CAMP - GI KIT

PROTOCOL

CAMP - GI KIT:

Part #: 62AM9PEB (1,000 tests), 62AM9PEC (20,000 tests), 62AM9PEJ (100,000 tests)

Version: 5 (January 2020) **Storage temperature:** 2-8°C

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

Cisbio Bioassays' cAMP - Gi assay is a competitive immunoassay intended to measure cyclic AMP (cAMP) accumulation in cells. It enables the direct pharmacological characterization of compounds acting on Gi-coupled receptors in either adherent or suspension cells.

The principle is based on HTRF® technology. Native cAMP produced by cells or unlabeled cAMP (standard curve) compete with Europium cryptate-labeled cAMP (Europium donor) for binding to monoclonal cAMP d2-labeled antibody (red acceptor). The specific signal is inversely proportional to the concentration of cAMP in the standard or sample (Fig. 1).

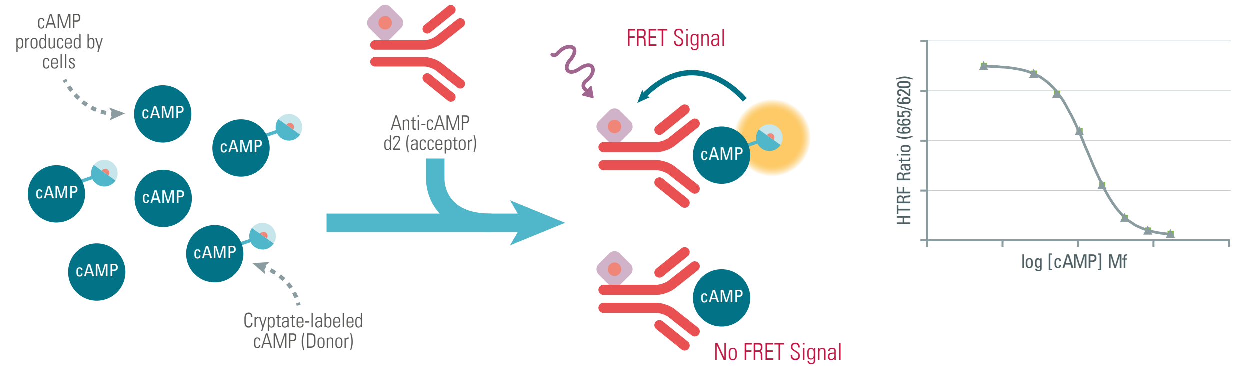


Figure 1: Principle of HTRF cAMP competitive binding assay.

As for all other HTRF assays, the calculation of the fluorescence ratio (665 nm/620 nm) eliminates any possible photophysical interferences and means the assay is unaffected by the experimental medium conditions (e.g. culture medium, serum, biotin, colored compounds, etc.).

MATERIALS & EQUIPMENT

MATERIALS PROVIDED:

NUMBER OF TESTS (CAT. #)	62AM9PEB (1,000 tests)	62AM9PEC (20,000 tests)	62AM9PEJ (100,000 tests)	REF# (when availabe separately)
cAMP d2 antibody (red acceptor, lyophilized)	1 vial	1 vial	5 vials	
cAMP Eu-cryptate reagent (Europium donor, lyophilized)	1 vial	1 vial	5 vials	
cAMP-Gs dynamic & Gi Standard Concentrate (lyophilized)	1 vial	1 vial	1 vial	62AM4CDA
Stimulation Buffer 1 (5X)*	1 vial (8 mL)	1 vial (100 mL)	5 vials (100 mL ea)	64SB1FDD (100 mL)
Lysis & Detection Buffer 8**	1 vial (13 mL)	1 vial (200 mL)	5 vials (200 mL ea)	64CL8FDG (200 mL)

* Lysis & Detection Buffer is used to prepare working solutions of acceptor and donor reagents.
** Stimulation Buffer 1 is used to prepare working standard solutions, cells & compounds
Note: Reagent preparation instructions are included below

PURCHASE SEPARATELY:

cAMP ASSAY SPARE REAGENTS		
IBMX[1] (PDE inhibitor, 500 mM)	62AMXADA (40 µL[3])	62AMXADC (500 µL[3])
Forskolin[2] (Adenylyl cyclase activator, 10 mM)	62AMYADA (40 µL[3])	62AMYADC (800 µL[3])

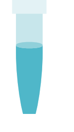
- Cell Culture Medium (appropriate for cell line used)
- Low volume white microplates[4]
- HTRF®-Certified Reader[5]

(1) IBMX is the most commonly used phosphodiesterase Pan-inhibitor. It guarantees high level of cAMP accumulation in the cells.
(2) Forskolin activates the adenylyl cyclase enzyme and increases the intracellular level of cAMP. It is used as a positive control for Gi 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.
(3) 100% DMSO Stock solution
(4) Assay volumes can be adjusted proportionally to run the assay in 96- or 1536-well microplates.For more information about microplate recommendations, please visit our website at: www.cisbio.com/content/microplates-recommendations
(5) For a list of HTRF-compatible readers and set-up recommendations, visit www.cisbio.com/readers

STORAGE AND STABILITY

Store the kit at 2-8°C until the expiration date indicated on the package.

Once reconstituted, stock solutions are stable at 2-8°C for one week. Stored at -16°C or below, they are stable for 6 months. Stock solutions may be frozen and thawed only once. Freeze in aliquots to avoid multiple freeze/thaw cycles.



Working solutions of conjugates (1X) and standards (for standard dose response curve) can be stored at 2-8°C for up to 24 hours. DO NOT FREEZE.

SPARE REAGENTS

Solutions of IBMX and Forskolin stored at 4°C are stable for 3 months. Stock solution may be frozen and thawed only once. Freeze in aliquots to avoid multiple freeze/thaw cycles.

REAGENT PREPARATION

BEFORE YOU BEGIN:

1. It is very important to prepare reagents in the buffer specified. Use of an incorrect diluent may affect reagent stability and assay results.
2. Allow the lyophilized reagents to warm up to room temperature for at least 30 mins before reconstitution.
3. Working solutions must be used within 24 hours. Do not freeze.
4. Stimulation buffer supplemented with IBMX must be prepared just before use
5. Forskolin (FSK) working solution must be prepared just before use
6. Conjugate solutions must be prepared in individual vials and dispensed separately for the assay.
7. cAMP standards (for standard curve) must be prepared in the same stimulation buffer as that used for the cell based assay supplemented with IBMX.
8. Stimulation Buffer 1 included in the kit should be replaced by cell culture medium if the compound stimulation time is above 2 h.
9. Take care to prepare stock and working solutions according to the directions below for the kit size you have purchased.
10. Be sure to set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader. (more information about HTRF® compatible readers and set-up recommendations available at www.cisbio.com/compatible-readers)

TO PREPARE STOCK SOLUTIONS:

If the kit is not going to be used at once, please consider aliquoting the stock solutions before freezing considering solutions can only be frozen/thawed once.

1,000 TESTS KIT	20,000/100,000 TESTS KITS
1. Reconstitute the cAMP Eu Cryptate reagent (donor conjugate) with 1.1 mL distilled water. Mix gently. *This is a 5X stock solution which can be stored for one week at 2-8°C or frozen up to 6 months.*	1. Reconstitute the cAMP Eu Cryptate reagent (donor conjugate) with 5 mL distilled water. Mix gently. *This is a 20X stock solution which can be stored for one week at 2-8°C or frozen up to 6 months.*
2. Reconstitute the cAMP-d2 antibody (acceptor conjugate) with 1.1 mL distilled water. Mix gently. *This is a 5X stock solution which can be stored for one week at 2-8°C or frozen up to 6 months.*	2. Reconstitute the cAMP-d2 antibody (acceptor conjugate) with 5 mL distilled water. Mix gently. *This is a 20X stock solution which can be stored for one week at 2-8°C or frozen up to 6 months.*
3. Reconstitute the cAMP Gs Dynamic & Gi Standard Concentrate using chosen stimulation buffer (e.g., DMEM, MEM, RPMI). See instructions on vial label for reconstitution volume. Mix gently after reconstitution. *The Standard Concentrate is stable frozen for 6 months.*	3. Reconstitute the cAMP Gs Dynamic & Gi Standard Concentrate using chosen stimulation buffer (e.g., DMEM, MEM, RPMI). See instructions on vial label for reconstitution volume. Mix gently after reconstitution. *The Standard Concentrate is stable frozen for 6 months.*

TO PREPARE WORKING SOLUTIONS:

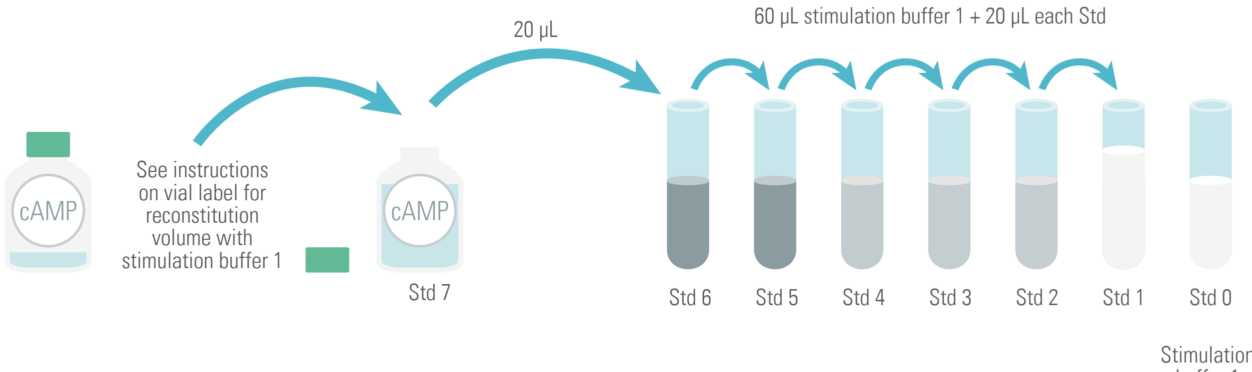
1,000 TESTS KIT	20,000/100,000 TESTS KITS
1. Stimulation Buffer 1: From the 5X stock solution: dilute 1 volume of Stimulation Buffer 1 (5X) in 4 volumes of distilled water	1. Stimulation Buffer 1: From 5X stock solution: dilute 1 volume of Stimulation Buffer 1 (5X) in 4 volumes of distilled water
2. Detection reagents: From the 5X stock solutions of cAMP Eu Cryptate reagent and cAMP-d2 antibody (reconstituted reagents) dilute in separate vials, 1 volume of each stock solutions in 4 volumes of Lysis & detection Buffer 8 (e.g., 0.5 mL of reconstituted reagent + 2 mL of Lysis & Detection Buffer).	2. Detection reagents: From the 20X stock solutions of cAMP Eu Cryptate reagent and cAMP-d2 antibody (reconstituted reagents) dilute in separate vials, 1 volume of each stock solutions in 19 volumes of Lysis & detection Buffer 8 (e.g., 0.5 mL of reconstituted reagent + 9.5 mL of Lysis & Detection Buffer)

CAMP ASSAY SPARE REAGENTS
3. IBMX 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. (e.g., 40 µL of IBMX stock solution + 39 960 µL of stimulation buffer)
4. Forskolin The forskolin stock solution is provided at 10 mM in DMSO. Working solution preparation : it is recommended to prepare 2 sequential dilutions to avoid Forskolin precipitation . First, 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 predilution in the stimulation buffer at the desired concentration.

STANDARD CURVE

Run a standard dose response curve to determine the linear dynamic range of the assay with your cell culture medium (Stimulation Buffer 1) in your laboratory. 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.

TO PREPARE WORKING STANDARDS:



STANDARD	SERIAL DILUTIONS	cAMP WORKING SOLUTION nM	cAMP FINAL CONCENTRATION nM
Std7	Reconstituted cAMP Standard	2848*	712
Std6	20 µL Std7 + 60 µL Stimulation Buffer 1	712	178
Std5	20 µL Std6 + 60 µL Stimulation Buffer 1	178	44.5
Std4	20 µL Std5 + 60 µL Stimulation Buffer 1	44.5	11.1
Std3	20 µL Std4 + 60 µL Stimulation Buffer 1	11.1	2.78
Std2	20 µL Std3 + 60 µL Stimulation Buffer 1	2.78	0.69
Std1	20 µL Std2 + 60 µL Stimulation Buffer 1	0.69	0.17
Std0 (positive control)	60 µL Stimulation Buffer 1	0	0

*The actual concentration of cAMP after reconstituting the provided vial is indicated on the label of the maximum standard

STANDARD CURVE ASSAY PROTOCOL

	STANDARD (STD7-STD0)	NEGATIVE CONTROL
Step 1	Dispense 5 µL of each cAMP standard (Std7-Std0) into each standard well, in triplicate.	Dispense 5 µL Stimulation Buffer 1 into negative control wells, in triplicate.
Step 2	Add 5 µL of Stimulation Buffer 1 into each standard well.	Add 5 µL of Stimulation Buffer 1 into negative control wells.
Step 3	Add 5 µL of cAMP Eu Cryptate reagent working solution into each standard well.	Add 5 µL of cAMP Eu Cryptate reagent working solution into negative control wells.
Step 4	Add 5 µL of cAMP d2 antibody working solution into each standard well.	Add 5 µL of Lysis & Detection Buffer 8 into negative control wells.
Step 5	Seal the plate and incubate 1 hour at room temperature.	Seal the plate and incubate 1 hour at room temperature.
Step 6	Remove the plate seal and read on an HTRF compatible reader.	Remove the plate seal and read on an HTRF compatible reader.

Suggested plate map:

	1	2	3
A	5 µL Std 0 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well A1	Repeat Well A1
B	5 µL Std 1 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well B1	Repeat Well B1
C	5 µL Std 2 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well C1	Repeat Well C1
D	5 µL Std 3 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well D1	Repeat Well D1
E	5 µL Std 4 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well E1	Repeat Well E1
F	5 µL Std 5 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well F1	Repeat Well F1
G	5 µL Std 6 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well G1	Repeat Well G1
H	5 µL Std 7 5 µL Stimulation. Buffer 1 5 µL cAMP Eu Cryptate reagent 5 µL cAMP d2 antibody	Repeat Well H1	Repeat Well H1
I	5 µL Stimulation. Buffer 1 5 µL Stimulation. Buffer 1 5 µL Lysis & Detection Buffer 5 µL cAMP Eu Cryptate reagent	Repeat Well I1	Repeat Well I1

Standard curve

Negative control (e.g. no cAMP-d2 antibody)

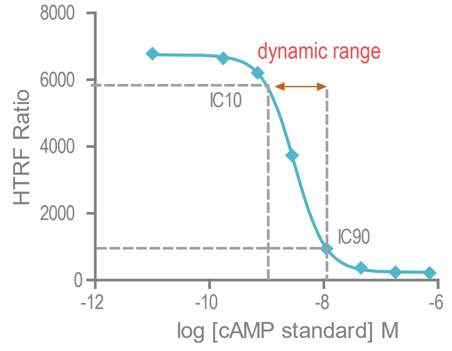
DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 104$$

2. Plot the HTRF ratio versus the cAMP concentrations.

For more information about data reduction, please visit www.cisbio.com/data-reduction



Characteristics of the assay relative to the IC_{50} and the signal over background.

Obtained using the PHERAstar Plus reader (BMG LABTECH).

A Guide to Optimizing Agonists Gi" and "A Guide to Optimizing Antagonists Gi

	IC_{50} (nM)	S/B
Incubation 1 hour at RT	3	31

CELL-BASED ASSAY

BEFORE RUNNING A CELL-BASED ASSAY:

1. *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.*
2. *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 (IC10 to IC90). If results fall outside the assay's linear range, the data will be inaccurate.*
3. *Compounds must be diluted in Stimulation Buffer 1 with the optimized IBMX concentration (recommended 0.5mM final) . Alternatively, for stimulation time above 2 hours, compounds could be diluted in cell culture medium supplemented with IBMX.*
4. *IBMX may have its own effect on cAMP accumulation. We recommend checking this effect by running experimental wells with and without IBMX in the stimulation buffer.*
5. *Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader. (more information about HTRF® compatible readers and set-up recommendations available at http://www.cisbio.com/compatible-readers)*

*A phosphodiesterase inhibitor must be added to the culture medium to prevent cAMP degradation and promote its accumulation.

Forskolin may be used as an optional control for your cell based assay.

For complete guidelines on fully optimizing your cAMP Gi assay, please refer to our Technical Guides: "A Guide to Optimizing Agonists Gi" and "A Guide to Optimizing Antagonists Gi".

CELL-BASED ASSAY PROTOCOL

	NEGATIVE CONTROL*	NON-STIMULATED CELLS**	STIMULATED CELLS
Step 1	Dispense 5 µL of cells into all wells.	Dispense 5 µL of cells into all wells.	Dispense 5 µL of cells into all wells.
Step 2	Add 4 µL Stimulation Buffer 1.	Add 4 µL Stimulation Buffer 1.	Add 4 µL of test compounds (2.5X).
Step 3	Seal the plate and incubate the appropriate time at 37°C.	Seal the plate and incubate the appropriate time at 37°C.	Seal the plate and incubate the appropriate time at 37°C.
Step 4	Add 1 µL Stimulation Buffer 1.	Add 1 µL of forskolin.	Add 1 µL of forskolin.
Step 5	Seal the plate and incubate the appropriate time at 37°C.	Seal the plate and incubate the appropriate time at 37°C.	Seal the plate and incubate the appropriate time at 37°C.
Step 6	Add 5 µL cAMP Eu-cryptate reagent.	Add 5 µL cAMP Eu-cryptate reagent.	Add 5 µL cAMP Eu-cryptate reagent.
Step 7	Add 5 µL Lysis & Detection Buffer.	Add 5 µL of Anti cAMP-d2 working solution.	Add 5 µL of Anti cAMP-d2 working solution.
Step 8	Seal the plate and incubate 1 hour at room temperature.	Seal the plate and incubate 1 hour at room temperature.	Seal the plate and incubate 1 hour at room temperature.
Step 9	Remove the plate sealer and read on an HTRF compatible reader.	Remove the plate sealer and read on an HTRF compatible reader.	Remove the plate sealer and read on an HTRF compatible reader.

*The Negative Control wells represent the non-specific fluorescence stemming from the Cryptate.
**Data with non-stimulated cells indicate the basal cellular level of cAMP before compound treatment.

DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Plot the HTRF ratio versus compound concentrations.

For more information about data reduction, please visit www.cisbio.com/data-reduction.

ASSAY FLEXIBILITY AND MINIATURIZATION

It is very easy to modify the assay (here described in 384-well, low volume format) to 1536-well (10 µL) or 96 half-well (100 µL) formats. Simply adjust the volume of each assay component proportionally in order to maintain the same reagent concentrations as the 20 µL assay. Assay performance is the same in 96 up to 1536-well plate format.

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cAMP - Gi
SHORT PROTOCOL

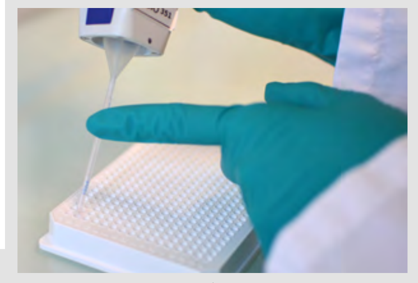
STANDARD CURVE PREPARATION

Warm up the lyophilized reagents to room temperature at least 30 minutes before reconstitution. We recommend running the standard curve in triplicate wells. Carefully identify **" Lysis & Detection Buffer"** and **"Stimulation Buffer 1*"**.

1 Reconstitute reagents following table below. Mix gently.

2 Prepare working solutions 1X (see table below) using ***Lysis & Detection Buffer 8*** *(detection reagents)* ***or distilled water*** *(Stimulation Buffer 1).*

3 Prepare standard curve ***(Std6-Std1)*** by making 1:4 serial dilutions from ***Std7*** in ***Stimulation Buffer 1X*** . Mix between each dilution.



4 Negative** Control wells: Add 5 µL of ***Stimulation Buffer 1X*** . Calibration curve wells: Add 5 µL of each Std (***Std0*** to ***Std7***).

5 All wells: Add 5 µL of ***Stimulation Buffer 1X.***

6 All wells: Add 5 µL of ***cAMP-Cryptate working solution.***

7 Negative Control wells: Add 5 µL of ***Lysis & Detection Buffer 8.*** Calibration curve wells: Add 5 µL of ***Anti-cAMP-d2 working solution.***

8 Seal the plate and incubate 1 hr at RT.

9 Remove the plate sealer and read on an HTRF® compatible reader.

RECONSTITUTION VOLUMES (DISTILLED WATER)				Reagent Concentrations after Reconstitution / Dilution
KIT SIZE	cAMP - Gi Kit			
	Anti-cAMP-Cryptate	cAMP-d2		
1,000	*1.1 mL*	*1.1 mL*	▶	*5X*
20,000 & 100,000	*5 mL*	*5 mL*	▶	*20X*
ALL	Dilute 5X Stimulation Buffer 1 in distilled water (1 volume of 5X stock solution in 4 volumes of distilled water)		▶	*1X*
	Reconstitute cAMP Standard Concentrate in Stimulation Buffer 1X*. See vial label for reconstitution volume.		▶	See label = Std7

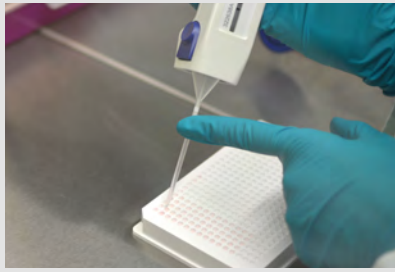
CELL-BASED ASSAY

Use cells previously optimized for cell density so that cAMP levels fall within the linear dynamic range of the assay (IC_{10} to IC_{90}).

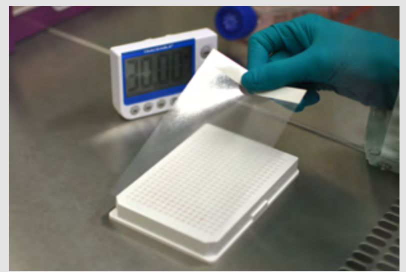
Follow Steps 1 and 2 (see reverse side) to reconstitute reagents and prepare working solutions.

1 Dispense 5 µL of ***cells*** into all wells.

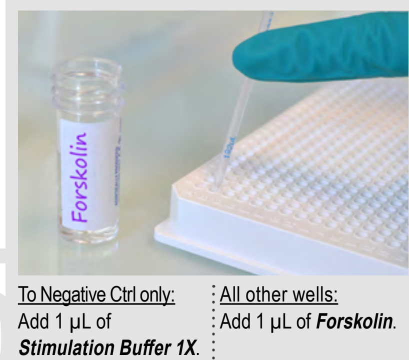
2 Negative Control** and non-stimulated** wells:
Add 4 µL ***Stimulation Buffer 1X**** supplemented with ***IBMX*** (0.5 mM final).



3 Stimulated cells wells:
Add 4 µL ***test compounds (2.5X) prepared in Stimulation Buffer 1X***.



4 Seal the plate (alternatively, use a cover) and incubate for appropriate time at 37°C. ***(Cell Stimulation Step)***



5 To Negative Ctrl only:
Add 1 µL of ***Stimulation Buffer 1X***.

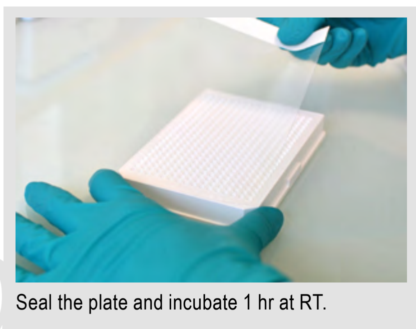
All other wells:
Add 1 µL of ***Forskolin***.

6 Seal the plate (alternatively, use a cover) and incubate for appropriate time at 37°C. ***(cAMP Accumulation step through Forskolin)***

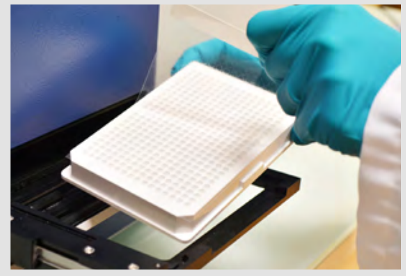
7 All wells:
Add 5 µL ***of cAMP-Cryptate working solution***

8 To Negative Ctrl only:
Add 5 µL ***Lysis & Detection Buffer 8***.

All other wells:
Add 5 µL of ***Anti-cAMP-d2 working solution***.



9 Seal the plate and incubate 1 hr at RT.



10 Remove the plate sealer and read on an HTRF® compatible reader.

* Stimulation Buffer = Stimulation buffer 1 (provided in the kit under 5X concentration) or cell culture medium used for cell stimulation, in both case supplemented with IBMX.

** The Negative Control wells represent the non-specific fluorescence stemming from the Cryptate.

*** Data with non-stimulated cells indicate the basal cellular level of cAMP = Non-stimulated.

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