MANUAL

LANCE™ *Ultra* cAMP Kit

For Laboratory Use Only
Research Chemicals for Research Purposes Only

I. Intended use

The LANCE™ *Ultra* cAMP kit is intended for the quantitative determination of 3′,5′-cyclic adenosine monophosphate (cAMP) in cell lysate and cellular membrane samples.

2. Provided reagents

Component	TRF0262 1,000 points*	TRF0263 10,000 points*	TRF0264 50,000 points*
cAMP standard, 50 µM	1 vial, 1 mL	1 vial, 1 mL	1 vial, 1 mL
Eu-cAMP tracer**§	1 vial, 110 µL	1 vial, 1 mL	5 vials, 1 mL each
U*Light*™-anti-cAMP**	1 vial, 37 µL	1 vial, 340 µL	1 vial, 1.68 mL
cAMP Detection Buffer	1 bottle, 25 mL	1 bottle, 250 mL	4 bottles, 250 mL each
BSA Stabilizer (7.5% solution)	1 vial, 1 mL	1 bottle, 10 mL	1 bottle, 50 mL

* When using the recommended protocols (20-µL assay in 384-well microplates).
** Centrifuge tubes for a few seconds before use to improve recovery of content.
§ Store the Eu-cAMP tracer aliquoted and frozen at -20°C. Avoid repeated freeze-thaw cycles.

3. Storage conditions

Upon receiving the kit, store the **Eu-cAMP tracer aliquoted and frozen at -20°C**, and all other reagents at 2-8°C protected from light. The expiration date of the kit is indicated on the box label.

4. Assay principle

The LANCE *Ultra* cAMP assay is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay designed to measure cAMP produced upon modulation of adenylyl cyclase activity by G-protein coupled receptors (GPCRs). The assay is based on the competition between the europium (Eu) chelate-labeled cAMP tracer and sample cAMP for binding sites on cAMP-specific monoclonal antibodies labeled with the U*Light*™ dye (Figure 1). When antibodies are bound to the Eu-labeled cAMP tracer, light pulse at 320 or 340 nm excites the Eu chelate molecule of the tracer. The energy emitted by the excited Eu chelate is transferred by FRET to U*Light* molecules on the antibodies, which in turn emit light at 665 nm. Residual energy from the Eu chelate will produce light at 615 nm. In the absence of free cAMP, maximal TR-FRET signal is achieved (Figure 1, left panel). Free cAMP produced by stimulated cells competes with the Eu-cAMP tracer for the binding to the U*Light*-mAb, causing a decrease in TR-FRET signal (Figure 1, right panel).

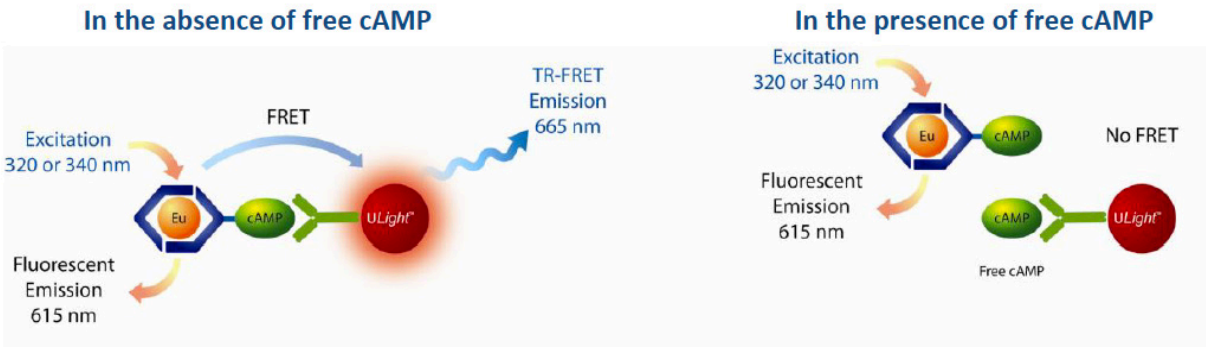


Figure 1. LANCE™ *Ultra* cAMP assay principle.

5. Reagents not supplied in the kit

Item	Recommended source	Product no
Hank's Balanced Salt Solution (HBSS) (1X) (no phenol red)	Invitrogen	14025-092
HEPES Buffer Solution (1 M) pH 7.2 to 7.5	Invitrogen	15630-080
Forskolin	Sigma	F6886
IBMX	Sigma	I7018
OptiPlate-384, white	Revvity	6007290 (pack of 50) 6007299 (pack of 200)
ProxiPlate-384 Plus, white	Revvity	6008280 (pack of 50) 6008289 (pack of 200)
OptiPlate-96, white	Revvity	6005290 (pack of 50) 6005299 (pack of 200)
OptiPlate-1536, white	Revvity	6004290 (pack of 50) 6004299 (pack of 200)
TopSeal™-A 384	Revvity	6005250

6. Assay optimization guidelines

The following protocol assumes that both the cell number and stimulation conditions have been optimized, as these parameters often vary for each receptor and cell line. It is therefore strongly recommended to generate either forskolin (Gs and Gi receptors) or full agonist (Gs receptors) concentration-response curves in order to determine the optimal cell number per well. We suggest testing from 250 to 5,000 cells per well in a 20-µL assay. The optimal cell number will be the one for which the forskolin or agonist concentration-response curve covers most of the dynamic range of the cAMP standard curve (IC_{10} – IC_{90}). This typically corresponds to the cell density giving the highest signal to background (S/B) ratio calculated using the maximal signal (untreated cells) and the minimal signal obtained with a saturating concentration of agonist or forskolin (fully activated cells). From the example presented below (Figure 2), the optimal cell concentration selected for subsequent experiments (ex. agonist dose-response curves) would be 1,000 cells/well. Note, however, that at 500 cells per well, the assay window is already acceptable and therefore, the optimal cell density will ultimately depend on your assay needs. Additional assay development guidelines are available on Revvity's website (www.revvity.com).

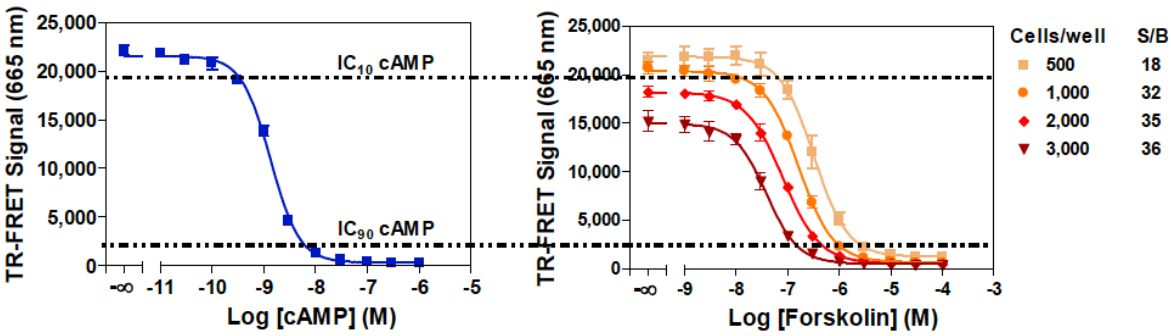


Figure 2. Determination of optimal cell density. Left panel: cAMP standard curve; right panel: cell and forskolin cross-titration.

7. Reagent preparation

7.1 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).** Make fresh.

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

- 14 mL of 1X HBSS (Invitrogen, cat. # 14025-092)
- 75 µL of 1M HEPES (Invitrogen, cat. # 15630-080)
- 30 µL of 250 mM IBMX dissolved in DMSO (Sigma, cat.# I7018)
- 200 µL of 7.5% BSA Stabilizer (included in the kit)
- Adjust pH to 7.4 with 0.1N NaOH and complete volume to 15 mL with 1X HBSS

NOTES:

- Alternative buffers such as cell culture medium containing 10% FBS and phenol red can also be used.
- For cAMP standard curves, addition of 0.5 mM IBMX to the Stimulation Buffer is optional. For cell- and membrane-based assays, IBMX could be replaced by another phosphodiesterase inhibitor (e.g., 100 µM RO-201724).
- Addition of BSA might not be essential for your cellular assay. However, if BSA is used, we strongly recommend the BSA Stabilizer (7.5% solution) included in the kit, as it is a highly purified preparation of BSA, free of europium and heavy metal ion contaminants.

7.2 cAMP standard serial dilutions in Stimulation Buffer

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	[Final] (M)	[4X] (M)	Volume of dilution	Stimulation Buffer
1	$1 X 10^{-6}$	$4 X 10^{-6}$	8 µL of 50 µM cAMP	92 µL
2	$3 X 10^{-7}$	$1.2 X 10^{-6}$	30 µL of 1	70 µL
3	$1 X 10^{-7}$	$4 X 10^{-7}$	30 µL of 2	60 µL
4	$3 X 10^{-8}$	$1.2 X 10^{-7}$	30 µL of 3	70 µL
5	$1 X 10^{-8}$	$4 X 10^{-8}$	30 µL of 4	60 µL
6	$3 X 10^{-9}$	$1.2 X 10^{-8}$	30 µL of 5	70 µL
7	$1 X 10^{-9}$	$4 X 10^{-9}$	30 µL of 6	60 µL
8	$3 X 10^{-10}$	$1.2 X 10^{-9}$	30 µL of 7	70 µL
9	$1 X 10^{-10}$	$4 X 10^{-10}$	30 µL of 8	60 µL
10	$3 X 10^{-11}$	$1.2 X 10^{-10}$	30 µL of 9	70 µL
11	$1 X 10^{-11}$	$4 X 10^{-11}$	30 µL of 10	60 µL
12 (ctrl)	0	0	-	70 µL

7.3 Eu-cAMP tracer solution in cAMP Detection Buffer

Prepare a **4X Eu-cAMP tracer working solution** by making a **1/50** dilution of the Eu-cAMP tracer stock solution in cAMP Detection Buffer.

Example: Add 5 µL of the Eu-cAMP tracer stock solution to 245 µL of cAMP Detection Buffer and mix gently.

7.4 U*Light*™-anti-cAMP solution in cAMP Detection Buffer

Prepare a **4X ULight-anti-cAMP working solution** by making a **1/150** dilution of the U*Light*-anti-cAMP stock solution in cAMP Detection Buffer.

Example: Add 5 µL of the U*Light*-anti-cAMP stock solution to 745 µL of cAMP Detection Buffer and mix gently.

NOTES:

- Working solutions can be stored up to 24 hours at 4°C.
- For optimal assay performance, do not modify the recommended dilutions for both the Eu-cAMP tracer and U*Light*-anti-cAMP.

8. Assay protocols for a 384-well plate (total assay volume of 20 µL)

In the protocols described in the table below, both the cells and tested compounds must be prepared in Stimulation Buffer (including 0.5 mM IBMX). cAMP Detection Buffer must be used only for the preparation of Eu-cAMP tracer and U*Light*-anti-cAMP working solutions.

cAMP standard curve	Gs Agonist	Gs Antagonist	Gi Forskolin titration	Gi Agonist	Gi Antagonist
5 µL 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 Agonist	2.5 µL Agonist	5 µL Forskolin	2.5 µL Forskolin	2.5 µL Forskolin/ Agonist
-	-	2.5 µL Antagonist	-	2.5 µL Agonist	2.5 µL Antagonist
Incubate 30 min at room temperature (optional step for cAMP standard curve)*					
5 µL 4X Eu-cAMP tracer working solution					
5 µL 4X U*Light*-anti-cAMP working solution					
Incubate 1 h at room temperature*					
Read on a TR-FRET microplate reader. Remove microplate seal prior to reading					

* Cover microplate with a TopSeal™-A film (Revvity, Inc. Cat. # 6005250) or another plate during incubations.

NOTES:

- Additional readings can be performed for at least 24 hours after addition of LANCE *Ultra* reagents without significant change in assay sensitivity.
- If preferred, in order to eliminate one addition step, 5 µL of cell suspension in Stimulation Buffer containing 4X U*Light*-anti-cAMP can be used. In this specific case, 10 µL of 2X Eu-cAMP tracer solution must be added in order to keep the 20-µL total assay volume.
- For 96- and 1536-well formats, adjust volume of each assay component proportionally in order to maintain the volume ratios used for the 384 plate format.
- Do not mix reagents from kits with different lot numbers in order to maintain assay performance between lots.

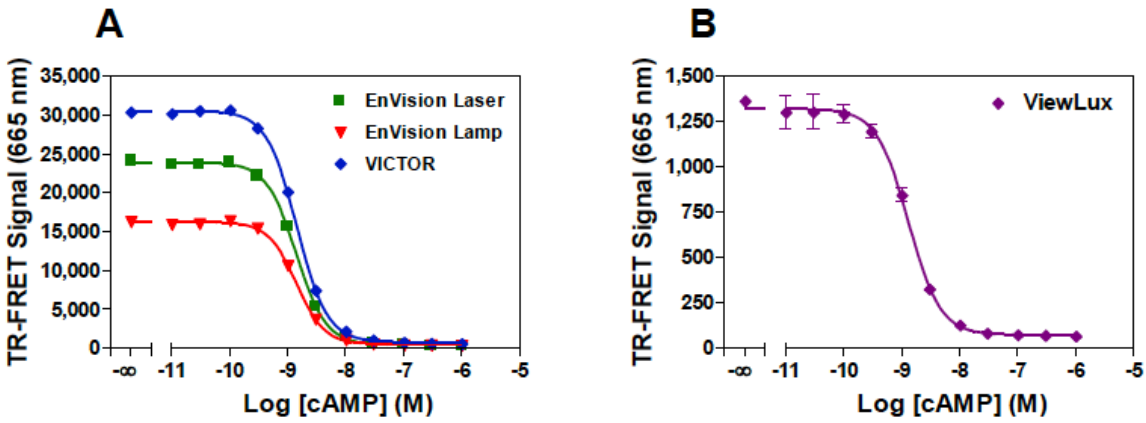
9. Instrument settings

Parameter	VICTOR™	EnVision™ Lamp/Laser	ViewLux™*
Flash Energy Area	High	N/A	N/A
Flash Energy Level	150	100%	600,000
Excitation Filter	320 / 340	Lamp: 111 (UV2 320)	DUG11 (UMB, AMC)
Integrator Cap	3	N/A	N/A
Integrator Level	2X LANCE High Count 615 and 665 (locked protocols)	N/A	N/A
Emission Filter	1) 615 2) 665	1) 203 (Eu 615) 2) 205 (APC 665)	1) 618/8 (Eu) 2) 671/8 (LANCE)
Delay Time	50 µs	50 µs	50 µs
Readout Speed, Gain and Binning	N/A	N/A	Medium, High and 2x
Number of Flashes	N/A	Lamp: 100 Laser: 20	N/A
Window	100 µs (200 µs**)	100 µs (200 µs**)	354 µs
Mirror Module	N/A	Lamp: 662, 462 or 412 Laser: 445 or 446	N/A
Cycle	2000 µs	Lamp: 2000 µs Laser: 16600 µs	N/A

* Measurement time of **20 seconds** recommended for the ViewLux® instrument.

** If signal too low with 100 µs.

10. Typical LANCE™ Ultra cAMP standard curves

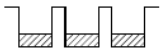
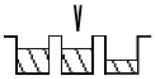
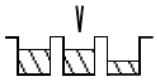


	EnVision Laser	EnVision Lamp	VICTOR	ViewLux
Max counts	24178	16352	30440	1366
Min counts	356	417	638	68
S/B	**67.9**	**39.2**	**47.7**	**20.1**
IC_{50} (nM)	**1.42**	**1.42**	**1.45**	**1.32**

Figure 3. Representative LANCE *Ultra* cAMP standard curves obtained on different instruments using the recommended settings. A white OptiPlate™-384 microplate with a single cAMP standard curve assay was incubated for 1 hour at room temperature and then read with the (A) EnVision® Multilabel reader (laser and lamp settings), VICTOR™ reader and (B) ViewLux®.

NOTE: Depending on the instrument, counts and S/B ratio may vary, but this will not affect significantly assay robustness or sensitivity (IC_{50}).

11. Assay volumes recommended for different plate formats

		½ AreaPlate™-96 (Cat. 6005560)	OptiPlate-384 (Cat. 6007290)	OptiPlate-1536 (Cat. 6004290)
Total Assay Volume		**40 µL**	**20 µL**	**8 µL**
Add cell suspension		10 µL	5 µL	2 µL
Add compound (s)		10 µL	5 µL	2 µL
Incubate		30 min at RT		
Add Eu-cAMP tracer		10 µL	5 µL	2 µL
Add U*Light*-anti-cAMP		10 µL	5 µL	2 µL
Incubate		1 h at RT		
Measure TR-FRET signal		See instrument settings in Section 9		

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