CAT.&Size: A010101 (1,000 tests)
A010012 (10,000 tests)
A010014 (100,000 tests)

Storage at: -60℃ or below

VKEYBIO-03-2025

For Research Use Only

Not For Diagnostic Or Therapeutic Use

KeyTec® cAMP TR-FRET KIT

Technical Manual

1. Introduction

KeyTec® cAMP TR-FRET KIT is designed for quantitative measurement of adenosine 3',5'-cyclic adenosine monophosphate (cAMP) in cell. The assay kit provides cAMP tracer (Streptavidin - Solar Eu*[1] and Biotin - cAMP pre-mix solution) , a cAMP-specific antibody labeled with KeyTec® TR-FRET Solar LA*[2] , and other relevant reagents. This assay is based on a competitive immunoassay method using KeyTec® TR-FRET technology, offering a simple, rapid, highly specific and sensitive, as well as reproducible detection process. The principle, outlined in Figure 1:

The binding of cAMP-specific antibody and cAMP tracer brings the TR-FRET donor and acceptor into close proximity, enabling resonance energy transfer (RET) upon excitation and generating a TR-FRET signal. Native cAMP produced by cells competes with cAMP tracer for binding to cAMP-specific antibody. The intensity of TR-FRET signal is inversely proportional to the concentration of cAMP.

*[1] KeyTec® TR-FRET Solar Eu: TR-FRET Donor

*[2] KeyTec® TR-FRET LA: TR-FRET Acceptor

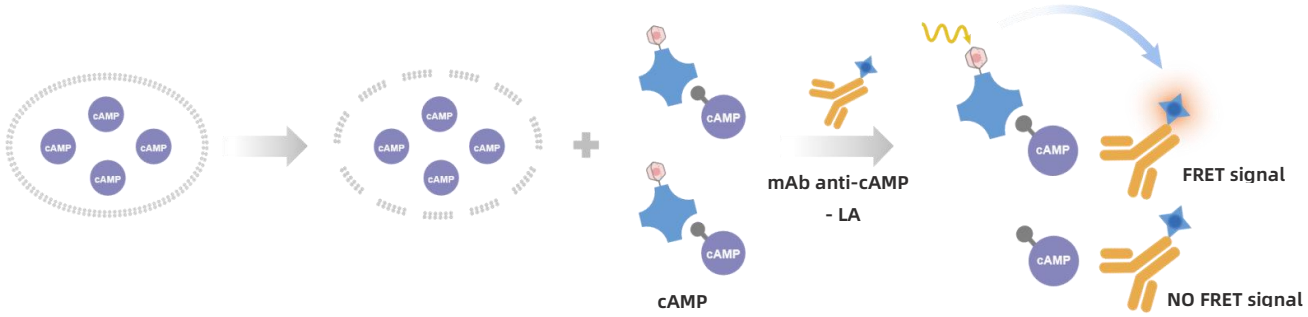

Figure1. The principle of KeyTec® TR-FRET cAMP detection

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2. Components

Components	Storage	A010101 (1,000 tests*3)	A010012 (10,000 tests*3)	A010014 (100,000 tests*3)
mAb anti-cAMP - LA (100X)	≤ -60 ℃	1 vial 50 μL/vial	1 vial 500 μL/vial	1 vial 5 mL/vial
cAMP Standard Lyophilized	2-8 ℃	1 vial 5 nmoles/vial A015011	2 vials 5 nmoles/vial A015011	6 vials 5 nmoles/vial A015011
cAMP tracer*4 (100X)	≤ -60 ℃	1 vial 50 μL/vial	1 vial 500 μL/vial	1 vial 5 mL/vial
Detection Buffer (5X)	≤ -60 ℃	1 bottle 2 mL/bottle	1 bottle 20 mL/bottle	1 bottle 200 mL/bottle

*3 tests refer to the number of assay wells that can be performed in 96-well or 384-well plates with 20 μL in total reaction volume. The reagents of the kit are suggested to use as recommended.

*4 cAMP tracer : Streptavidin - Solar Eu and Biotin - cAMP pre-mix solution.

3. Storage

- Store all reagents according to the recommended conditions. The products are stable for one year from the date of receipt.
- After thawing, Aliquot the stock into single-use volumes (recommended minimum: 10μL) to avoid repeated freeze-thaw cycles. Store these aliquots at -60 °C and below.

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4. Required Components (Not Supplied)

Material	Brand	CAT.
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
KeyTec® TR-FRET IBMX[*5]	VKEY-BIO	A1010033
KeyTec® TR-FRET Forskolin [*6]	VKEY-BIO	A1010021S
384-well Microplate	VKEY-BIO	M2000102N
Top sealing film	VKEY-BIO	M1000102N

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

*[6] Forskolin 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 Gi coupled receptor study as preactivation step to show cAMP level inhibition upon cell stimulation.

5. Procedure

5.1 Reaction System

Components	Volume[*7] (20 μL)
Test samples or positive control	5 μL
Compound solution or Stimulation Buffer（1X）	5 μL
cAMP tracer working solution (1X)	5 μL
mAb anti-cAMP - LA working solution (1X)	5 μL

*[7] Recommended Format: Shallow-well 384-well microplate; For 96-well or 1536-well microplates, proportionally scale the reaction system.

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5.2 Reagent preparation

- After thawing on ice, Aliquot the stock into single-use volumes (recommended minimum: 10μL) to avoid repeated freeze-thaw cycles. Store these aliquots at -60 °C and below.
- Before use, equilibrate all reagents to RT.
- Use the provided/recommended buffers to prepare sample and detection reagents.

6. Working Solution Preparation

6.1 Buffer Preparation

- Stimulation Buffer (1X): 1X HBSS, 5 mM HEPES, 0.1% BSA（pH 7.4) and IBMX（refer to 6.3).
- Use cell culture medium (e.g., RPMI or DMEM) to replace Stimulation Buffer if the compound stimulation time is above 2 hours
- Detection Buffer（1X）: dilute 1 volume of Detection Buffer（5X）with 4 volume of Ultra-pure water

6.2 Donor and Acceptor Preparation

- mAb anti-cAMP - LA working solution (1X): For example, Prepare 500 μL working solution(1X), dilute 5 μL of 100X mAb anti-cAMP - LA stock solution with 495 μL of Detection Buffer(1X).
- cAMP Tracer working solution (1X): For example, Prepare 500 μL working solution(1X), dilute 5 μL of 100X cAMP Tracer stock solution and 495 μL of Detection Buffer(1X).

6.3 Additive

- IBMX concentration optimization: In order to minimize the effect of IBMX , we recommend to optimize the IBMX concentration. Dilute IBMX stock solution (500mM in 100% DMSO) in 1X stimulation buffer at the desired concentration. An empirical concentration of working solution at 0.5mM is recommended. For example: mix 10 μL of the 500mM IBMX stock solution with 9.99 mL of 1X Stimulation Buffer. Note: The Stimulation Buffer with IBMX must be prepared

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before use.

- Forskolin concentration optimization: In order to increase the intracellular level of cAMP , we recommend to optimize the Forskolin concentration. Dilute Forskolin stock solution (10mM in 100% DMSO) in 1X stimulation buffer at the desired concentration. 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 at the desired concentration. Note: Forskolin working solution in 1X Stimulation Buffer medium must be prepared before use.

6.4 Standard

- Run a cAMP standard curve to determine the linear range of the assay. This curve is the mathematical model used to convert the sample's TR-FRET signal into a cAMP concentration. In particular, the IC10 and IC90 will be useful to optimize the cell density of the cell lines. If the cAMP levels of unstimulated and stimulated cells fall outside the assay's linear range, the data will be inaccurate.

- **cAMP standard stock solution(50 µM)**: Before reconstitution, centrifuge cAMP standard to pellet the powder to the bottom (850×g,1-2 minutes). Add 100 µL of Ultra-pure water to the powder and mix gently.

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Standard	cAMP Final Conc. (nM)	cAMP Working Conc. (nM)	Dilution
STD-9	1,000	4,000	8 μL standard stock solution + 92 μL Stimulation Buffer[*8]
STD-8	300	1,200	30 μL STD-9 + 70 μL Stimulation Buffer[*8]
STD-7	100	400	30 μL STD-8 + 60 μL Stimulation Buffer[*8]
STD-6	30	120	30 μL STD-7 + 70 μL Stimulation Buffer[*8]
STD-5	10	40	30 μL STD-6 + 60 μL Stimulation Buffer[*8]
STD-4	3	12	30 μL STD-5 + 70 μL Stimulation Buffer[*8]
STD-3	1	4	30 μL STD-4 + 60 μL Stimulation Buffer[*8]
STD-2	0.3	1.2	30 μL STD-3 + 70 μL Stimulation Buffer[*8]
STD-1	0.1	0.4	30 μL STD-2 + 60 μL Stimulation Buffer[*8]
STD-0 (PC)	0	0	70 μL Stimulation Buffer[*8]

[*8] Stimulation Buffer can be replaced by the cell culture media.

6.5 Parameter Optimization

- Assay parameters -including cell density, IBMX concentration, agonist (or Forskolin) concentration , stimulation time and incubation temperature (room temperature or 37 ℃)- must be optimized for each GPCR cellular model.

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7. Procedure

- Follow the steps in the table below.

	Standard Curve	G_s Agonist	G_i Agonist	G_s Antagonist	G_i Antagonist
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[*8]	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 plate to prevent liquid evaporation, Incubation（RT or 37 ℃）for appropriate time				
Step 5	5 µL cAMP tracer（1X working solution）				
Step 6	5 µL mAb anti-cAMP - LA（1X working solution）				
Step 7	Seal the plate to prevent liquid evaporation, Incubation（RT）for 1-2 h				
Step 8	Record the data in plate reader with top sealing film				

[*8] Stimulation Buffer can be replaced by the cell culture media.

8. Data Analysis

- Calculate the 665 nm/620 nm Ratio（TR-FRET Ratio）and the percentage coefficient of variation (CV %) for each well.

$$\text{TR-FRET Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10{,}000$$

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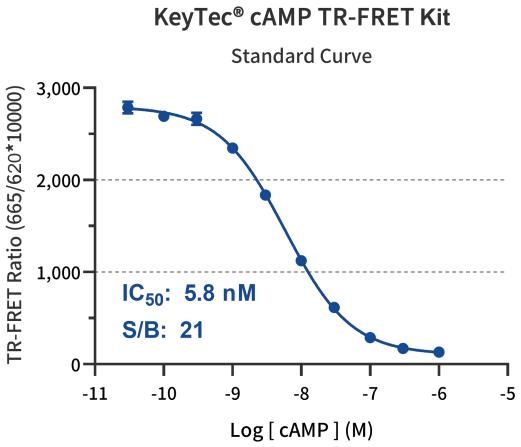
9. Summary

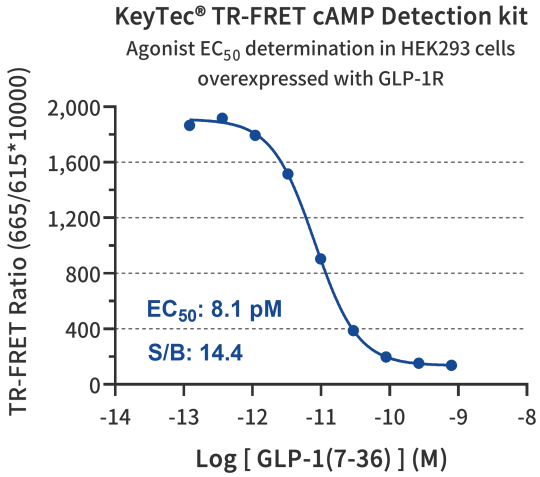
9.1 Standard Curve

Standard curve	cAMP (nM)	TR-FRET Ratio	CV%
STD-9	1,000	132	1.3
STD-8	300	172	2.4
STD-7	100	288	3.4
STD-6	30	616	3.5
STD-5	10	1,123	2.3
STD-4	3	1,853	0.9
STD-3	1	2,330	1.8
STD-2	0.3	2,664	2.5
STD-1	0.1	2,691	1.7
STD-0	0	2,787	2.3



9.2 Results

- **GLP-1(7-36) stimulates cAMP production in GLP-1R-HEK293 cells in a dose-dependent manner.** GLP-1R over-expressing HEK293 cells (10K cells/well, duplicate) were treated with increasing concentrations of GLP-1(7-36) for 30 min at 37 ℃. and intracellular cAMP was measured. The curve represents a four-parameter logistic fit of the data.



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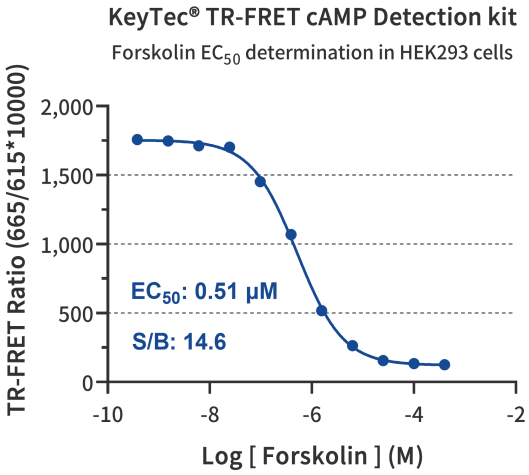
- **Forskolin stimulates cAMP production in HEK293 cells in a dose-dependent manner.** HEK293 cells (10K cells/well, duplicate) were treated with increasing concentrations of Forskolin for 30 min at 37 ℃. and intracellular cAMP was measured. The curve represents a four-parameter logistic fit of the data.



Note: Exemplary data shown. Results are instrument-dependent.

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