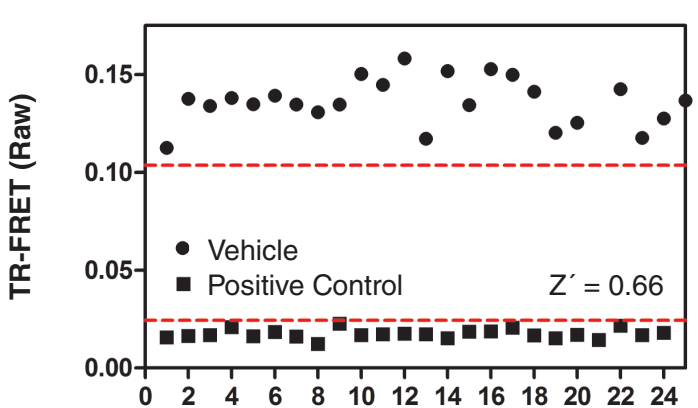

Figure 4. Typical Z´ data for the L3MBTL1 MBT Domains TR-FRET Assay Kit. Data are shown from 24 replicate wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z´ from this experiment was 0.66. The red lines correspond to three standard deviations from 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
Low fluorescence signal	A. Incompatible sample matrix B. L3MBTL1 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 C. Perform the assay using a filter-based instrument

References

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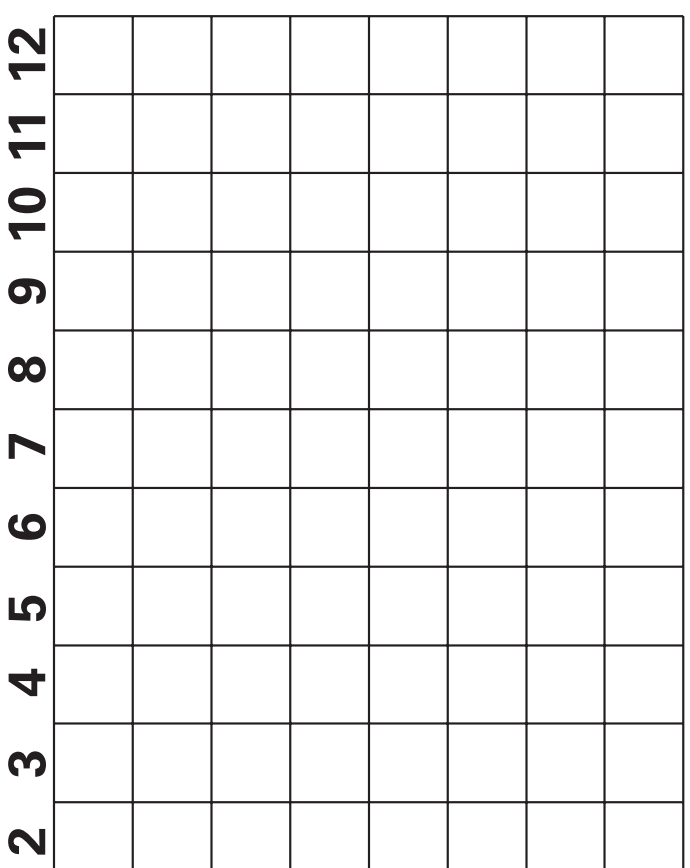

NOTES

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.