TECHNICAL DATA SHEET

THUNDER™ cAMP TR-FRET Assay Kit

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CATALOG NUMBERS KIT-CAMP-1000 (1000 tests)
KIT-CAMP-5000 (5000 tests)
KIT-CAMP-20000 (20000 tests)

Store at -80°C.
For research use only.
Not for use in diagnostic procedures.

Visit product page

PRODUCT DESCRIPTION

This assay kit is designed for the quantitative determination of adenosine 3',5'-cyclic monophosphate (cAMP) in cell lysates using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit is compatible with both adherent and suspension cells.

The THUNDER™ cAMP TR-FRET assay is highly sensitive (IC_{50}: 3 nM) and robust. The assay signal is stable for at least 18 hours at room temperature, with little loss in sensitivity. These characteristics make this kit ideal for the pharmacological characterization and the high throughput screening of Gs- and Gi-protein-coupled receptors (GPCRs).

SPECIFICITY

This assay kit contains a specific and selective antibody that recognizes cAMP in cell lysates.

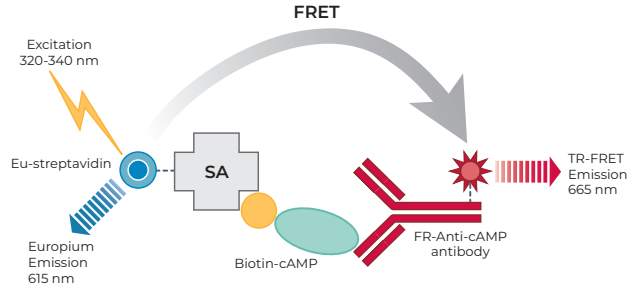
SPECIES REACTIVITY

All species expected.

TR-FRET ASSAY PRINCIPLE

The cAMP assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) competitive immunoassay designed to measure cAMP produced by cells, following treatment with GPCRs agonists or antagonists (Figure 1). GPCRs that couple with adenylate cyclase will increase or decrease intracellular cAMP levels. The THUNDER™ cAMP assay workflow consists of 2 steps (Figure 2). Cells are first stimulated to either increase or decrease the cAMP levels. cAMP is then detected during the lysis step in a simple "add-incubate-measure" format (two-step reagent addition; no wash steps). The assay is based on the competition between a europium-labeled cAMP tracer complex and sample cAMP for binding to a cAMP-specific monoclonal antibody labeled with a far-red acceptor fluorophore (FR-anti-cAMP antibody). The tracer complex is formed by the tight interaction between biotinylated cAMP (biotin-cAMP) and streptavidin labeled with a europium chelate donor (Eu-SA). In the absence of free cAMP, the FR-anti-cAMP antibody is bound to the Eu-SA/biotin-cAMP tracer, which brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a maximal TR-FRET signal at 665 nm (Figure 1, left panel). Residual energy from the Eu chelate generates light at 615 nm. Free cAMP from test samples (standard or cell lysate) competes with the Eu-SA/biotin-cAMP tracer for the binding to the FR-anti-cAMP antibody, causing a decrease in TR-FRET signal (Figure 1, right panel). As a result, the specific signal at 665 nm will be inversely proportional to the cAMP concentration in the sample. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

In the absence of free cAMP



In the presence of free cAMP

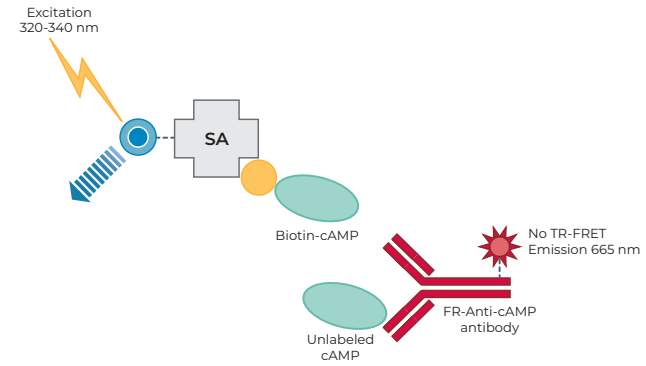


Figure 1 Schematic representation of the THUNDER™ cAMP assay principle.

STEP 1	STEP 2
Cell treatment	**Lysis & cAMP detection**
· Seed cells in white assay plate · Add compound · Incubate for optimized time	· Add **1X acceptor-labeled anti-cAMP Antibody Working Solution** · Add **1X Europium-SA/biotin-cAMP Mix** · Incubate for **1 h** · Read TR-FRET signal

Figure 2 THUNDER™ cAMP assay workflow.

THUNDER™ cAMP ASSAY PROTOCOL

Upon receipt, store the kit in the dark at -80°C

This protocol must be read in its entirety prior to beginning the assay.

- We cannot guarantee the performance of the product outside the conditions detailed in this Assay Protocol.
- Bring all reagents to room temperature prior to running the assay.
- Centrifuge all tubes before use to improve recovery of content (2000x g, 10-15 sec).
- Use ultrapure water (Milli-Q® grade water; 18 MΩ cm) to prepare the buffers.
- It is recommended to test all standards in triplicate and samples at least in duplicate.

KIT COMPONENTS

	1,000 points*	5,000 points*	20,000 points*
cAMP Standard (50 µM)	1 vial, 100 µL	1 vial, 100 µL	1 vial, 100 µL
Biotin-cAMP (200X)	1 vial, 25 µL	1 vial, 125 µL	1 vial, 500 µL
Eu-labeled streptavidin (200X)	1 vial, 25 µL	1 vial, 125 µL	1 vial, 500 µL
FR-anti-cAMP antibody (100X)	1 vial, 50 µL	1 vial, 250 µL	1 vial, 1 mL
cAMP Detection Buffer (5X)**	2 vials of 1 mL	1 bottle, 10 mL	1 bottle, 40 mL

* The number of assay points is based on an assay volume of 20 µL in low-volume 384-well assay plates using the kit components at the recommended concentrations.

** Extra cAMP Detection Buffer can be ordered separately (cat # TRFRET-CAMPDB).

ADDITIONAL MATERIALS REQUIRED

	Recommended source	Catalog No.
Stimulation buffer	See page 4 for preparation	NA
Hank's Balanced Salt Solution (HBSS) (1X) (calcium, magnesium, no phenol red)	Gibco	14025-092
Versene 1X	Gibco	15040-066
HEPES Buffer Solution (1 M)	Gibco	15630-080
Ultrapure laboratory grade water	Many options available	NA
BSA (protease free, fatty acid free; globulin free)	Sigma	A7030
Forskolin	Sigma	F6886
IBMX (3-Isobutyl-1-Methylxanthine)	Sigma	I5879
DMSO	Sigma	I7018
Low-volume 384-well microplate, white	PerkinElmer Greiner Corning	6007290 784075 4513
Adhesive sealing film for plates	Many options available	NA
Single channel pipettors	Many options available	NA
A plate reader equipped with the TR FRET option	Many options available	NA

ASSAY DEVELOPMENT WORKFLOW

The THUNDER™ TR-FRET cAMP assay should be developed and optimized following the assay workflow described in the table below.

Step	Purpose
1	Select a suitable cell line or primary cells.
2	Determine the sensitivity (IC_{50} value) and working range (IC_{10} – IC_{90}) of the cAMP assay by running a standard curve.
3	Determine the optimal cell density giving the highest S/B ratio while staying within the assay working range (IC_{10} – IC_{90}). • Gs assay: run a full-agonist or forskolin concentration-response experiment at different cell densities. • Gi assay: run a forskolin concentration-response experiment at different cell densities. Convert signals to cAMP levels to determine the forskolin EC_{50} value to be used for the agonist assay.
4	Determine agonist potency (EC_{50} value) by running an agonist concentration-response curve • Gi assay: use the EC_{50} forskolin concentration (based on cAMP levels)
5	Determine antagonist potency (IC_{50} value) by running an antagonist concentration-response curve. • Gs assay: use the EC_{50} agonist concentration (based on cAMP levels) • Gi assay: use the EC_{50} forskolin and EC_{50} agonist concentrations (based on cAMP levels)

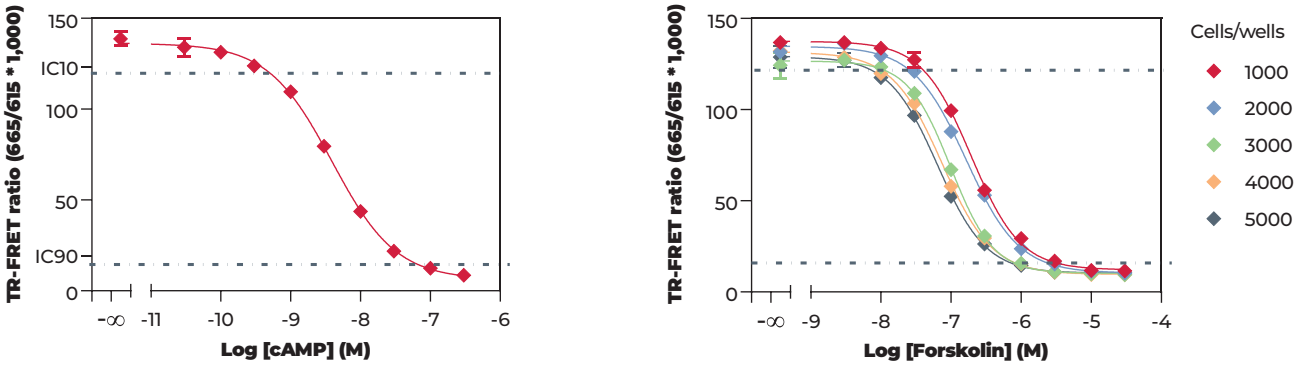
ASSAY DEVELOPMENT GUIDELINES

1. **IBMX concentration:** IBMX is a widely used non selective inhibitor of cAMP phosphodiesterases. We recommend using IBMX at a concentration of 0.5 mM in the Stimulation Buffer. At this concentration, IBMX does not reduce the signal in cAMP standard curves. The effect of IBMX on compound potency and the optimal concentration should be determined according to the assays and cellular models being used. IBMX can be prepared at high concentration (500 mM) and aliquoted and stored at -20°C to avoid multiple freeze-thaw cycles.

2. **cAMP Standard Curve:** A cAMP standard curve should be included with each run both to verify that the assay generates the expected IC_{50} value and S/B ratio, and to convert the TR-FRET signals into cAMP levels. It is essential that the assay conditions (cell density, forskolin concentration, agonist concentration) are optimized so that the measured TR-FRET signals fall within the working range of the standard curve (defined as cAMP concentrations between IC_{10} – IC_{90}). Due to the sigmoidal relationship between the signal and the cAMP concentrations, signals at the top region of the standard curve are very sensitive to variations in cAMP concentrations, whereas signals at the bottom region are insensitive to the variations.

3. **Cell Number:** It is strongly recommended to generate either forskolin (Gs- and Gi-coupled receptors) or full agonist (Gs receptors) concentration-response curves at different cell densities in order to determine the optimal cell number per well. We suggest testing from 1,000 to 10,000 cells per well. The optimal cell number will be the one for which the forskolin or agonist concentration-response curve covers most of the working range of the cAMP standard curve, while maximizing the assay S/B ratio. In the example presented below (Figure 3), the optimal cell concentration selected for subsequent experiments would be 4,000 cells/well.

ASSAY DEVELOPMENT GUIDELINES (CONTINUES)



Cells/well	1000	2000	3000	4000	5000
S/B	12	12	13	14	12
EC_{50} Forskkolin (nM)	192	164	99	75	64

Selected cell density: 4,000 cells/well

Figure 3. Determination of optimal cell density. Left panel: cAMP standard curve; right panel: cell and forskolin cross-titration. In this example, the forskolin concentration-response curve obtained shows that 4,000 cells/well provides a response that falls within the working range of the cAMP curve, while maximizing the assay S/B ratio.

4. **Forskolin Concentration:** An optimized forskolin concentration is required to produce Gi agonist concentration-response curves. We recommend using forskolin at its EC_{50} concentration based on cAMP levels. This value should correspond to the forskolin EC_{80}-EC_{90} value based on the TR-FRET signal. Note that the chosen forskolin concentration should not exceed the working range (IC_{10}) of the cAMP standard curve.

5. **Agonist Concentration:** The presence of an agonist is required to produce Gs and Gi antagonist concentration-response curves. We recommend using the agonist at its EC_{50} concentration based on cAMP levels. This value should correspond to the agonist EC_{80}-EC_{90} value based on the TR-FRET signal. Note that the chosen agonist concentration should not exceed the working range (IC_{10} for a Gs receptor; IC_{90} for a Gi receptor) of the cAMP standard curve.

6. **Stimulation Time and Temperature:** We recommend a 30-minute stimulation time at room temperature (RT). However, this step should be optimized by evaluating stimulation responses from 15 to 60 minutes at either RT or 37°C.

REAGENT PREPARATION

- The instructions described below are for testing the entire number of assay points in the 1,000-point kit. Adjust volumes accordingly for testing of fewer or more points.
- Prepare only as much reagent as is needed on the day of the experiment.

STEP 1 PREPARATION OF STIMULATION BUFFER

The recommended Stimulation Buffer for cell-based assays is:
1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA (pH 7.4).

To prepare 15 mL of Stimulation Buffer, add the following to a tube:

A. 14 mL of 1X HBSS (Gibco, cat. # 14025-092)
B. 75 µL of 1 M HEPES (Gibco, cat. # 15630-080)
C. 15 µL of 500 mM IBMX dissolved in DMSO (Sigma, cat. # I7018)
D. 200 µL of 7.5% BSA (Sigma cat. # A7030)
E. Adjust pH to 7.4 with 0.1 N NaOH and complete volume to 15 mL with 1X HBSS

NOTES

- *Addition of BSA might not be essential for your cellular assay.*
- *Alternative buffers such as cell culture medium containing 10% FBS and phenol red can also be used.*
- *The IBMX concentration may need further optimization when working with different cell lines.*

STEP 2 PREPARATION OF cAMP STANDARD SERIAL DILUTIONS IN STIMULATION BUFFER

- Prepare working standards just before use and use within one hour. Do not store working standards.
- Each well requires 5 µL of working standard.
- Change tip between each standard dilution.
- Mix each tube thoroughly before the next transfer.
- The following section describes the preparation of a standard curve for triplicate measurements (recommended).

Prepare the **4X cAMP standard serial dilutions** in Stimulation Buffer from the 50 µM cAMP standard supplied with the kit, as indicated in the table below.

Dilution	Volume of cAMP	Volume of Stimulation Buffer	[4X] (M)	[cAMP] in standard curve (M)
1	3 µL of 50 µM cAMP	122 µL	1.2E-06	3.0E-07
2	30 µL of 1	60 µL	4.0E-07	1.0E-07
3	30 µL of 2	70 µL	1.2E-07	3.0E-08
4	30 µL of 3	60 µL	4.0E-08	1.0E-08
5	30 µL of 4	70 µL	1.2E-08	3.0E-09
6	30 µL of 5	60 µL	4.0E-09	1.0E-09
7	30 µL of 6	70 µL	1.2E-9	3.0E-10
8	30 µL of 7	60 µL	4.0E-10	1.0E-10
9	30 µL of 8	70 µL	1.2E-10	3.0E-11
10	0	70 µL	0	0

STEP 3 PREPARATION OF 1X cAMP DETECTION BUFFER

- Mix end-over-end the 5X cAMP Detection Buffer before use.
- The thawed 5X cAMP Detection Buffer can be stored at 4°C for 1 month. For longer periods of time, buffer should be stored at -80°C.
- Unused 1X cAMP Detection Buffer can be stored at 4°C for 2 weeks.

Dilute the 5X cAMP Detection Buffer to 1X with ultrapure water.
For example: add 2 mL of 5X Detection Buffer to 8 mL of ultrapure water.

NOTE: *The 1X cAMP detection buffer is designed to lyse the cells and must be used only for the preparation of 1X FR-anti-cAMP antibody Solution and 1X Eu-SA/biotin-cAMP Mix.*

STEP 4 PREPARATION OF FR-ANTI-cAMP ANTIBODY WORKING SOLUTION IN 1X cAMP DETECTION BUFFER

- Prepare and mix just before use.
- Each well requires 5 µL of 1X FR-anti-cAMP antibody.
- Do not vortex solutions containing the FR-anti-cAMP antibody but instead mix gently.
- The thawed 100X FR-anti-cAMP Antibody stock can be stored at 4°C for 2 days. For longer periods of time, the antibody should be stored at -80°C.
- The unused 1X FR-anti-cAMP Antibody Working Solution may be stored at 4°C for 1 day or aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles.

Dilute the 100X FR-anti-cAMP Antibody to 1X with the 1X cAMP Detection Buffer.

For example: add 50 µL of the 100X FR-anti-cAMP Antibody solution to 4950 µL of 1X cAMP Detection Buffer and mix by inverting the tube up and down.

STEP 5 PREPARATION OF EU-SA/BIOTIN-cAMP MIX IN 1X cAMP DETECTION BUFFER

- Prepare and mix just before use.
- Each well requires 5 µL of Eu-SA/biotin-cAMP Mix.
- The unused 200X Eu-SA may be stored at 4°C for 2 days or aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles.
- The unused 200X biotin-cAMP may be stored at 4°C for 2 days or aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles.
- The unused 1X Eu-SA/biotin-cAMP Mix may be stored at 4°C for 1 day.

Co-dilute the 200X Eu-SA and the 200X biotin-cAMP to 1X with the 1X cAMP Detection Buffer.

For example, to prepare 5 mL of 1X Eu-SA/biotin-cAMP Mix, add the following to a tube:

A. Add 4950 µL of 1X cAMP Detection Buffer in a tube
B. Add 25 µL of Eu-SA stock
C. Mix gently
D. Add 25 µL of biotin-cAMP stock
E. Mix gently
F. Incubate for at least 15 minutes at room temperature

CELL PREPARATION

A. Harvest cells with a non-enzymatic cell dissociation solution (like Versene).
B. Wash cells once with HBSS.
C. Resuspend the cells in Stimulation Buffer at the desired concentration. It is recommended to test 1,000 to 10,000 cells per assay.
D. Prepare a "no cell" control (Stimulation Buffer alone).

TEST COMPOUND PREPARATION

A. Prepare intermediate 2X or 4X (depending on the type of cAMP assay) dilution series of test compound(s) by serially diluting compound(s) across 12 wells of a polypropylene 96 well plate into Stimulation Buffer.

NOTE: *It is recommended conducting a 12-point, half-log interval concentration-response curve in at least duplicate for an accurate estimation of the EC_{50} or IC_{50}.*

B. Alternatively, for hydrophobic, DMSO-soluble test compounds, perform the initial dilutions in 100% DMSO, and then dilute the compound dilution series into Stimulation Buffer, resulting in a 2X (2% DMSO) or 4X (4% DMSO) dilution. Most cells can tolerate up to 1% DMSO in the 10 µL cell treatment step.

NOTE: *The assay tolerance to DMSO must be established before conducting a test compound titration in DMSO vehicle. It is important to keep equal solvent concentrations between treated and untreated cells. In addition, when testing serial dilutions of compounds, the solvent concentrations should always remain constant across the dilution series.*

ASSAY PROCEDURE

- The following protocol assumes that both the cell density and stimulation conditions have been optimized during assay development.
- The assays are performed in low-volume 384-well microplates in a total volume of 20 µL. However, assays can be conducted in half-area 96-well plates (40 µL) and 1536-well plates by adjusting the volumes of each assay component proportionally.
- The following pipetting protocols were developed for assays using suspension cells. However, the assay can be adapted easily for adherent cells.
- It is strongly recommended to include a cAMP standard curve every time you run a cAMP assay to verify that the assay generates the expected S/B ratio and IC_{50} value, and to convert the signal into cAMP levels.
- Samples and standards must be assayed at least in duplicate each time the assay is performed.
- Both the cells and test compounds must be prepared in Stimulation Buffer (including 0.5 mM IBMX).
- Addition of the 1X cAMP Detection Buffer lyses the cells. The 1X cAMP Detection Buffer must be used only for the preparation of 1X FR-anti-cAMP antibody Solution and 1X Eu-SA/biotin-cAMP Mix.
- **Do NOT premix the FR-anti-cAMP and the Eu-SA/biotin-cAMP tracer complex in order to eliminate an addition step.**

IMPORTANT NOTE
Optimal cell densities should be determined during assay development in cell titration experiments such that the measured signal falls within the IC_{10}-IC_{90} range of the cAMP standard curve.

ASSAY PROCEDURE (CONTINUES)

The following table summarizes the pipetting protocol for conducting different cAMP assays:

cAMP standard curve	Gs Agonist	Gs Antagonist	Gi Forskolin	Gi Agonist	Gi Antagonist
5 µL 4X cAMP standard	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension
5 µL Stimulation Buffer	5 µL 2X agonist*	2.5 µL 4X agonist*	5 µL 2X forskolin*	2.5 µL 4X forskolin*	2.5 µL 4X forskolin/4X agonist mix*
-	-	2.5 µL 4X antagonist*	-	2.5 µL 4X agonist*	2.5 µL 4X antagonist*
Seal the plate and incubate for 30 min at RT					
5 µL FR-anti-cAMP Antibody Working Solution in 1X cAMP Detection Buffer					
5 µL Eu-SA/biotin-cAMP Mix in 1X cAMP Detection Buffer					
Seal the plate and incubate 1 h at RT**					
Remove plate sealer and read plate on a TR-FRET compatible microplate reader					

** The untreated cells receive the same volume of stimulation buffer and are incubated for the same amount of time, and the same temperature, as the treated cells.*

***Additional readings can be performed for at least 18 hours without any negative effect on the assay performance.*

PLATE READER SETTINGS

We recommend reading the TR-FRET assays at two wavelengths, detecting both the emission from the Europium chelate donor fluorophore at 615 nm, and the acceptor fluorophore at 665 nm. The following instrument settings are provided as guidelines.

	TR-FRET Compatible Reader	
Parameter	**Flash lamp excitation**	**Laser Excitation**
Excitation filter	320 nm (or 340 nm)	Not applicable
Emission filter	615 nm (or 620 nm)	615 nm (or 620 nm)
Dely time	90 µs	50 µs
Flash energy level	100% or High	100%
Number of flashes	100	20
Window (integration time)	300 µs	100 µs

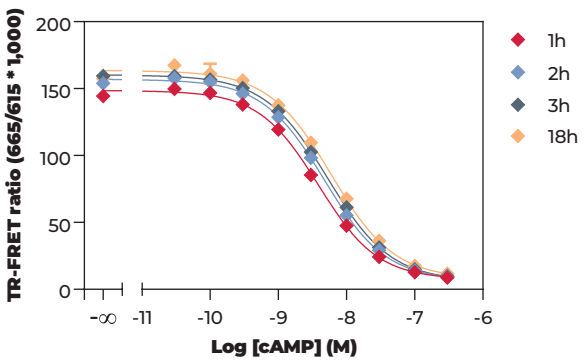
DATA ANALYSIS

- TR-FRET data are typically calculated and presented ratiometrically using the following formula: [(665 nm/615 nm) x 1,000]
- Alternatively, the signals at 665 nm can be used directly to process your data.
- Calculate the TR-FRET ratio for each well.
- Since TR-FRET signal is read in a time-resolved mode, background subtraction is usually not necessary.
- Create a cAMP standard curve by analyzing data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting.

IMPORTANT: *Convert the results from the reader (TR-FRET ratio or signals at 665 nm) into cAMP levels using the cAMP standard curve.*

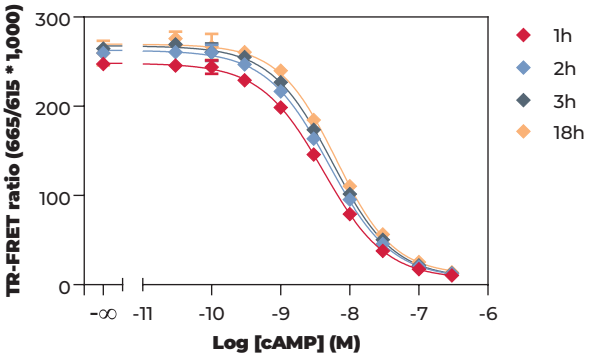
TYPICAL STANDARD CURVES

LAMP



Detection time (h)	1	2	3	18
IC_{50} (nM)	3.8	4.7	5.3	5.9
S/B	17	17	16	14

LASER



Detection time (h)	1	2	3	18
IC_{50} (nM)	4.1	4.9	5.5	6.3
S/B	24	21	21	18

Figure 4. Typical nonlinear THUNDER™ cAMP standard curves in Stimulation Buffer. Left: lamp excitation, Right: laser excitation. Data represent the mean ± standard deviation of triplicate measurements (3 wells) for each standard. The assay working range is defined as IC_{10} to IC_{90}. The data was generated using a low-volume 384-well white plate read on the EnVision® equipped with TR-FRET option after 1 hour and overnight incubation. Please note that depending on the instrument and the lot of kit used, the level of counts and S/B ratio may slightly vary without affecting the assay robustness.

VALIDATION DATA

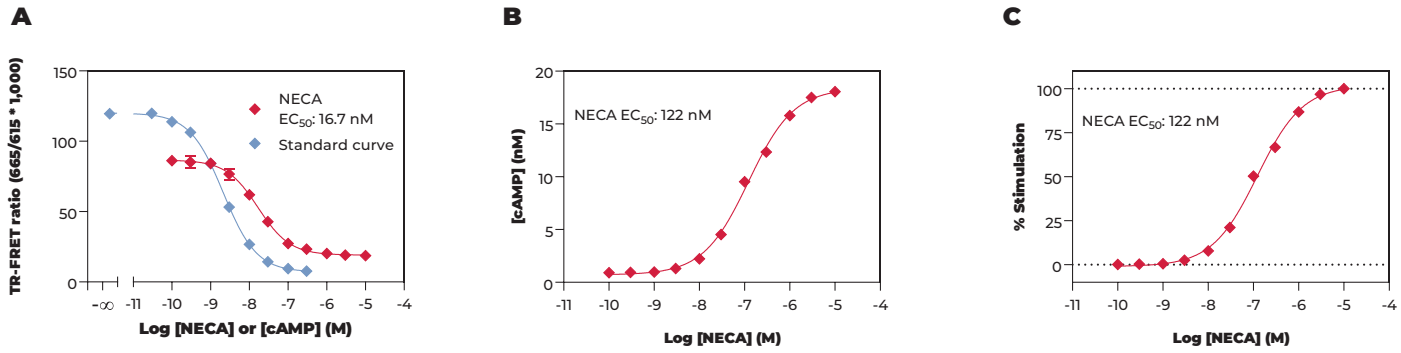


Figure 5. An example of agonist concentration-response curves in U266B1 cells (2,000 cells/well) expressing the endogenous Gs-coupled Adenosine A2a receptor. Panel A: the agonist response is plotted as the TR-FRET ratio (665/615 × 1000) along with the cAMP standard curve. Note that the agonist produces signals that are within the working range of the standard curve. Panel B: the agonist response is plotted as the cAMP level to determine the EC_{50} value. Panel C: the same agonist response is plotted as % stimulation.
Note: *IBMX, an antagonist of Adenosine receptors, was omitted from the stimulation buffer to improve assay sensitivity.*

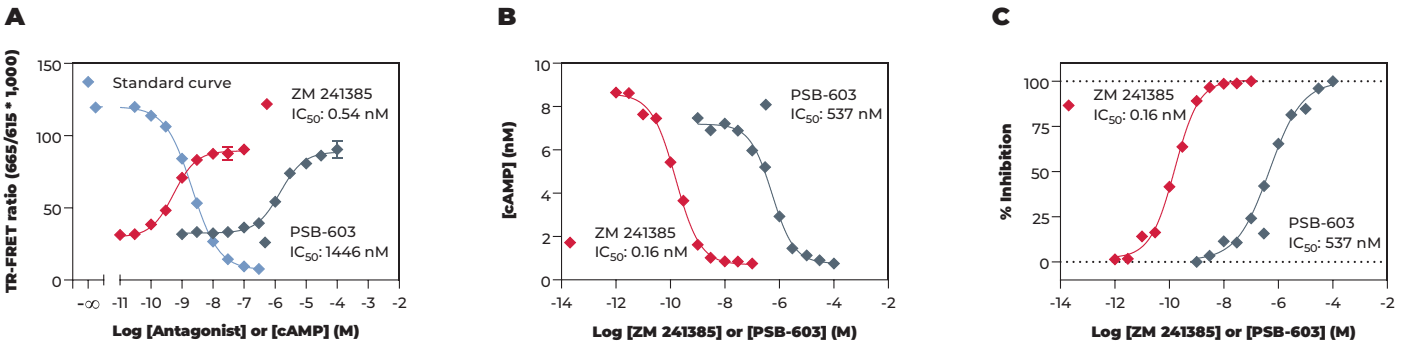


Figure 6. An example of antagonist concentration-response curves in U266B1 cells (2,000 cells/well) expressing the endogenous Gs-coupled Adenosine A2a receptor. Panel A: the antagonist responses are plotted as the TR-FRET ratio (665/615 × 1000) along with the cAMP standard curve. Note that the antagonists produce signals that are within the working range of the standard curve. Panel B: the antagonist responses are plotted as the cAMP level to determine the IC_{50} values. Panel C: the same antagonist responses are plotted as % inhibition.

Note 1: *IBMX, an antagonist of Adenosine receptors, was omitted from the stimulation buffer to improve assay sensitivity.*
Note 2: *ZM 241385 is a potent antagonist of Adenosine A2a receptor, while PSB-603 is selective to Adenosine A2b receptor.*

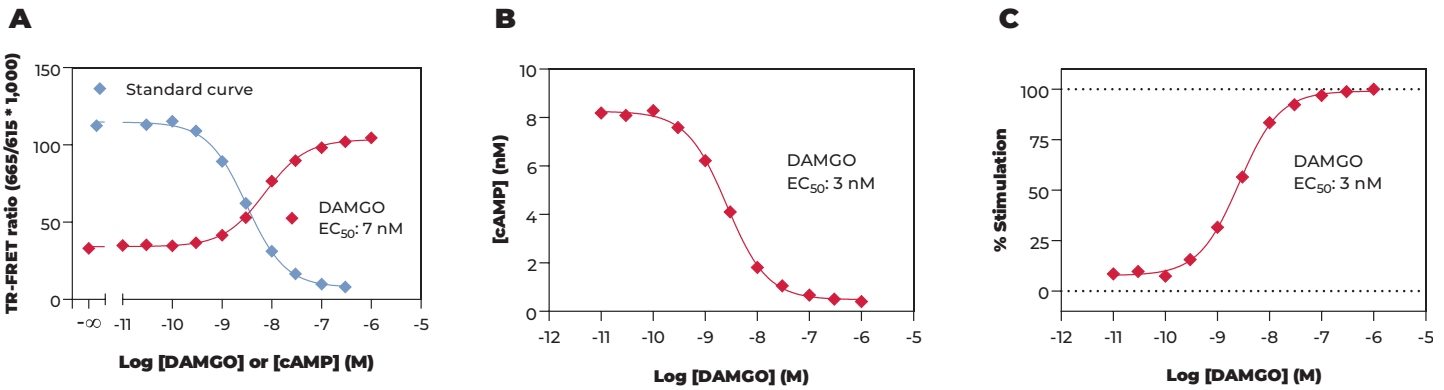


Figure 7. An example of agonist concentration-response curves in CHO cells (2,500 cells/well) expressing the Gi-coupled human Opioid Mu (OP3) receptor. Panel A: the agonist response is plotted as the TR-FRET ratio (665/615 × 1000) along with the cAMP standard curve. Note that the agonist produces signals that are within the working range of the standard curve. Panel B: the agonist response is plotted as the cAMP level to determine the EC_{50} value. Panel C: the same agonist response is plotted as % stimulation.

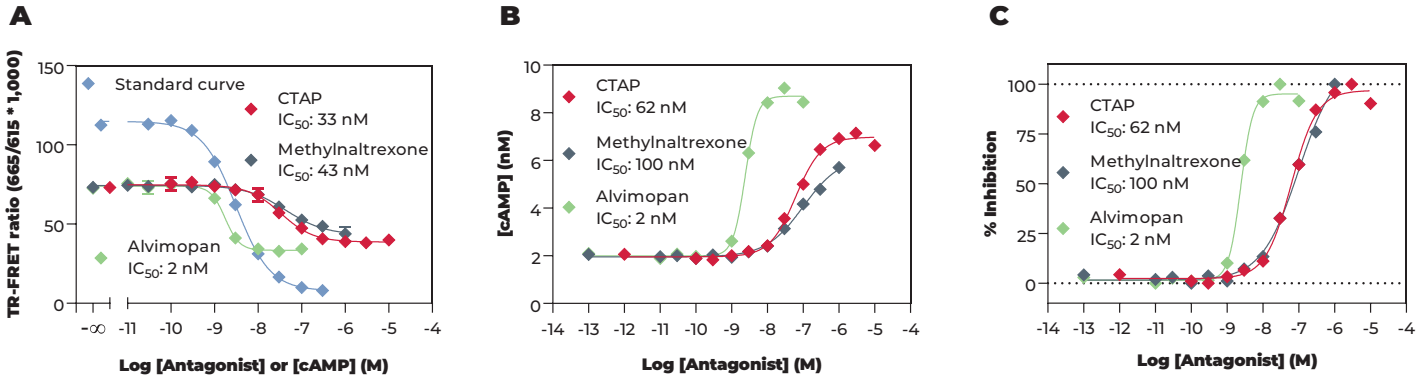


Figure 8. An example of antagonist concentration-response curves in CHO cells (2,500 cells/well) expressing the Gi-coupled human Opioid Mu (OP3) receptor. Panel A: The antagonist responses are plotted as the TR-FRET ratio (665/615 × 1000) along with the cAMP standard curve. Note that the antagonists produce signals that are within the working range of the standard curve. Panel B: The antagonist responses are plotted as the cAMP level to determine the IC_{50} values. Panel C: The same antagonist responses are plotted as % inhibition.