



## JMJD2A Tudor Domains TR-FRET Assay Kit

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Item No. 700960

[www.caymanchem.com](http://www.caymanchem.com)

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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 |
|-------------|--|-------------------------|---------------------------|---------------------------|---------|
| 700961      | JMJD2A Tudor Domains Europium Chelate            | 1 vial/<br>420 wells    | 5 vials/<br>420 wells     | 5 vials/<br>2,100 wells   | -80°C   |
| 700962      | JMJD2A Tudor Domains Ligand/APC Acceptor Mixture | 1 vial/<br>420 wells    | 5 vials/<br>420 wells     | 5 vials/<br>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   |
| 700965      | JMJD2A Tudor Domains Positive Control            | 1 vial/<br>2.5 nmol     | 1 vial/<br>12.5 nmol      | 5 vials/<br>12.5 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 JMJD2A Tudor 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

## 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.<sup>1-3</sup> Collectively, the combinatorial array of chromatin PTMs forms the foundation of the “Histone Code”.<sup>4</sup> 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.<sup>5,6</sup> Unlike many protein-protein interaction domains, chromatin readers appear to be particularly tractable for drug development.<sup>7</sup> 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

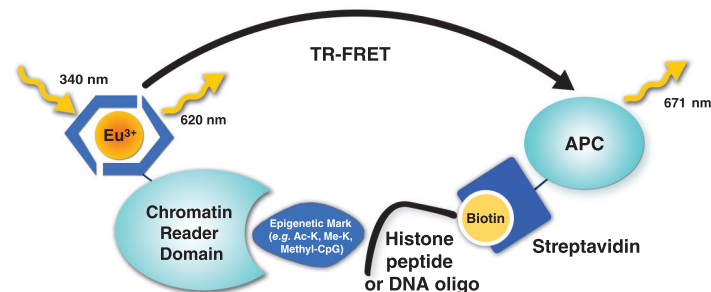
JMJD2A is a member of the JMJD2 class of histone demethylases. Members of this family contain the histone demethylase domains JmjN and JmjC as well as two PHD fingers and two tudor domains.<sup>8</sup> JMJD2A forms a complex with Class I histone deacetylases and retinoblastoma protein (pRb) and together this complex elicits transcriptional repression.<sup>8-10</sup> These complexes are selectively recruited to chromatin bearing methylated H3K4 *via* interactions mediated by the Tudor domains of JMJD2A.<sup>8</sup> JmjC-domain containing proteins are attractive drug targets due to their potential role in several disease states, including androgen-dependent prostate cancer, obesity, and X-linked mental retardation.<sup>11-13</sup> This assay kit designed to characterize inhibitors of the JMJD2A tudor domains.

Cayman’s JMJD2A Tudor Domains TR-FRET Assay Kit is a homogeneous, Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay method amenable to rapid characterization of tudor domain inhibitors in a high-throughput format. The ‘donor’ fluorophore in this assay consists of JMJD2A tudor domains (human amino acids 888-1023) directly labeled with a europium (Eu<sup>3+</sup>) chelate. A biotinylated peptide containing target a methylated lysine residue serves as the ligand for JMJD2A tudor 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 tudor domain/peptide interaction displaces tudor-Eu<sup>3+</sup> from the APC/avidin resulting in a loss of TR-FRET signal. The JMJD2A Tudor 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 fluorophore 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 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<sup>th</sup> 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  $\text{Eu}^{3+}$  or  $\text{Tb}^{3+}$ ) 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.

## 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  $\mu\text{m}$  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 2 to a total volume of 100 ml with UltraPure water. For best results, filter completed 1X Assay Buffer with a 0.22  $\mu\text{m}$  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  $\mu\text{M}$  final assay concentration, a 4  $\mu\text{M}$  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.

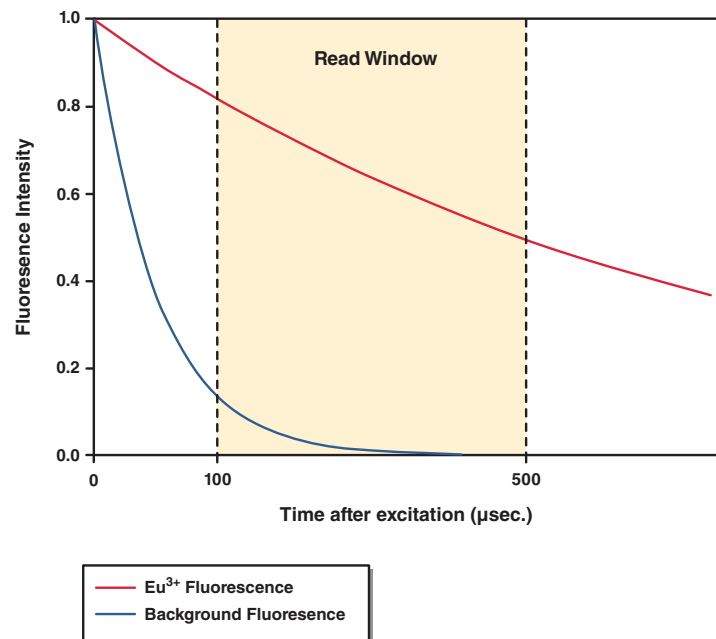


Figure 2. The extended fluorescence lifetimes of  $\text{Eu}^{3+}$ -based fluorophores allows the samples to be analyzed after background fluorescence has decayed, improving signal to noise and reducing spectral artifacts.

## Preparation of Assay-Specific Reagents

### JMJD2A Tudor Domains Europium Chelate (Item No. 700961)

#### 420 well vial JMJD2A Tudor Domains Europium Chelate (384- or 1,920-well kit):

On ice, thaw one tube of JMJD2A Tudor Domains Europium Chelate (420 well, Item No. 700961) 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 JMJD2A Tudor Domains Europium Chelate (9,600-well kit):

On ice, thaw one tube of JMJD2A Tudor Domains Europium Chelate (2,100 well, Item No. 700961) 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.

### JMJD2A Tudor Domains Ligand/APC Acceptor Mixture (Item No. 700962)

#### 420 well vial JMJD2A Tudor 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 JMJD2A Tudor Domains Ligand/APC Acceptor Mixture (420 well, Item No. 700962) 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 JMJD2A Tudor 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 JMJD2A Tudor Domains Ligand/APC Acceptor Mixture (2,100 well, Item No. 700962) 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.

### JMJD2A Tudor Domains Positive Control (Item No. 700965)

#### 2.5 nmol vial JMJD2A Tudor Domains Positive Control (384- or 1,920-well kit):

For each 384 well plate, add 200  $\mu$ l of 1X TR-FRET Assay Buffer 2 to one tube containing the JMJD2A Tudor Domains Positive Control (2.5 nmol, Item No. 700965) and vortex gently. Unused solutions may be stored at -20°C for approximately two weeks.

OR

#### 12.5 nmol vial JMJD2A Tudor 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 JMJD2A Tudor Domains Positive Control (12.5 nmol, Item No. 700965) 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 JMJD2A Tudor Domains Europium Chelate to equilibrate to room temperature prior to performing the assay. Keep the JMJD2A Tudor Domains Europium Chelate on ice until just prior to use. NOTE: Volumes indicated below are for a 384-well plate format with a 20  $\mu$ l 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  $\mu$ M is desired, prepare a 4  $\mu$ M solution). This solution may contain up to 4% of an organic solvent (e.g., DMSO). Add 5  $\mu$ l 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<sub>50</sub> value of the inhibitor.

### 2. Positive and Negative Control Samples

For positive (inhibitor control) control wells, add 5  $\mu$ l of JMJD2A Tudor Domains Positive Control to the desired wells. This will provide a final assay concentration of 3 $\mu$ M.

For negative (no inhibition) control wells, add 5  $\mu$ l 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  $\mu$ l of the diluted JMJD2A Tudor 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 JMJD2A Tudor Domains Europium Chelate. *Protect from light.*

### 5. JMJD2A Tudor Domains Ligand/APC Acceptor Mixture

Add 5  $\mu$ l of the reconstituted JMJD2A Tudor 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  $\mu$ s delay and a 500  $\mu$ s 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            | JMJD2A Tudor Domains Positive Control (μl) | 1X TR-FRET Assay Buffer 2 (μl) | Test Sample (μl) | JMJD2A Tudor Domains Europium Chelate (μl) | JMJD2A Tudor 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<sub>50</sub> values.

### Performance Characteristics

#### Z' Factor:

Z' factor is a term used to describe the robustness of an assay,<sup>14</sup> 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 JMJD2A Tudor Domains TR-FRET Assay Kit was determined to be 0.87.

#### 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.

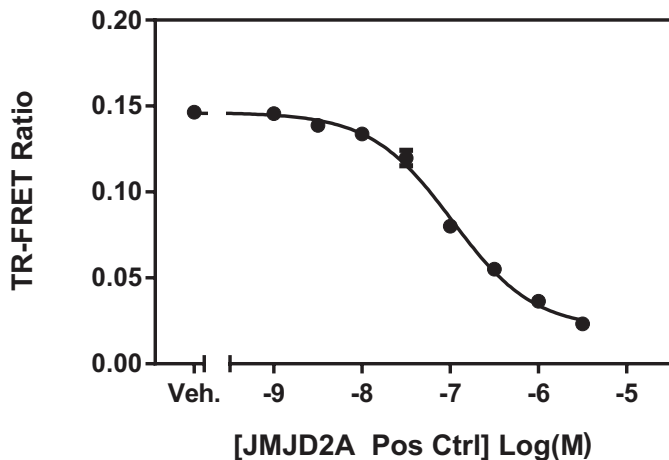


Figure 3. Typical inhibition curves for the displacement of the methylated peptide from JMJD2A Tudor Domains by the JMJD2A Tudor Domains Positive Control. "Veh." represents compound vehicle control.

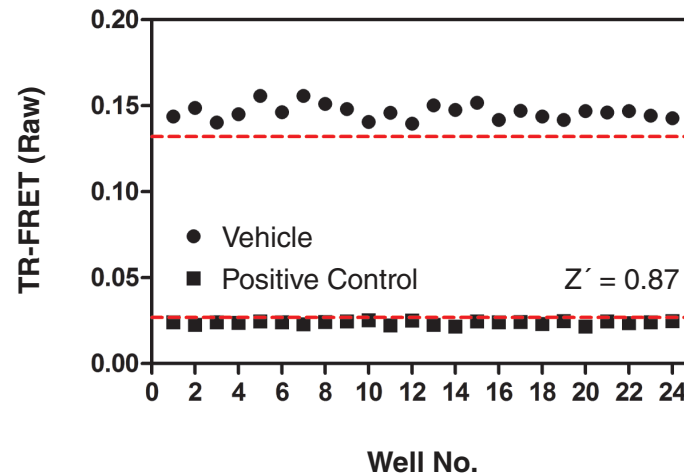


Figure 4. Typical Z' data for the JMJD2A Tudor Domains TR-FRET Assay Kit. Data are shown from 24 replicate wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z' from this experiment was 0.87. The red lines correspond to three standard deviations from the mean for each control value.

## Troubleshooting

| Problem                                  | Possible Causes   | Recommended Solutions  |
|--|---|--|
| Erratic values; dispersion of duplicates | A. Bubble in the well<br>B. Poor pipetting/technique          | A. Centrifuge the plate briefly  |
| Low fluorescence signal                  | A. Incompatible sample matrix<br>B. JMJD2A handled improperly | A. Test sample matrix for interference before running samples in the assay<br>B. Keep the protein frozen at -80°C until ready to use; thaw protein and keep on ice until adding to assay<br>C. Perform the assay using a filtered based instrument |

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|    | A | B | C | D | E | F | G | H |

## 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.

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