VKEYBIO-03-2024
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KeyTec® cAMP TR-FRET

Instruction Manual

1. Introduction

KeyTec® cAMP TR-FRET KIT designed for quantitative measurement of adenosine 3',5'-cyclic adenosine monophosphate (cAMP) in cell lysates. It is based on competitive immunoassay model and utilizes TR-FRET technology, known for its ease of use, homogeneity (no wash), low background, wide range, robustness.

The detection principle is based on TR-FRET technology. Streptavidin and anti-cAMP antibodies, each labeled with KeyTec® TR-FRET Solar Eu*1 and KeyTec® TR-FRET LA*2, Streptavidin-Solar Eu form a complex with Biotin–cAMP referred to as the cAMP tracer. When cAMP tracer binds to mAb anti-cAMP–LA, excitation of the donor will result in the generation of the TR-FRET signal at 665 nm. Native cAMP produced by cells or unlabeled cAMP (standard curve) competes with the cAMP tracer for binding to anti-cAMP antibodies. The specific signal is inversely proportional to the concentration of cAMP in the standard or sample. (Figure 1)

*1 KeyTec® TR-FRET Solar Eu: TR-FRET Donor Molecule

*2 KeyTec® TR-FRET LA/HX: TR-FRET Acceptor Molecule

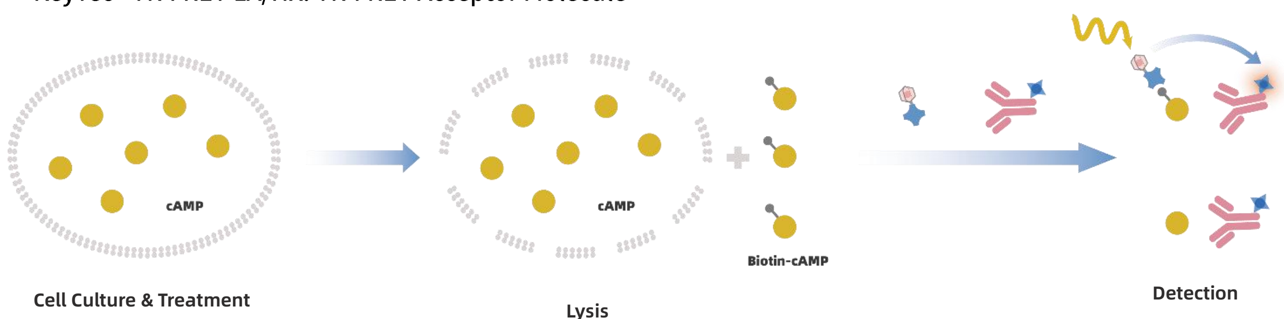

Figure 1. KeyTec® cAMP TR-FRET Assay Principle

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2. Components

Components	Storage	A010101 (1,000 tests*3)	A010012 (10,000 tests*3)
mAb anti-cAMP-LA (100X)	⩽ -60 °C	1 vial 50 μL/vial	1 vial 500 μL/vial
Biotin-cAMP (200X)	⩽ -60 °C	1 vial 25 μL/vial	1 vial 250 μL/vial
cAMP Standard Solution (50 μM)	⩽ -60 °C	1 vial 100 μL/vial	1 vial 100 μL/vial
Streptavidin-Solar Eu (200X)	⩽ -60 °C	1 vial 25 μL/vial	1 vial 250 μL/vial
Detection Buffer (5X)	⩽ -60 °C	2 vials 1 mL/vial	1 bottle 20 mL/bottle

A010013 (50,000 tests) and A010014 (100,000 tests) are customized sizes.

*3 The tests are sufficient in a 384-well microplate assay format, with 20 μL per well.

3. Storage Conditions

- Upon receipt, store the kit below -60 °C. Kit components remain stable under appropriate storage conditions as recommended.
- When first thaw, aliquot the components as needed to avoid multiple freeze-thaw cycles
- Up to 1 years from date of receipt, when stored and handled as recommended.

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4. Materials Required But Not Supplied

Materials Required But Not Supplied	Recommended source	CAT. & Size
Hank's Balanced Salt Solution (HBSS) (1X) (calcium, magnesium, no phenol red)	Gibco	14025-092
HEPES Buffer Solution (1 M)	Gibco	15630-080
BSA (protease free, fatty acid free; globulin free)	Sigma	A7030
IBMX*4	Sigma	I5879
Forskolin*5	Sigma	F6886
KeyTec® 384-Well White Flat Low-Volume Microplates, PS, Solid, Non-treated, No lid	VKEY-BIO	M2000102N
KeyTec® Fluorescent High-Transparency Microplate Top Seals	VKEY-BIO	M1000102N

*4 IBMX is the most commonly used phosphodiesterase (PDE) Pan-inhibitor. It guarantees high level of cAMP accumulation in the cells.

*5 Forskolin activates the adenylyl cyclase enzyme and increases the intracellular level of cAMP. It is used as a positive control for Gs applications (biological models validation, maximal intracellular cAMP modulation determination). It is highly used for G_i coupled receptor study as preactivation step to show cAMP level inhibition upon cell stimulation

5. Assay Procedure

5.1 Assay Format

Assay Format	Total Volume (20 µL*6)
Standard or samples	5 µL
Stimulation buffers +/- compounds	5 µL
mAb anti-cAMP-LA working solution (1X)	5 µL
cAMP tracer working solution (1X)	5 µL

*6 The system accommodates 384-well low-volume microplates, and assay volumes can be adjusted proportionally to perform in 96- or 1536-well microplates.

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5.2 Reagents Handling

1) Buffers

- Thaw the buffer at room temperature and equilibrate to room temperature before use. Aliquot the reagents as needed and store the reagents below -60 °C.
- The recommended Stimulation Buffer for cell-based assays is：1X HBSS，5 mM HEPES，0.5 mM IBMX，0.1% BSA (pH 7.4).
- If the compound stimulation time is above 2 hours, use cell culture medium (e.g., RPMI or DMEM) to replace Stimulation Buffer for diluting compounds, IBMX, and cAMP standard
- Dilute the 5X Detection 1X Buffer to Detection Buffer by ddH_2O.
- Use the specified buffer to prepare reagents to avoid affecting assay results.

2) Conjugates

- **Thaw reagents** other than buffers on ice and equilibrate to room temperature before use. It is recommended to aliquot the reagents as needed to avoid multiple freeze-thaw cycles.
- **mAb anti-cAMP-LA Working Solution:** Dilute the 100X mAb anti-cAMP-LA to 1X with the 1X Detection Buffer. Each well requires 5 µL of 1X mAb anti-cAMP–LA. Prepare and mix just before use. For example: mix 5 µL of the 100X mAb anti-cAMP-LA stock solution with 495 µL of 1X Detection Buffer for a 500 µL 1X working solution.
- **cAMP tracer Working Solution:** Co-dilute the 200X SA-solar Eu and the 200X biotin-cAMP to 1X with the 1X Detection Buffer, then incubate for at least 15 minutes at room temperature. Each well requires 5 µL of 1X cAMP tracer Working Solution. Prepare and mix just before use. For example: mix 2.5 µL of the 200X SA-Eu and 2.5 µL of the 200X biotin-cAMP with 495 µL of 1X Detection Buffer for a 500 µL 1X working solution.

3) Additive

- IBMX can be stocked at 500 mM in 100% DMSO at -20 °C, avoiding multiple freeze-thaw cycles. We recommend using IBMX at 0.5 mM (working solution) in the Stimulation Buffer / cell culture medium. For example: mix 10 µL of the 500 mM IBMX with 9.99 mL of 1X Stimulation Buffer / cell culture medium for a 10 mL 1X working solution. Note: The Stimulation Buffer containing IBMX must be prepared prior to use.
- Forskolin can be stocked at 10 mM in 100% DMSO at -20 °C, avoiding multiple freeze-thaw cycles. The concentration of forskolin to be used in the assay must be optimized. First, prepare an intermediate dilution of 1/5 in 100% DMS0 (e.g.,40 µL of stock solution + 160 µL of DMSO), followed by a second dilution from this pre-dilution in the stimulation buffer / cell culture medium at the desired concentration. Note: Forskolin working solution in 1X Stimulation Buffer / cell culture medium must be prepared prior to use.

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4) Standard

- Run a standard dose response curve to determine the linear dynamic range of the assay Stimulation Buffer / cell culture medium (containing IBMX). This will also verify that your assay is generating the expected S/B and IC50. In particular, the IC10 and IC90 will be useful in experiments to optimize the cell density of the cell lines you will stimulate with compounds.
- Prepare the 4X cAMP standard serial dilutions in Stimulation Buffer / cell culture medium (containing IBMX) using the 50 µM cAMP standard supplied with the kit following ***Table-1***. Determine the total amount of standard to be prepared based on assay requirements; the amounts in ***Tale-1*** are for reference only.

Table-1: Standard curve working solution preparation.

Standard	Final Concentration cAMP (nM)	Working Concentration cAMP (nM)	Serial Dilution
STD-9	300	1200	3 µL Standard stock Solution + 122 µL Stimulation Buffer
STD-8	100	400	30 µL STD-9 + 60 µL Stimulation Buffer
STD-7	30	120	30 µL STD-8 + 70 µL Stimulation Buffer
STD-6	10	40	30 µL STD-7 + 60 µL Stimulation Buffer
STD-5	3	12	30 µL STD-6 + 70 µL Stimulation Buffer
STD-4	1	4	30 µL STD-5 + 60 µL Stimulation Buffer
STD-3	0.3	1.2	30 µL STD-4 + 70 µL Stimulation Buffer
STD-2	0.1	0.4	30 µL STD-3 + 60 µL Stimulation Buffer
STD-1	0.03	0.12	30 µL STD-2 + 70 µL Stimulation Buffer
STD-0 (PC)	0	0	60 µL Stimulation Buffer

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5.3 Cell-Based Assay

1) Before You Running

- Various cell parameters must be optimized that depend on the type of compound to be screened (agonist or antagonist) and on the particular G protein coupled to the GPCR (G_i or G_s). These parameters include cell density, IBMX* concentration, agonist concentration (for antagonist mode assay), and stimulation time.
- IBMX is the most commonly used phosphodiesterase (PDE) Pan-inhibitor. It guarantees high level of cAMP accumulation in the cells. IBMX has no effect on standard curve if used at the recommended concentration of 0.5 mM. It may have its own effect on cAMP accumulation. We recommend checking this effect by running experimental wells + / - IBMX in the stimulation buffer / cell culture medium.
- Compounds must be diluted in Stimulation Buffer / cell culture medium (containing IBMX).
- Cell density must be also optimized to ensure that cAMP levels of unstimulated and stimulated cells fall within the linear dynamic range of the assay (IC_{10} to IC_{90}). If results fall outside the assay's linear range, the data will be inaccurate.
- For the G_i coupled receptor study assay, the Forskolin needs to be optimized. The preferred optimization method is to make a Forskolin dose curve at different cell densities, and a full dose-response of Forskolin alone (basal) or pre-treated with a maximal dose of the agonist (treated cells). The ratio of treated cells to basal provides the maximal assay window achievable under each condition.
- Antagonist screening requires stimulation with appropriate concentrations of agonist. In general, the EC90 concentration can be selected for antagonist detection.
- In general, optimal results can be obtained with 30 min room temperature stimulation. However, it is important to optimize the stimulation time for different cell lines, choosing room temperature or 37 ° C.

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2) Assay Format

- Add the reagents to the microplates in turn by following the steps shown in the following table.

	Standard Curve	Agonists G_s	Agonists G_i	Antagonist G_s	Antagonist G_i
Step 1	5 µL Standards	5 µL Cell Suspension	5 µL Cell Suspension	5 µL Cell Suspension	5 µL Cell Suspension
Step 2	5 µL Stimulation Buffer[*7]	5 µL Agonist (2X)	2.5 µL Agonist (4X)	2.5 µL Antagonist (4X)	2.5 µL Antagonist (4X)
Step 3	-	-	2.5 µL Forskolin (4X)	2.5 µL Agonist (4X)	2.5 µL Forskolin + Agonist premixed (4X)
Step 4	Seal the microplate and incubate optimized time at room temperature or 37 ° C				
Step 5	5 µL mAb anti-cAMP - LA (1X working solution) [*8]				
Step 6	5 µL cAMP tracer (1X working solution) [*8]				
Step 7	Seal the microplate by “KeyTec® Fluorescent High-Transparency Microplate Top Seals” and incubate 1 h at room temperature				
Step 8	(no need to remove the High-Transparency plate sealer) Read on the TR-FRET compatible reader				

[*7] 1X Stimulation Buffer / cell culture medium

[*8] mAb anti-cAMP-LA (1X working solution) and cAMP tracer (1X working solution) should be dispensed separately in distinct steps for the assay. Do not pre-mix.

5.4 Data Calculating

- Calculate the ratio of 665 nm/615 nm (TR-FRET Ratio) and the CV for each individual well.

$$\text{TR-FRET Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 615 nm}} \times 10{,}000$$

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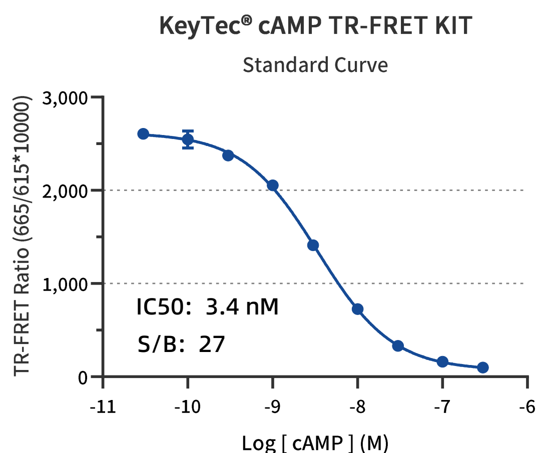
6. Performance

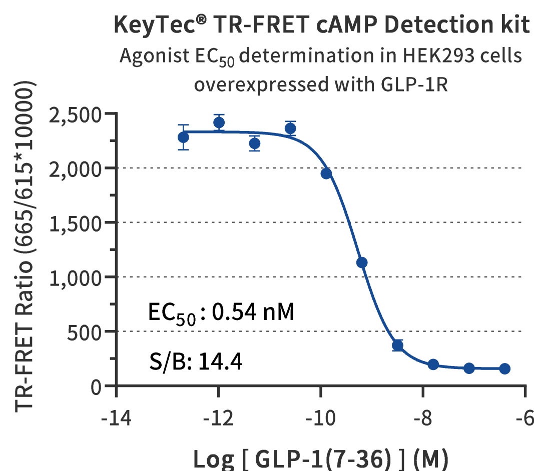
6.1 Standard curve

Standard curve	cAMP (nM)	TR-FRET Ratio	CV%
STD-9	300.00	97.5	0.73
STD-8	100.00	161.5	3.06
STD-7	30.00	343.5	1.44
STD-6	10.00	727.0	5.06
STD-5	3.00	1,410.0	1.10
STD-4	1.00	2,054.5	1.96
STD-3	0.30	2,372.5	1.40
STD-2	0.10	2,546.0	3.61
STD-1	0.03	2,605.5	0.03
STD-0	0.00	2,643.5	0.29



6.2 Results of Agonist GLP-1(7-36) dose-response in HEK293 overexpressed with GLP-1R

- HEK293 cell line overexpressed with GLP-1R (2K cells/well, in duplicate) were incubated with serial dilutions of GLP-1(7-36) for 30 min at 37 °C. The data show that treatment of HEK293 cells with GLP-1(7-36) stimulates the GLP-1R signaling pathway, leading to an increase in cAMP levels.



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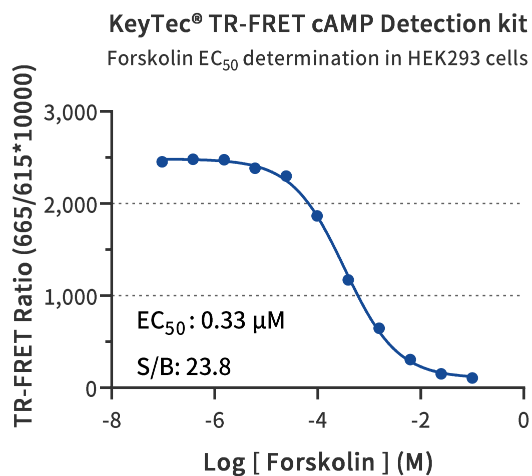
6.3 Results of Forskolin for dose-response in HEK293 cell line



- HEK293 cell line（10K cells/well, in duplicate）were incubated with serial dilutions of Forskolin for 30 min at 37 °C. The data show that treatment of HEK293 cells with Forskolin induces to an increase in cAMP levels.

Tip: The data provided above is for reference only. Actual results may vary depended on the performance of the microplate reader used.

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