Add&Read cAMP Detection Kit

DD2801

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User Manual

Version 24.1

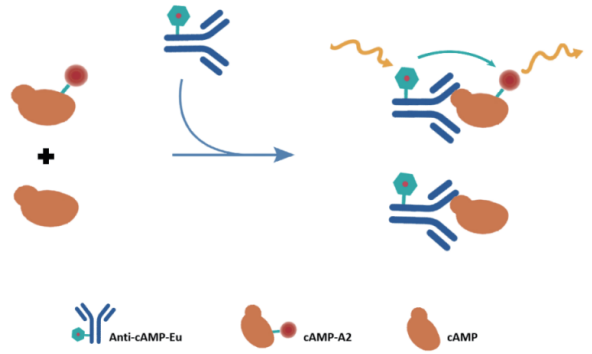
Contents

For Research Use Only. Not for use in diagnostic procedures.

01/Product Description

cAMP Detection Kit is a competitive immunoassay intended to measure cyclic AMP (cAMP) accumulation in cells. The kit contains an antibody that specifically recognizes cAMP, labeled with a fluorescent donor Eu (Anti-cAMP-Eu), and cAMP is labeled with a fluorescent acceptor A2 (cAMP-A2).

In the solution, Anti-cAMP-Eu binds with cAMP-A2, which can lead to Fluorescence Resonance Energy Transfer (FRET). When excited with light at 320 nm, the fluorescent donor Eu emits light at 620 nm. This 620 nm light, in turn, excites the fluorescent acceptor A2, causing it to emit light at 665 nm. After cell lysis, the cAMP produced within the cells is released into the lysate sample, which competes for binding with Anti-cAMP-Eu, disrupting the FRET phenomenon.The FRET signal value (the ratio of light intensity at 665 nm to 620 nm) is inversely proportional to the concentration of cAMP in the standard or sample.



02/Components

Components	DD2801-01 (1,000 tests)	DD2801-02 (5,000 tests)	DD2801-03 (20,000 tests)
cAMP Standard	1 ml	2 × 1 ml	5 × 1 ml
Anti-cAMP-Eu (100 ×)	60 µl	300 µl	2 × 600 µl
cAMP-A2 (100 ×)	60 µl	300 µl	2 × 600 µl
Stimulation Buffer (5 ×)	16 ml	60 ml	2 × 100 ml
Lysis Detection Buffer	20 ml	70 ml	3 × 100 ml

03/Storage Conditions

Store at -30 ~ -15℃ and transport at ≤ 0℃.

▲ cAMP Standard, Anti-cAMP-Eu, and cAMP-A2 are stored at -30 ~ -15℃. Upon first use, freeze in aliquots to avoid multiple freeze/thaw cycles.

▲ Stimulation Buffer and Lysis Detection Buffer are stored at -20 ~ 4℃.

04/Applications

Suitable for all cell lines.

05/Self-prepared Materials

Reagent: IBMX (MCE#HY-12318), Forskolin (MCE #HY-15371);
Consumables: 96-well shallow plates (Vazyme #DD2601), 384-well shallow plates (Thermo #264706);
Equipment: Microplate reader (equipped with HTRF/TR-FRET module).

06/Notes

1. Use the Stimulation Buffer and Lysis Detection Buffer provided in the kit to ensure the accuracy and stability of the experimental results.
2. Before starting the cell experiment, prepare the IBMX, Forskolin, and other related reagents from the self-supplied materials.

07/Experiment Process

07-1/Stimulation Buffer Preparation

The Stimulation Buffer stock solution is 5 ×, and it needs to be diluted to 1 × with ddH_2O before use.

1. Preparation of 1 × Stimulation Buffer (stock solution is 5 ×):

a. Dilute 1 volume of Stimulation Buffer(5 ×) in 4 volumes of distilled water, and mix well.
▲The 1 × Stimulation Buffer must be prepared immediately before use and used fresh.

07-2/Anti-cAMP-Eu & cAMP-A2 Preparation

The reaction volume for 96/384 well shallow plates is 20 µl, and it is recommended to add 5 µl each of Anti-cAMP-Eu and cAMP-A2 working solution to every 20 µl system. Before preparation, calculate the volume of Anti-cAMP-Eu and cAMP-A2 required for this experiment: V= (number of wells × 5/100) µl

1. Preparation of Anti-cAMP-Eu working solution (stock solution is 100 ×):

a. Allow the Anti-cAMP-Eu to warm up to room temperature for at least 30 mins before use, and mix well using a pipette or vortex mixer.
b. Dilute 1 volume Anti-cAMP-Eu in 99 volumes of Lysis Detection Buffer, and mix well.
▲When calculating the number of wells for sample preparation, consider the pipetting loss, and it is generally recommended to prepare: Number of sample wells = actual number of detection wells × 110%.

2. Preparation of cAMP-A2 working solution (stock solution is 100 ×):

a. Allow the cAMP-A2 to warm up to room temperature for at least 30 mins before use, and mix well using a pipette or vortex mixer.
b. Dilute 1 volume cAMP-A2 in 99 volumes of Lysis Detection Buffer, and mix well.
▲Stock solutions may be frozen and thawed 7 times. Freeze in aliquots to avoid multiple freeze/thaw cycles.

07-3/Additive Preparation

1. Preparation of IBMX Working Solution

IBMX stock solution is provided at 500 mM in 100% DMSO.Dilute IBMX stock solution in the stimulation buffer at the desired concentration.An empirical concentration of working solution at 0.5 mM is recommended. For instance, by adding 10 µl of the 500 mM IBMX storage solution to 9,990 µl of Stimulation Buffer or the corresponding cell culture medium, you will obtain 10 ml of 0.5 mM IBMX working solution.
▲The stimulation buffer with added IBMX must be prepared immediately before use and used fresh.

2. Preparation of Forskolin Working Solution

The Forskolin stock solution is provided at 10 mM in 100% DMSO. It is recommended to prepare 2 sequential dilutions to avoid Forskolin precipitation :

a. Dilute with 100% DMSO to make a 2 mM intermediate storage solution; for example, take 5 µl of the 10 mM Forskolin storage solution and add it to 20 µl of DMSO solution to obtain 25 µl of 2 mM intermediate storage solution.
b. Dilute the intermediate storage solution to the desired concentration with Stimulation Buffer or the corresponding cell culture medium.
▲The Forskolin working solution must be prepared immediately before use and used fresh.

07-4/Standard Preparation

1. cAMP standards must be prepared in the same stimulation buffer as that used for the cell based assay supplemented with IBMX.
2. Before starting the cell experiments, it is recommended to conduct a biochemical standard curve test to evaluate the linear dynamic range of the detection system under the current laboratory conditions (which may be affected by the performance of the microplate reader). This helps to verify whether the analytical method can achieve the expected signal-to-background ratio (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.
3. Please dilute and prepare the standards according to the concentrations shown in the table below. Use the Stimulation Buffer or the corresponding cell culture medium to prepare the standard curve. To reduce matrix effect interference and obtain a more accurate cAMP back-calculated concentration, it is recommended to use the same solution as that used for

cell compound treatment to prepare the standard curve. For example, if the cell treatment uses DMEM medium with added compound (stored in DMSO solution) and IBMX, then use DMEM medium with the same proportion of DMSO and IBMX for standard dilution. Determine the total amount of standard preparation according to the experimental requirements; the amount shown in the table is for reference only.

4. The reaction volume for 96/384 well shallow plates is 20 µl, and each well requires 5 µl of cAMP Standard. Calculate the required volume of cAMP Standard before preparation. The following steps will yield 60 µl of cAMP Standard.
5. Allow the cAMP Standard to warm up to room temperature for at least 30 mins before use.Mix well using a pipette or vortex mixer and take 80 µl of cAMP Standard; this is Std 8.
6. Take 20 µl of Std 8 and add 60 µl of 1 x Stimulation Buffer, mix well to obtain Std 7.
7. Continue the 4-fold gradient dilution in the same manner to obtain Std 7 to Std 1.

Standard	Dilution Step	Working Concentration of cAMP (nM)	Final Concentration of cAMP (nM)
Std 8	-	2,848	712
Std 7	20 µl Std 8 + 60 µl Stimulation Buffer	712	178
Std 6	20 µl Std 7 + 60 µl Stimulation Buffer	178	44.5
Std 5	20 µl Std 6 + 60 µl Stimulation Buffer	44.5	11.13
Std 4	20 µl Std 5 + 60 µl Stimulation Buffer	11.13	2.78
Std 3	20 µl Std 4 + 60 µl Stimulation Buffer	2.78	0.70
Std 2	20 µl Std 3 + 60 µl Stimulation Buffer	0.70	0.17
Std 1	20 µl Std 2 + 60 µl Stimulation Buffer	0.17	0.04
Std 0	60 µl Stimulation Buffer	0	0

▲ The cAMP Standard should be aliquoted and stored at -30 ~ -15℃ to avoid repeated freeze-thaw cycles.

07-5/Cell Treatment Steps

1. Harvest fresh cells by centrifugation at 3,200 × rpm (1,000 × g) for 10 minutes. Discard the culture medium and resuspend the cells in Stimulation Buffer containing 0.5mM
2. IBMX.
   Dilute the cells to the appropriate cell count and the compounds to the appropriate
3. concentrations (agonists or antagonists).
   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
4. (Gs or Gi).
   IBMX is a widely used, non-selective phosphodiesterase inhibitor that can inhibit the degradation of cAMP, leading to its accumulation at higher levels for experimental detection. At the recommended concentration, it should not affect the standard curve. However, the impact of IBMX on compound activity and the optimal concentration should
5. be assessed based on actual detection conditions.

A cAMP standard curve should be performed in each experiment, which not only allows for the assessment of whether the expected IC50 and S/B can be obtained but also converts the TR-FRET signal into cAMP concentration. Initial optimization of cell density, Forskolin concentration, agonist concentration, stimulation time, and temperature is necessary to ensure that the measured TR-FRET signal falls within the working range of the standard curve (cAMP concentration between IC10 and IC90). This is primarily because the standard curve is an S-shaped curve, with the high concentration plateau region being very sensitive to changes in cAMP concentration, while the low concentration plateau region's signal is insensi-

6. tive to changes, and data from this region are inaccurate.
   For Gi-type receptor detection, Forskolin needs to be optimized. The preferred method of optimization is to perform a dose-response curve of Forskolin at different cell densities, and determine the maximum detection window under each condition by calculating the ratio of
7. cells pre-treated with the maximum dose of agonist to cells treated with Forskolin alone.
   When testing antagonists, it is necessary to stimulate with an appropriate concentration of agonist. Generally, the EC50 of the agonist should be determined first, and then an agonist
8. concentration at the EC90 can be used for antagonist detection.
   Generally, stimulating at room temperature for 30 minutes will yield satisfactory results. However, different cell lines and drugs may require optimization based on actual conditions, and you can choose between room temperature or 37℃.

07-6/Reaction System

1.Sample Addition

For 96/384 well shallow plates with a reaction volume of 20 µl, samples should be added according to the experimental groups and reaction systems as outlined in the table below:

	Standard	Positive Control	Negative control	Cell	Cell Positive Control	Cell Negative Control
cAMP Standard	5 µl	-	-	-	-	-
Cell	-	-	-	5 µl	5 µl	5 µl
Compounds				5 µl	-	-
Seal the plate and incubate for appropriate time at room temperature or 37℃ . *						
cAMP-A2	5 µl	5 µl	-	5 µl	5 µl	
Anti-cAMP-Eu	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Stimulation Buffer	5 µl	10 µl	10 µl		5 µl	5 µl
Lysis Detection Buffer	-	-	5 µl			5 µl

*The duration of agonist stimulation significantly affects the experimental outcomes. The incubation time requires optimization, with a recommendation for 30 minutes at 37℃.

2. Reagent Addition

a. Add 5 μl of cAMP Standard/Cell to the 96/384 well shallow plate;
b. Add 5 μl of Stimulation Buffer/Compounds, gently mix twice within the well using a pipette, seal the plate and incubate for appropriate time at room temperature or 37°C;
c. Add 5 μl of cAMP-A2, gently mix twice within the well using a pipette;
d. Add 5 μl of Anti-cAMP-Eu, gently mix twice within the well using a pipette (this reagent should be added last);
e. Incubate at room temperature or 25℃ for 1 hour, then detect using a microplate reader (equipped with HTRF/TR-FRET module), with excitation at 320 nm, and measure the emission at two wavelengths (665 nm and 620 nm).

08/Data/Result Calculation

1. Standard Curve Fitting

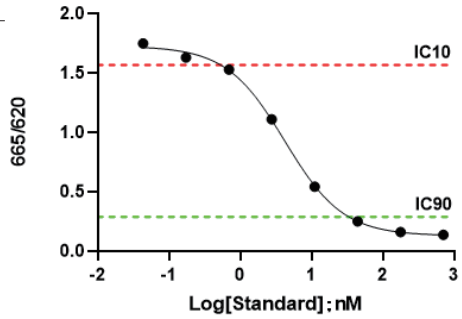
Divide the fluorescence value at 665 nm by that at 620 nm to obtain the 665/620 ratio. Plot the Log_{10}[Sample Concentration] on the x-axis and the 665/620 values on the y-axis, and perform a four-parameter fit to create the curve.

2. Calculation of IC10 and IC90

Use Graphpad Prism software to calculate IC10 and IC90; in the data table, click on "Analyze," select "Nonlinear regression," then choose "Equation pane": Dose-response—Specific, X as Log(concentration); then select "Log(agonist) vs. response—Find ECanything."
The parameter F must be constrained to a constant value between 0 and 100. If fitting for IC90, set F to 10; the report will display ECF, where ECF represents the concentration rather than the logarithmic concentration.

09/Product Performance Indicators

Std No.	[Standard], nM	665/620
Std 8	712	0.14
Std 7	178	0.16
Std 6	44.5	0.25
Std 5	11.13	0.54
Std 4	2.78	1.11
Std 3	0.70	1.53
Std 2	0.17	1.63
Std 1	0.04	1.75
Std 0	0	1.75
Negative Control	-	0.09



▲Due to variations among different laboratories and microplate readers, discrepancies in detection results may occur across different lab settings.

09-1/Analytical Sensitivity

Determine the detection limit by repeating the measurement of Standard 0 twenty times and the quantification limit by repeating the measurement of 0.75 nM twenty times.

Type	Stimulation Buffer
Limit of Detection (LOD)	0.43 nM
Limit of Quantitation (LOQ)	0.75 nM

▲Due to variations among different laboratories and microplate readers, discrepancies in detection results may occur across different lab settings.

09-2/Precision

This kit uses five known concentrations of quality control (QC) samples to assess intra-assay precision by measuring them in triplicate on the same microplate. It also uses five known concentrations of QC samples to assess inter-assay precision, measured in six independent analytical batches by two technicians.

Table1. Intra-assay Precision

Sample	Number of Times (N)	Mean Concentration (nM)	SD	CV
Sample at the Upper Limit of Quantitation (ULOQ)	3	44.18	4.34	9.82%
High Concentration Sample	3	34.78	3.55	10.20%
Medium Concentration Sample	3	3.89	0.33	8.48%
Low Concentration Sample	3	1.92	0.14	7.30%
Sample at the Lower Limit of Quantitation (LLOQ)	3	0.71	0.10	13.75%

Table 2. Intra-assay Precision

Sample	Number of Times (N)	Mean Concentration (nM)	SD	CV
Sample at the Upper Limit of Quantitation (ULOQ)	6	44.08	4.54	10.30%
High Concentration Sample	6	33.47	3.30	9.85%
Medium Concentration Sample	6	3.94	0.31	7.75%
Low Concentration Sample	6	1.99	0.15	7.31%
Sample at the Lower Limit of Quantitation (LLOQ)	6	0.75	0.08	10.89%

09-3/Accuracy

This kit uses five quality control (QC) samples with known concentrations, each measured in triplicate on the same microplate, to assess accuracy by comparing the measured concentrations to their theoretical concentrations.

Sample	Theoretical Concentration (nM)	Number of Times (N)	Mean Measured Concentration (nM)	Accuracy (80%–120%)
Sample at the Upper Limit of Quantitation (ULOQ)	44.5	3	44.08	99%
High Concentration Sample	35	3	33.47	96%
Medium Concentration Sample	4	3	3.94	99%
Low Concentration Sample	2	3	1.99	99%
Sample at the Lower Limit of Quantitation (LLOQ)	0.75	3	0.75	101%

09-4/Specificity

This kit is designed to detect both native and recombinant cAMP. Specificity testing was conducted on the following factors at a concentration of 100 ng/ml, and no significant cross-reactivity was observed.

100 ng/ml			
ATP	CTP	UTP	GTP
ADP	AMP	GMP	Adenosine