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## BRD4 bromodomain 1 TR-FRET Assay Kit

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Item No. 600520

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

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## TABLE OF CONTENTS

<b>GENERAL INFORMATION</b>	3	Materials Supplied
	4	Safety Data
	4	Precautions
	5	If You Have Problems
	5	Storage and Stability
	5	Materials Needed but Not Supplied
<b>INTRODUCTION</b>	6	Background
	7	About This Assay
	8	Introduction to TR-FRET
<b>PRE-ASSAY PREPARATION</b>	11	Buffer Preparation
	11	Sample Preparation
<b>ASSAY PROTOCOL</b>	12	Preparation of Assay-Specific Reagents
	14	Performing the Assay
	16	Effects of Solvent
<b>ANALYSIS</b>	17	Calculations
	17	Performance Characteristics
<b>RESOURCES</b>	20	Troubleshooting
	21	References
	23	Notes
	23	Warranty and Limitation of Remedy

## GENERAL INFORMATION

### Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	384 wells Quantity/ Size	1,920 wells Quantity/ Size	9,600 wells Quantity/ Size	Storage
600523	BRD4 bromodomain 1 Europium Chelate	1 vial/ 420 wells	5 vials/ 420 wells	5 vials/ 2,100 wells	-80°C
600524	BRD4 bromodomain 1 Ligand/APC Acceptor Mixture	1 vial/ 420 wells	5 vials/ 420 wells	5 vials/ 2,100 wells	-80°C
600503	TR-FRET Assay Buffer (10X)	1 vial/2 ml	1 vial/10 ml	5 vials/10 ml	-20°C
600504	TR-FRET Assay Buffer Additive	1 vial/200 mg	1 vial/1 g	5 vials/1 g	-20°C
600505	(+)-JQ1 Positive Control*	1 vial/8 nmol	5 vials/8 nmol	5 vials/40 nmol	-80°C
600854	384-Well Solid Plate (low volume; white)	1 plate	5 plates	25 plates	RT
400023	Foil Plate Covers	1 ea	5 ea	25 ea	RT

\*Manufactured, marketed, and sold with authorization from Tensha Therapeutics, Inc. Patent Pending relating to PCT Publ. No. WO/2011/143669, and any related U.S. and foreign patents and patent applications.

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 BRD4 bromodomain 1 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

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 if stored as directed 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 665 nm
2. Adjustable pipettes and a multichannel pipettor
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. DMSO

## Background

The acetylation of histone lysine residues plays a crucial role in the epigenetic regulation of gene transcription.<sup>1</sup> Acetylated lysine residues are recognized by a small protein domain known as a bromodomain. These domains recruit regulatory complexes to acetylated nucleosomes, thereby controlling chromatin structure and gene expression.<sup>2</sup> The BET family of proteins, a class of proteins that contain tandem bromodomains and an extra terminal domain, include BRD2, BRD3, BRD4, and BRDT. These proteins play a key role in many cellular processes, including inflammatory gene expression, mitosis, and viral-host interactions.<sup>3-5</sup> The isolated individual or tandem bromodomains of BRD2 and BRD4 have been shown to bind acetylated histone tails, serving to couple histone acetylation marks to the transcriptional regulation of target promoters.<sup>4,6-9</sup>

Small molecule inhibitors of bromodomain-histone interactions, exemplified by I-BET and JQ1, hold promise as useful therapeutics for human disease.<sup>10-12</sup> I-BET binds to the bromodomains of BET proteins with high-affinity ( $K_d = 32.5-42.5$  nM) and disrupts the formation of chromatin complexes involved in the expression of specific inflammatory genes in activated macrophages.<sup>13</sup> Through these actions, I-BET provides protection against bacteria-induced sepsis and lipopolysaccharide-triggered endotoxic shock.<sup>13</sup> Enantiomerically pure (+)-JQ1 binds to BRD4 bromodomain 1 and BRD4 bromodomain 2 with  $K_d$  values of ~50 and 90 nM, respectively, whereas the (-)-JQ1 stereoisomer has no appreciable affinity to BET bromodomains.<sup>10</sup> In a bromodomain-peptide displacement assay, ( $\pm$ )-JQ1 inhibits BRD2 and BRD4 binding with  $IC_{50}$  values of 18 and 77 nM, respectively.<sup>14</sup> (+)-JQ1 has been used as a chemical probe to investigate the role of BET bromodomains in the transcriptional regulation of oncogenesis.<sup>10,15-17</sup>

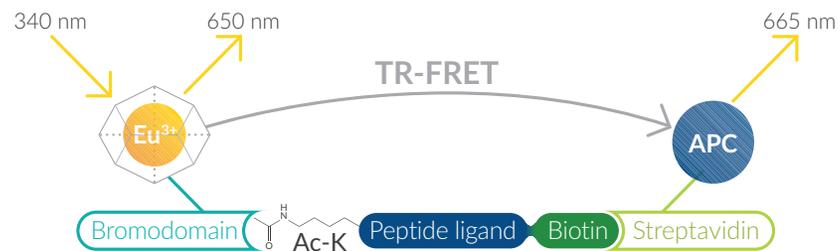
## About This Assay

Cayman's BRD4 bromodomain 1 TR-FRET Assay Kit is a homogeneous, time-resolved Förster resonance energy transfer (TR-FRET) assay method amenable to rapid characterization of inhibitors of the bromodomain-acetylated peptide interaction in a high-throughput format. The donor fluorophore in this assay consists of BRD4 bromodomain 1 (human amino acids 49-170) directly labeled with a europium ( $\text{Eu}^{3+}$ ) chelate. A biotinylated peptide containing acetylated lysines (BRD-Eu<sup>3+</sup>) serves as the ligand for BRD4 bromodomain 1. 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 BRD-peptide interaction displaces BRD-Eu<sup>3+</sup> from the APC-avidin conjugate resulting in a loss of TR-FRET signal. The BRD4 bromodomain 1 TR-FRET Assay Kit is robust ( $Z' = >0.85$ ) and 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 2% 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 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<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 the bromodomain TR-FRET Assay Kit.** 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.

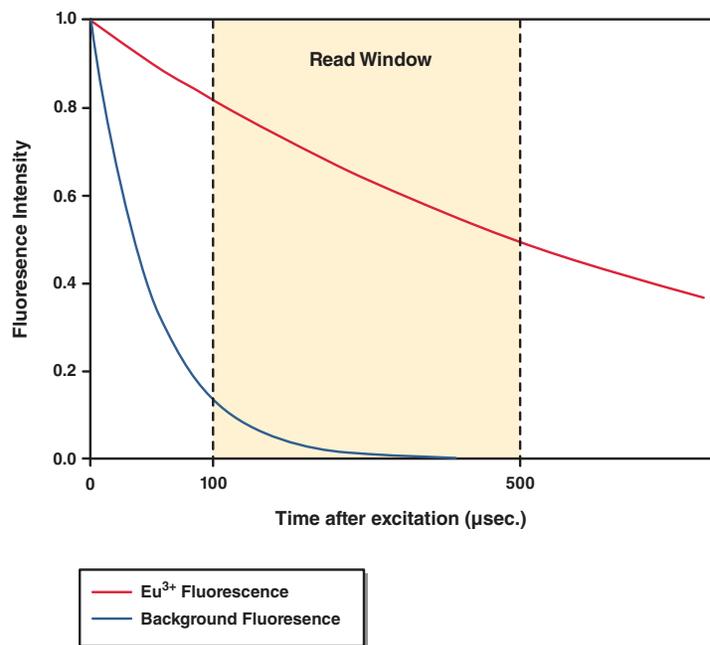


Figure 2. The extended fluorescence lifetimes of Eu<sup>3+</sup>-based fluorophores allow the samples to be analyzed after background fluorescence has decayed, improving the signal-to-noise ratio and reducing spectral artifacts.

## PRE-ASSAY PREPARATION

### Buffer Preparation

**2 ml vial TR-FRET Assay Buffer (10X) (384-well kit; Item No. 600503):** Add 18 ml of ultrapure water to the vial. Add 200 mg of TR-FRET Assay Buffer Additive (Item No. 600504) and allow to dissolve. 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 ultrapure water.* Store the diluted buffer at 4°C; it will be stable for approximately one month.

OR

**10 ml vial TR-FRET Assay Buffer (10X) (1,920- or 9,600-well kit; Item No. 600503):** For five 384-well plates, dilute 10 ml TR-FRET Assay Buffer (10X) to a total volume of 100 ml with ultrapure water. Add 1 g of TR-FRET Assay Buffer Additive (Item No. 600504) and allow to dissolve. 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 ultrapure 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 at a concentration 4X the desired final assay concentration (e.g., for 1 µM final assay concentration, a 4 µM solution should be made). This solution may contain up to 8% organic solvents such as DMSO, dimethyl formamide (DMF), or short-chain alcohols. The final concentration of organic solvents in the assay will then be <2%. Avoid using high concentrations of metal chelating agents or phosphate buffers.

## Preparation of Assay-Specific Reagents

### BRD4 bromodomain 1 Europium Chelate (Item No. 600523)

#### 420-well vial BRD4 bromodomain 1 Europium Chelate (384- or 1,920-well kit):

On ice, thaw one tube of BRD4 bromodomain 1 Europium Chelate (420 wells, Item No. 600523) per 384-well plate and briefly centrifuge before opening. Dilute contents to a final volume of 4.2 ml in 1X TR-FRET Assay Buffer. Content volume is indicated on the label. Mix gently (do not vortex) and keep on ice. Diluted protein should be used within the same day.

OR

#### 2,100-well vial BRD4 bromodomain 1 Europium Chelate (9,600-well kit):

On ice, thaw one tube of BRD4 bromodomain 1 Europium Chelate (2,100 wells, Item No. 600523) per five 384-well plates and briefly centrifuge before opening. Dilute contents to a final volume of 21 ml in 1X TR-FRET Assay Buffer. Content volume is indicated on the label. Mix gently (do not vortex) and keep on ice. Diluted protein should be used within the same day.

### BRD4 bromodomain 1 Ligand/APC Acceptor Mixture (Item No. 600524)

#### 420-well vial BRD4 bromodomain 1 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 to one vial of the BRD4 bromodomain 1 Ligand/APC Acceptor Mixture (420 wells, Item No. 600524) 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 BRD4 bromodomain 1 Ligand/APC Acceptor Mixture (9,600-well kit):

For five 384-well plates, add 3 ml of 1X TR-FRET Assay Buffer to one vial of the BRD4 bromodomain 1 Ligand/APC Acceptor Mixture (2,100 wells, Item No. 600524) 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.

### (+)-JQ1 Positive Control (Item No. 600505)

#### 8 nmol vial (+)-JQ1 Positive Control (384- or 1,920-well kit):

For each 384-well plate, add 15  $\mu$ l of DMSO (not supplied) to one tube containing the (+)-JQ1 Positive Control (8 nmol, Item No. 600505) and vortex gently. Briefly centrifuge and add 185  $\mu$ l of 1X TR-FRET Assay Buffer and vortex briefly. Unused solutions may be stored at -20°C for approximately two weeks.

OR

#### 40 nmol vial (+)-JQ1 Positive Control (9,600-well kit):

For five 384-well plates, add 75  $\mu$ l of DMSO to one tube containing the (+)-JQ1 Positive Control (40 nmol, Item No. 600505) and vortex gently. Briefly centrifuge and add 925  $\mu$ l of 1X TR-FRET Assay Buffer 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 the TR-FRET ratio in the assay. Allow all reagents except for the BRD4 bromodomain 1 Europium Chelate to equilibrate to room temperature prior to performing the assay. Keep the BRD4 bromodomain 1 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. Scale as needed for higher or lower density plate formats.

#### 1. Inhibitor Samples

Dilute inhibitor samples in 1X TR-FRET Assay Buffer 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 8% of an organic solvent (e.g., DMSO). Add 5  $\mu$ l of this solution 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 40  $\mu$ M (+)-JQ1 Positive Control to the desired wells. This will provide a final assay concentration of 10  $\mu$ M (+)-JQ1.

For negative (no inhibition) control wells, add 5  $\mu$ l of 1X TR-FRET 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. BRD4 bromodomain 1 Europium Chelate

Add 10  $\mu$ l of the diluted BRD4 bromodomain 1 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 BRD4 bromodomain 1 Europium Chelate. *Protect from light.*

#### 5. BRD4 bromodomain 1 Ligand/APC Acceptor Mixture

Add 5  $\mu$ l of the reconstituted BRD4 bromodomain 1 Ligand/APC Acceptor Mixture to every well.

#### 6. Incubation of the Plate

Seal the plate with the Foil Plate Cover (Item No. 400023) 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 a time-resolved format by exciting the sample at 340 nm and reading emissions at 620 and 665 nm, using a 100  $\mu$ s delay and a 500  $\mu$ s read window. To ensure optimal assay sensitivity, it is strongly recommended that a filter-based instrument be used to perform TR-FRET measurements. The plate reader used at Cayman Chemical employs a 330/80 nm excitation filter and 620/10 nm and 665/8 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 (665 nm emission/620 nm emission).

Well Type	(+)-JQ1 Positive Control (μl)	1X Assay Buffer (μl)	Test Sample (μl)	BRD4 bromodomain 1 Europium Chelate (μl)	BRD4 bromodomain 1 Ligand/APC Acceptor Mixture (μl)
Positive Control	5	-	-	10	5
Negative Control	-	5*	-	10	5
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%. 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 (665 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 four-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>18</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 BRD4 bromodomain 1 TR-FRET Assay Kit was determined to be 0.85.

### 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.

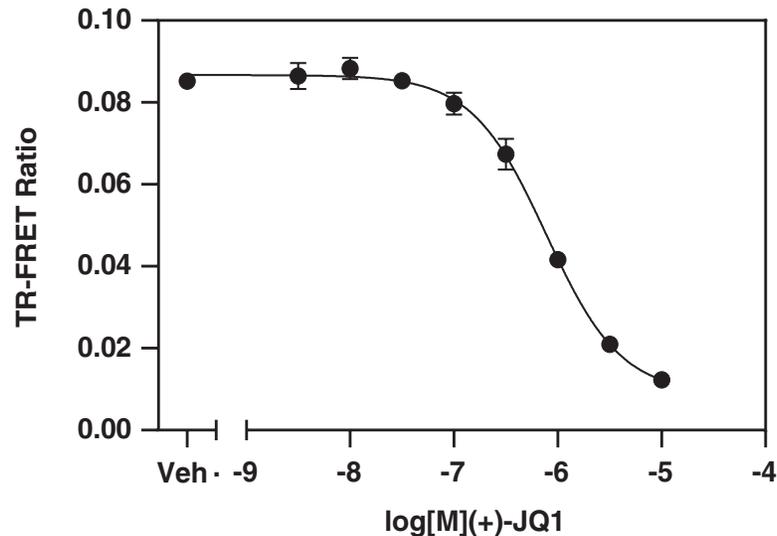


Figure 3. Typical inhibition curves for the displacement of the acetylated peptide from BRD4 bromodomain 1 by (+)-JQ1. "Veh." represents compound vehicle control.

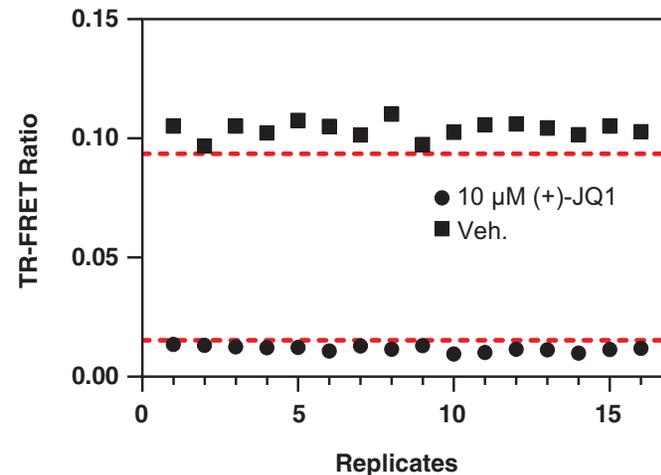


Figure 4. Typical Z' data for the BRD4 bromodomain 1 TR-FRET Assay Kit. Data are shown from 16 replicate wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z' factor from this experiment was 0.85. The red lines correspond to three standard deviations from the mean for each control value.

## Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Bubble in the well B. Poor pipetting/technique	A. Centrifuge the plate briefly
Low fluorescence signal	A. Incompatible sample matrix B. BRD protein handled improperly C. Monochromater-based instrument used for data acquisition	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. Analyze the assay using a filter-based plate reader

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