

PerkinElmer Life and Analytical Sciences, Inc.



LANCE™-cAMP 384 Kit

AD0262 – 500 Assay points
AD0262E – 1,000 Assay points
AD0263 – 10,000 Assay points
AD0264 – 50,000 Assay points

For Laboratory Use

CAUTION: A research chemical for research purposes only.

INTENDED USE

This LANCE™ cAMP 384 kit is intended for the quantitative determination of adenosine 3',5'-cyclic monophosphate (cAMP) in cell culture and cell membrane samples.

INTRODUCTION

cAMP is one of the most important second messengers, mediating diverse physiological responses of neurotransmitters, hormones, and drugs. Intracellular concentration of cAMP is tightly regulated by two membrane-bound enzymes, adenylyl cyclase (AC) and phosphodiesterase (PDE). AC activity promotes the synthesis of cAMP from ATP while PDE degrades cAMP to AMP. The activity of AC is controlled through various G-protein-coupled receptors (GPCRs), via their interaction with one of two distinct GTP binding proteins, $G_{\alpha s}$ and $G_{\alpha i}$. These G proteins are heterotrimeric molecules composed of the subunits G_{α} (s or i), G_{β} and G_{γ} . Agonist activation of GPCRs leads to the binding of GTP to the G_{α} subunit, causing a conformational change that leads to the dissociation of the trimer into G_{α} and $G_{\beta\gamma}$. Upon dissociation, $G_{\alpha s}$ is primarily involved in AC stimulation whereas $G_{\alpha i}$ and $G_{\beta\gamma}$ are inhibitory. The measurement of intracellular cAMP is thus an ideal method for measuring the effect of test compounds on GPCR-mediated AC activation or inhibition.

LANCE is a trademark of PerkinElmer, Inc.
Alexa Fluor® 647 is a registered trademark of Molecular Probes, Inc.
(Invitrogen Corp.).

PRINCIPLES OF THE ASSAY

The LANCE 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 GPCRs. The assay is based on the competition between a europium-labeled cAMP tracer complex and sample cAMP for binding sites on cAMP-specific antibodies labeled with the dye Alexa Fluor® 647. The europium-labeled cAMP tracer complex is formed by the tight interaction between Biotin-cAMP (b-cAMP) and streptavidin labeled with Europium-W8044 chelate (Eu-SA).

When antibodies are bound to the Eu-SA/b-cAMP tracer, light pulse at 340 nm excites the Eu-chelate molecules of the tracer. The energy emitted by the Eu-chelate is transferred to an Alexa molecule on the antibodies, which in turn emits light at 665 nm (figure 1). The fluorescence intensity measured at 665 nm will decrease in the presence of cAMP from test samples and resulting signals will be inversely proportional to the cAMP concentration of a sample.

This assay is intended for the detection of cAMP produced by live cells or from cellular membrane preparations stimulated with GPCR agonists.

For $G_{\alpha s}$ -coupled receptors, agonist stimulation of cells or membrane preparations results in an increase in cAMP levels, and to a concomitant decrease in fluorescence at 665 nm. Addition of an antagonist will reverse the response, resulting in an increase in signal. In the case of $G_{\alpha i}$ -coupled receptors, cells are stimulated by the AC activator forskolin and by the agonist simultaneously. An agonist will inhibit the forskolin-induced cAMP production, therefore an increase in LANCE signal will be observed compared with samples treated with forskolin alone. In $G_{\alpha i}$ -coupled systems, antagonists will block the effect of the agonist, resulting in a decrease in LANCE signal toward forskolin-induced levels. In cell-based assays, cells are lysed after stimulation.

The LANCE cAMP assay is extremely sensitive and robust. The assay reactions are stable for at least 20 hours at room temperature, with little loss in sensitivity. These characteristics make this kit ideal for HTS screening campaigns.

This kit insert describes procedures to perform LANCE cAMP assays in 384-well microplates in a total assay volume of 24 μ L. The assays can be run in different final volumes to accommodate the 96 and 1536 formats if the concentration ratio of the reagents is maintained. (See Summary Protocol sheet on pg 30).

The LANCE cAMP assay can be also performed using GPCR expressed in cell membranes and using adherent cells.

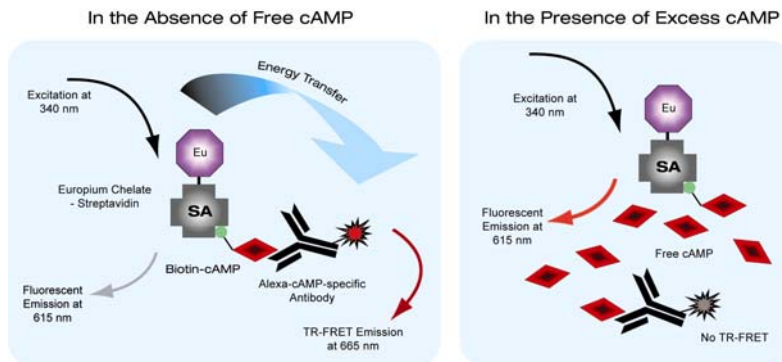


Figure 1. LANCE cAMP Assay Principle. Light pulse at 340 nm excites the Europium-chelate of the Eu-SA/b-cAMP tracer. The energy emitted from the Eu-chelate is transferred to the Alexa Fluor[®] 647 labeled anti-cAMP antibodies bound to the tracer, generating a TR-FRET signal at 665 nm. Residual energy from the Eu-chelate will produce light at 615 nm. cAMP of a sample competes with the tracer for antibody binding sites and causes signal reduction.

KIT CONTENTS

The reagents are sufficient for 500, 1,000, 10,000 or 50,000 assay points when using the suggested protocols (20 μ L or 24 μ L reactions in 384-well microplates).

The expiration date of the complete package is stated on the outer label. Store at +2 to +8°C protected from light.

Reagents

Component	AD0262 500 points	AD0262E 1,000 points	AD0263 10,000 points	AD0264 50,000 points
cAMP Standard	1 vial, 1 mL	1 vial, 1 mL	1 vial, 1 mL	1 vial, 1 mL
Biotin-cAMP	1 vial, 25 µL	1 vial, 25 µL	1 vial, 170 µL	1 vial, 830 µL
LANCE Eu-W8044 labeled streptavidin	1 vial, 25 µL	1 vial, 25 µL	1 vial, 60 µL	1 vial, 280 µL
Alexa Fluor [®] 647-anti cAMP antibody	1 vial, 40 µL	1 vial, 80 µL	1 vial, 670 µL	2 vials, 1.70 mL
cAMP Detection Buffer	1 bottle, 25 mL	2 bottles, 25 mL	1 bottle, 250 mL	5 bottles, 250 mL
BSA Stabilizer (7.5% solution)	<i>Not provided</i>	1 vial, 1 mL	<i>Not provided</i>	<i>Not provided</i>
White Optiplate-384	<i>Not provided</i>	1 micro-plate	<i>Not provided</i>	<i>Not provided</i>
White Proxiplate-384 Plus	<i>Not provided</i>	1 micro-plate	<i>Not provided</i>	<i>Not provided</i>
LANCE Positive Control	<i>Not provided</i>	1 vial, 2 mL	<i>Not provided</i>	<i>Not provided</i>
LANCE Negative Control	<i>Not provided</i>	1 vial, 2 mL	<i>Not provided</i>	<i>Not provided</i>

Description of Kit Components

The **cAMP Standard** (50 $\mu\text{mol/L}$) is supplied in 50 mmol/L sodium acetate with 0.09% sodium azide as preservative.

The **Biotin-cAMP** tracer is supplied in 10 mmol/L Tris-HCl buffered (pH 8.0) salt solution with 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% bovine serum albumin (BSA), and 0.05% sodium azide as preservative.

The **LANCE Eu-W8044 labeled streptavidin** is supplied in 50 mmol/L Tris-HCl buffered (pH 7.8) salt solution with 0.9% sodium chloride (NaCl), 0.1% BSA, and 0.05% sodium azide as preservative.

The **Alexa Fluor[®] 647-anti cAMP antibody** is supplied in 50 mmol/L Tris-HCl buffered (pH 7.8) salt solution with 0.9% NaCl, 0.1% BSA, and 0.05% sodium azide as preservative.

Ready-for-use **cAMP Detection Buffer** (pH 7.4) composed of 50 mmol/L HEPES, 10 mmol/L calcium chloride (CaCl_2), and 0.35% Triton X-100¹.

The **BSA stabilizer** is supplied in 50 mmol/L Tris buffered (pH 7.8) salt solution with 7.5 % BSA, and <0.1% sodium azide as preservative.

The **white OptiPlate-384** and **white ProxiPlate-384 Plus** are microplates suitable for luminescence- and fluorescence-based assays. They have been validated for LANCE cAMP assays.

The **LANCE Positive Control** is a ready-for-use solution composed of Eu-W1024-biotin and SA-APC in polarization buffer.

The **LANCE Negative Control** is a ready-for-use solution composed of Eu-W1024-biotin and SA in polarization buffer.

¹Triton is a registered trademark of Rohm and Haas Co.

MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE KIT

The LANCE cAMP 384 kit requires the following items:

Item	Suggested source*	Product no.
Stimulation buffer	See page 12	
Hank's Balanced Salt Solution (HBSS) (1X) (no phenol red)	Invitrogen Corp.	14025-092
Versene	Invitrogen Corp.	15040-066
HEPES Buffer Solution (1 mol/L)	Invitrogen Corp.	15630-080
Milli-Q water		
BSA Stabilizer (7.5% solution)***	PerkinElmer, Inc.	CR84-100
Forskolin	Sigma-Aldrich, Inc.	F 6886
IBMX**	Sigma-Aldrich, Inc.	I 5879
OptiPlate-384, white***	PerkinElmer, Inc.	6007290 (pack of 50) 6007299 (pack of 200)
ProxiPlate-384 Plus, white***	PerkinElmer, Inc.	6008280 (pack of 50) 6008289 (pack of 200)
OptiPlate-96, white	PerkinElmer, Inc.	6005290 (pack of 50) 6005299 (pack of 200)
OptiPlate-1536, white	PerkinElmer, Inc.	6005228 (pack of 20)
TopSeal-A 384	PerkinElmer, Inc.	6005250
LANCE Controls***	PerkinElmer, Inc.	
Liquid handling system (FlexDrop™, Evolution™ EP ³ , or equivalent)	PerkinElmer, Inc.	
TRF detection readers (ViewLux™, Fusion™, VICTOR™, EnVision™, or equivalent)	PerkinElmer, Inc.	

* Equivalent sources can be substituted

** 3-Isobutyl-1-Methylxanthine

*** These items are provided in the AD02662E - 1,000 assay points Kit

² Milli-Q is a registered trademark of Millipore Corp.

FlexDrop, Evolution, ViewLux, Fusion, VICTOR, and EnVision are trademarks of PerkinElmer, Inc.

WARNINGS AND PRECAUTIONS

This LANCE cAMP 384 kit is intended for research use only.

Reagents contain sodium azide (NaN_3) as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

Disposal of all waste should be in accordance with local regulations.

INSTRUMENT SETTINGS & CALIBRATION

It is critical to ensure that the instruments possess the right filters (excitation at 320 or 340 nm; emission at 615 and 665 nm). For the VICTOR and EnVision instruments, modifications to locked protocols according to the table below are recommended. Adjustments to the locked protocols can be made after copying them under a new name (e.g. Copy LANCE High Count 615 and 665 labels...).

To perform the flatfield calibration on the ViewLux instrument, we recommend using the LANCE positive control as the reference sample (LANCE Controls, prod. no. AD0163) rather than the LANCE cAMP 384 kit reagents. The LANCE positive control should be used diluted 1:5 in water. The volume of the sample should be the same as the assay sample volume (20 μL or 24 μL as recommended in the assay protocol). The flatfield calibration is performed using the calibration wizard for both 615 nm and 665 nm channels. Details of the protocol can be found in the ViewLux Reference Manual.

The ability of the readers to detect LANCE chemistry can be assessed using the LANCE Positive and Negative Controls (provided in the 1,000 assay points kits). The volume of the sample should be the same as the assay sample volume (20 μ L or 24 μ L as recommended in the assay protocol). We describe below the protocol for a 24 μ L assay:

Allow the vials to reach room temperature before use.

Dilute the controls 1:5 in water.

Dispense 24 μ L of the diluted Positive Control in 12 wells of a white Optiplate-384.

Similarly dispense 24 μ L of the diluted Negative Control in 12 another wells.

For 96-well and 1536-well microplates, use dispense volumes of 40 μ L and 8 μ L, respectively. For a ProxiPlate-384 Plus, use dispense volumes of 20 μ L.

Measure fluorescence within 30 minutes using the LANCE protocol.

Typically, in Optiplate-384 microplates, the LANCE Positive Control gives a signal higher than 100,000 counts, while the LANCE Negative Control produces less than 5,000 counts. The signal-to-noise (Positive/Negative) ratio is usually around 50.

Parameter	VICTOR	EnVision	ViewLux*
Flash Energy Area	High	N/A	N/A
Flash Energy Level	150	100%	600,000
Excitation Filter	320 / 340	UV2 320	DUG11 (UMB, AMC)
Integrator Cap	3	N/A	N/A
Integrator Level	2x LANCE High Count 615	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 μ s	60 μ s	50 μ s
Readout speed, gain and binning	N/A	N/A	Medium, High and 2x
Number of Flashes	N/A	100	N/A
Window	100 μ s (200 μ s**)	100 μ s (200 μ s**)	354 μ s
Mirror module	N/A	462 (D400/D630) or 412 (D400)	N/A
Cycle	2000 μ s	2000 μ s	N/A

* Measurement time of **20 seconds** recommended for the ViewLux

** If signal too low with 100 μ s

Similar settings as the VICTOR settings can be applied for Fusion instruments, but longer window time (e.g. 300 μ s) and 340 nm excitation filter are needed.

STANDARD CURVE ASSAY PROTOCOL

1. Preparation of Stimulation Buffer

Make fresh. Prepare only the amount needed within one day.

We recommend a Stimulation Buffer made of HBSS 1X solution (Invitrogen Corp.) and containing 5 mmol/L HEPES buffer (Invitrogen Corp.) and 0.01%–0.1% BSA (pH 7.4). Alternative buffers such as PBS with 0.1% BSA can be used as well. In cell-based assays, the Stimulation Buffer should include IBMX (**see page 20**).

Important Note : The use of BSA is mandatory in LANCE cAMP assays. We recommend using the BSA stabilizer 7.5% solution (CR84-100; included in the 1,000 assay points kit), which is a highly purified preparation of BSA, free of Europium & heavy metal ions contaminants.

Protocol to make 15 mL of Stimulation Buffer HBSS 1X containing 5 mM HEPES, 0.1% BSA, pH 7.4

To a tube, add 14 mL of HBSS 1X, 75 μ L of 1M HEPES, 200 μ L of 7.5% BSA Stabilizer. Adjust the pH to 7.4 and complete the volume to 15 mL with HBSS 1X.

2. Preparation of cAMP Standard Serial Dilutions in Stimulation Buffer

Dilute the cAMP Standard solution (50 $\mu\text{mol/L}$) in Stimulation Buffer according to the table below.

Dilution	[final in assay] (mol/L)	[intermediate] (mol/L)	Volume of dilution	Stimulation Buffer
1	1×10^{-6}	4×10^{-6}	8 μL of 50 $\mu\text{mol/L}$	92 μL
2	3×10^{-7}	1.2×10^{-6}	30 μL of dil 1	70 μL
3	1×10^{-7}	4×10^{-7}	30 μL of dil 2	60 μL
4	3×10^{-8}	1.2×10^{-7}	30 μL of dil 3	70 μL
5	1×10^{-8}	4×10^{-8}	30 μL of dil 4	60 μL
6	3×10^{-9}	1.2×10^{-8}	30 μL of dil 5	70 μL
7	1×10^{-9}	4×10^{-9}	30 μL of dil 6	60 μL
8	3×10^{-10}	1.2×10^{-9}	30 μL of dil 7	70 μL
9	1×10^{-10}	4×10^{-10}	30 μL of dil 8	60 μL
10	3×10^{-11}	1.2×10^{-10}	30 μL of dil 9	70 μL
11	1×10^{-11}	4×10^{-11}	30 μL of dil 10	60 μL
12	-	-	-	70 μL

3. Preparation of Alexa Fluor® 647-anti cAMP Antibody Solution in Stimulation Buffer

Prepare the antibody solution by making a **1/100** dilution of the Alexa Fluor® 647-anti cAMP antibody solution in the Stimulation Buffer.

Example: Add 5 µL of the antibody solution to 495 µL of Stimulation Buffer, and mix by inverting the tube up and down.

4. Preparation of Detection Mix in Detection Buffer

Step 1. Dilute the LANCE Eu-W8044 labeled streptavidin (Eu-SA) in cAMP Detection Buffer	Add 5 µL of Eu-W8044 labeled streptavidin (Eu-SA) to 85 µL of cAMP Detection Buffer.
Step 2. Dilute the Biotin-cAMP (b-cAMP) in cAMP Detection Buffer	Add 5 µL of Biotin-cAMP (b-cAMP) to 25 µL of cAMP Detection Buffer.
Step 3. Prepare the Detection Mix from Step 1 and Step 2 dilutions	a) Add 615 µL of Detection Buffer to a tube b) Add 5 µL of Eu-W8044 dilution (from Step 1) c) Mix gently d) Add 5 µL of b-cAMP dilution (from Step 2) e) Mix gently f) Incubate for at least 15 minutes at room temperature

5. Standard Curve Assay Protocol

The assays are performed in triplicates in a white OptiPlate-384 or ProxiPlate-384 Plus microplate in a total volume of 20 μL or 24 μL . For other assay formats, please refer to the protocol table presented on Page 30.

For OptiPlate-384 microplates (total assay volume of 24 μL):

- a) Add 6 μL of cAMP Standard dilutions.
- b) Add 6 μL of Antibody Solution.
- c) Incubate for 30–60 minutes at room temperature.
- d) Add 12 μL of Detection Mix.
- e) Cover microplate with another plate or with a TopSeal-A film and incubate for 60 minutes at room temperature.
- f) Read on a TRF detection instrument. **Remove the TopSeal-A prior to reading.**
- g) Additional readings can be performed for up to 20 h after tracer addition without significant decrease in assay sensitivity. See **Recommendations on the Read-Time for the LANCE cAMP Assay** (page 18).

For OptiPlate-384 and ProxiPlate-384 microplates (total assay volume of 20 μL):

- a) Add 5 μL of cAMP Standard dilutions.
- b) Add 5 μL of Antibody Solution.
- c) Incubate for 30–60 minutes at room temperature.
- d) Add 10 μL of Detection Mix.

- e) Cover microplate with another plate or with a TopSeal-A film and incubate for 60 minutes at room temperature.
- f) Read on a TRF detection instrument. **Remove the TopSeal-A prior to reading.**
- g) Additional readings can be performed for up to 20 h after tracer addition without significant decrease in assay sensitivity. See **Recommendations on the Read-Time for the LANCE cAMP Assay** (page 18).

Representative cAMP standard curves generated in Optiplate-384 microplates in a total volume of 24 μ L are presented in Figures 2 and 3. IC_{50} values of 2-3 nM with a detection limit of approximately 1 fmol/well are determined. Similar results are obtained in ProxiPlate-384 Plus microplates using an assay volume of 20 μ L (data not shown). Please note that depending on the instrument and the lot of kit used, the level of counts and S/B ratio may slightly vary without affecting the assay robustness.

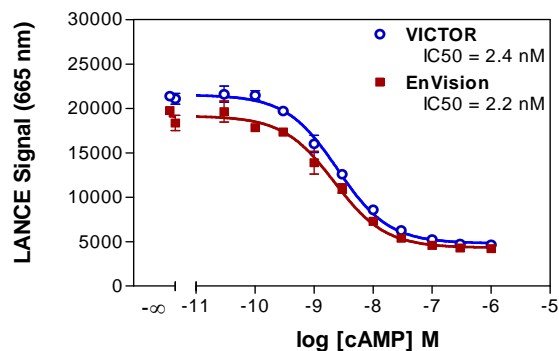


Figure 2. Typical LANCE cAMP standard curves obtained on EnVision and VICTOR instruments. A single plate with cAMP standard dilutions was read with both the EnVision and VICTOR instruments after 1 h incubation.

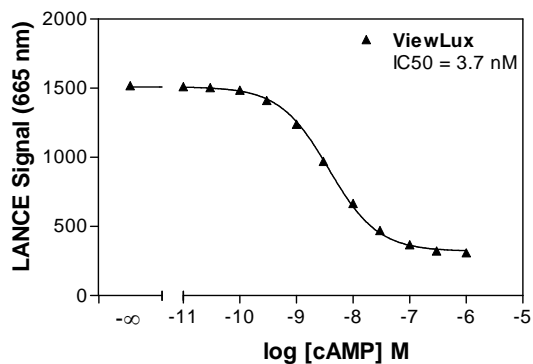


Figure 3. Typical LANCE cAMP standard curve obtained on a ViewLux instrument. A plate with cAMP standard dilutions was read with the ViewLux instrument after 1 h incubation.

6. Recommendations on the Read-Time for the LANCE cAMP Assay

Following the addition of the assay detection mixture, equilibrium binding is established over a period of time between the cAMP produced in the assay, the cAMP tracer, and the Alexa-labeled anti-cAMP antibody. During the time that this equilibrium is being established, the IC_{50} or EC_{50} measured for the assay will shift slightly. The time-course of this shift needs to be taken into account when choosing the read-time for the assay post detection mixture addition.

Figure 4 shows the time-course of the change in the pIC_{50} of the standard curve on the same plate read at one hour intervals over 1-6 hours and then again after 20 hours. The plate was covered with a plate seal for the overnight incubation to prevent evaporation. As this figure shows, the shift in the pIC_{50} occurs more rapidly during the first 1 to 2 hours following the addition of the detection mixture. The shift then slows down as the incubation time progresses.

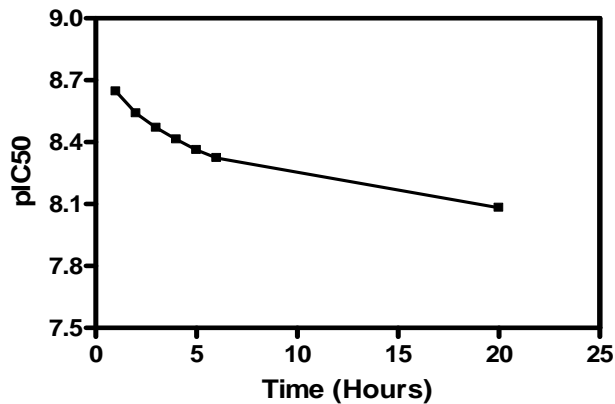


Figure 4. Time-Course for the standard curve in the LANCE cAMP Assay. The assay was measured on the ViewLux.

If the assay is read at a consistently specific time following the addition of the detection mixture, the IC_{50} or EC_{50} obtained is highly reproducible. Figure 5 shows the superposition of the standard curve obtained from ten separate plates when the plates were each read following 3 hour incubation.

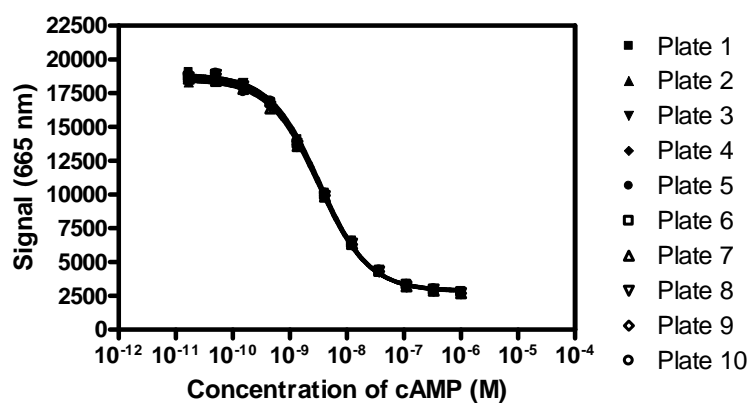


Figure 5. LANCE cAMP standard curve generated in multiple microplates and read on EnVision instrument after 3 hour incubation.

Therefore, if highly reproducible IC_{50} or EC_{50} values are required from plate-to-plate and day-to-day it is recommended that a consistent read-time is maintained. If the read-time is consistent, it can be any time at least one hour post addition of the detection mixture. However, if precise timing is not practical for reasons such as instrument availability or scheduling conflicts, it is recommended that the read-time be increased into the range of 2-6 hours or overnight, when the equilibrium is more fully established.

CELL ASSAY OPTIMIZATION GUIDELINES

Development of a LANCE cAMP assay for a specific receptor requires the optimization of the following parameters:

- 1. IBMX Concentration:** We recommend using IBMX at a concentration of 0.5 mmol/L in Stimulation Buffer. At this concentration, IBMX does not reduce the signal in cAMP standard curves. The IBMX concentration may need further optimization in cell-based assays when working with different cell lines.
- 2. Cell Number:** It is strongly recommended to generate forskolin dose-response curves in order to establish the optimal cell number per well. We suggest testing from 1,000 to 12,000 cells per well in a 24 μ L assay. The optimal cell number will be the one for which the forskolin dose-response curve covers most of the linear region of a cAMP standard curve.

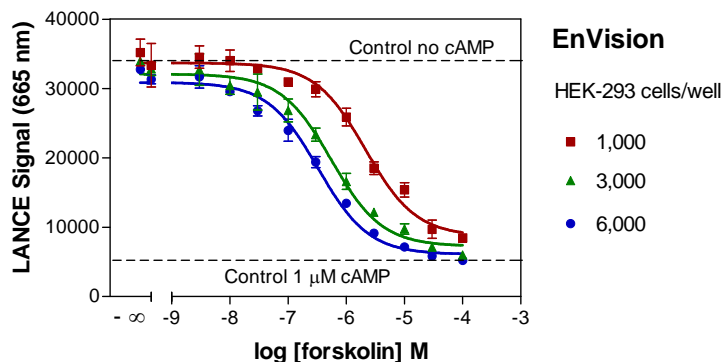


Figure 6. Forskolin dose-response curves obtained with HEK-293 cells. Assay measured on the EnVision.

The LANCE signal is plotted as a function of the log of the concentration of forskolin for each of the 3 cell concentrations tested (Figure 6). The controls signals are plotted to provide the minimum and maximum values of the possible signal range. The optimal cell number will be the one for which the forskolin dose-response curve covers most of the linear region of a cAMP standard curve (see Figure 7).

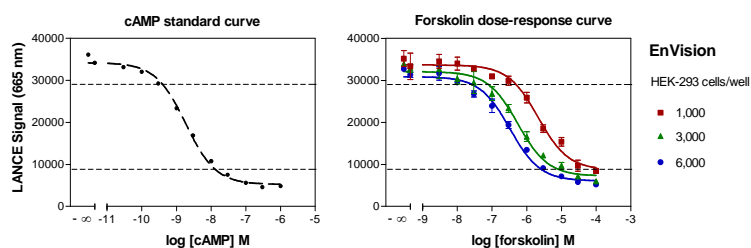


Figure 7. Determination of the optimal HEK-293 cell number

In this example, the forskolin dose-response obtained with HEK cells shows that 3,000 cells/well provides a response that falls within the linear region of the cAMP curve which is presented on the left part of figure 7.

- 3. Forskolin Concentration:** An optimized forskolin concentration is required to produce $G_{\alpha i}$ agonist dose-response curves. Forskolin is typically used at a concentration producing either 50% (EC_{50}) or 80% (EC_{80}) of maximal adenylyl cyclase activation. To determine the EC_{50} or EC_{80} , the forskolin signal inhibition curve should be transformed to a cAMP production curve, using a cAMP standard curve run in parallel. Assays performed at the EC_{50} will be more sensitive to weak agonists compared to those performed at the EC_{80} . On the other hand, assays performed using a forskolin concentration equivalent to the EC_{80} , allow an enhanced signal window.

4. **Agonist Concentration:** The presence of an agonist is required to produce G_{α_s} and G_{α_i} antagonist dose-response curves. The optimal concentration for the agonist is determined as follows: Two agonists dose-response curves are generated, one in the absence of antagonist, and the other with an excess of a reference antagonist (~10 $\mu\text{mol/L}$). The two curves are plotted on a same graph. The agonist concentration at which the difference between the two curves is the largest is selected. This is often observed at the EC80 of the agonist, which is the concentration of agonist leading to 80% of the maximal response. We do not recommend an agonist concentration higher than the EC80 for antagonist dose-response curves.
5. **Stimulation Time:** We recommend a 30-minute stimulation time. Optimization can be performed by evaluating stimulation responses from 15 to 60 minutes.
6. **Read Time:** See Recommendations on the Read-Time for the LANCE cAMP Assay on page 18.

GENERAL CELL-BASED ASSAY PROTOCOL

NOTE: In cell based-assays, IBMX should be added to the Stimulation Buffer (see page 20).

DMSO at concentrations up to 2% during cell stimulation does not affect assay performance. However, it is recommended to titrate DMSO with each cell line used.

Cell and Compound Preparation

1. Harvest cells with a non-enzymatic cell dissociation solution (like Versene).
2. Wash cells once with HBSS.
3. Resuspend the cells in Stimulation Buffer at a concentration of 2×10^6 cells per mL (12,000 cells per 6 μL). Further dilute cells to obtain the cell number needed. It is recommended to test 12,000, 6,000, 3,000 and 1,000 cells per 6 μL . Prepare a "no cell" control or a cAMP Standard curve in parallel (see page 13).

4. Add the Alexa Fluor[®] 647-labeled antibodies to the final cell suspension by making a **1/100** dilution of the stock solution in the cell suspension.
5. Make test compounds serial dilutions at 2-fold stimulation concentrations in Stimulation Buffer. If more than one test compound is added (e.g. agonist-forskolin, antagonist-agonist, or forskolin-antagonist-agonist), mix the compounds in Stimulation Buffer in order for all of them to be at a 2-fold concentration during the stimulation step.

LANCE cAMP Assay

The assay is performed in a white OptiPlate-384 or ProxiPlate-384 Plus microplate in a final assay volume of 24 or 20 μL .

For OptiPlate-384 microplates (total assay volume of 24 μL):

1. Add 6 μL of the test compound dilutions.
2. Add 6 μL of cell suspension (containing the Alexa-labeled antibodies).
3. Stimulate cells for 30–60 minutes at room temperature.
4. Add 12 μL of Detection Mix (see preparation on page 14).
5. Cover plate with another plate or with a TopSeal-A film and incubate for 60 minutes at room temperature.
6. Read on a TRF detection instrument. **Remove the TopSeal-A film from the microplate prior to reading**
7. Additional readings can be performed for up to 20 hours after tracer addition without significant decrease in assay sensitivity. See **Recommendations on the Read-Time for the LANCE cAMP Assay** (page 18).

For OptiPlate-384 and ProxiPlate-384 Plus microplates (total assay volume of 20 μ L):

1. Add 5 μ L of the test compound dilutions.
2. Add 5 μ L of cell suspension (containing the Alexa-labeled antibodies).
3. Stimulate cells for 30–60 minutes at room temperature.
4. Add 10 μ L of Detection Mix (see preparation on page 14).
5. Cover plate with another plate or with a TopSeal-A film and incubate for 60 minutes at room temperature.
6. Read on a TRF detection instrument. **Remove the TopSeal-A film from the microplate prior to reading.**
7. Additional readings can be performed for up to 20 hours after tracer addition without significant decrease in assay sensitivity. See **Recommendations on the Read-Time for the LANCE cAMP Assay** (page 18).

Please note that alternative orders of addition of reagents or cell suspensions may give satisfactory performance, but should be validated in each case.

DATA ANALYSIS

The LANCE signal obtained at 665 nm can be used directly for data analysis of cAMP standard curves, Z' determination and compound library screening. Counts at 665 nm obtained in cAMP standard curves will allow the determination of the amount of cAMP produced in stimulated cells.

The signal at 615 nm is useful to identify dispensing or quenching problems. Normally, the signal at 615 nm varies by about 10–15% in a cAMP standard curve. Counts at 615 nm are higher when the signal at 665 nm is minimal (e.g. at the highest cAMP concentration) and are lower when the signal at 665 nm is maximal (i.e. in the absence of competing cAMP). A test compound giving lower signal at both 665 nm and 615 nm wavelengths may be quenching fluorescence, or might indicate a dispensing problem.

Some test compounds may interfere the signal in the LANCE cAMP assay, giving either false positive or false negative signals. There are compounds which may quench signal by absorbing light or by other mechanisms. For minimizing false positives and false negatives in the primary screen a quench correction is recommended.

Quench correction can be performed using the blank-corrected ratio 665 nm/615 nm. The following equation gives the corrected signal:

$$F_{665,CS} = [(F_{665,S} - F_{665,BL}) \times F_{615,MAX}] / F_{615,S}$$

The blank value is separately measured by adding buffer to the wells to obtain blank reading at 665 nm ($F_{665,BL}$, e.g. 80 counts). This reading is specific for the plate type, reader and measurement protocol.

Signals at 665 nm ($F_{665,S}$) and at 615 nm ($F_{615,S}$) are measured from sample wells containing all the assay components including test compounds. The non-quenched signal at 615 nm is obtained from the highest cAMP concentration in the standard curve ($F_{615,MAX}$).

Most of the quenched samples are corrected using this equation. For more information, please see Application note 1234-9860 "Quench Correction for LANCE Time-Resolved Fluorescence Resonance Energy Transfer".

IMPORTANT NOTES

1. A thorough understanding of this package insert is necessary for successful use of the LANCE cAMP 384 kit. The reagents supplied with this kit are intended for use as an integral unit. **Do not mix identical reagents from kits having different lot numbers in order to maintain a consistency in the total signal between lots.** Do not use kit reagents after the expiration date printed on the kit label.
2. Microfuge vials of reagents for a few seconds to improve recovery of content.
3. **Do not vigorously vortex solutions containing cAMP antibody but instead mix gently.**
4. The type of plate used is critical to the assay. White OptiPlate-384 microplates are strongly recommended. Black plates will produce less signal but acceptable S/B ratios.
5. It is critical to **remove** the TopSeal-A film from the plate prior to reading.

6. It is strongly recommended to use the BSA stabilizer solution (7.5% solution) available from PerkinElmer (prod. no. CR84-100) to make the Stimulation Buffer. This solution is specifically designed for the LANCE assays.
7. When preparing the Detection Mix, always dilute the Eu-SA component first, and then add the Biotin-cAMP component to the Eu-SA solution.

WARRANTY

Purchase of the product gives the purchaser the right to use this material in his own research, development, and investigational work. The product is not to be injected into humans or used for diagnostic procedures. PerkinElmer Life and Analytical Sciences reserves the right to discontinue or refuse orders to any customer who plans to use these products for any other purposes.

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All information supplied with the product and technical assistance given is believed to be accurate, but it remains the responsibility of the investigator to confirm all technical aspects of the application. We appreciate receiving any additions, corrections, or updates to information supplied to the customer.

LITERATURE

Scientific Papers:

Hemmila I. (1999): LANCE™: Homogeneous Assay Platform for HTS. *J Biomol Screen.* **4** (6), 303–308.

Application Notes:

1234-9860 Quench Correction for LANCE™ Time-Resolved Fluorescence Resonance Energy Transfer

Posters:

Hurt, S., Harney, H., Xie, H., Le, H., Stanaker, R., Illy, C., and Manchanda, R. (2005): Automation of a GPCR cell-based functional assay on the Cellular Workstation system using the LANCE cAMP technology. Presented at SBS 2005.

Kasila, P. and Harney, H. (2005): LANCE™ cAMP – Increased sensitivity, stability and overall assay performance with G_{α_s} and G_{α_i} coupled receptors in a miniaturized 1536-well format. Presented at ScreenTech 2005.

Kasila, P. and Xie, H. (2005): A comprehensive comparison of state of the art cAMP assays. Presented at SBS 2005.

Kasila, P., Xie, H., Harney, H., Raneri, A., and Warner, G. (2005): LANCE™ cAMP + Evolution™ P³ + ViewLux™: uHTS. Presented at SBS 2005.

Kasila, P. and Xie, H. (2005): LANCE™ cAMP - A uHTS cAMP detection assay for use with membranes. Presented at SBS 2005.

For a complete documentation listing, please consult our Web site: <http://las.perkinelmer.com>



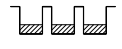
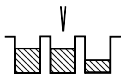
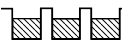

PATENTS

LANCE™-type assays and certain reagents used therefore may be covered by one or more of the following patents:

US 4,925,804, US 5,637,509, EP 0324323,
DE 3877179, AT 84150, US 4,761,481,
US 6,242,268, JP 2086269, EP 0525825,
EP 0195413, DE 3650706, DE 3689288,
CA1276028, US 4,920,195, EP 0203047,
DE 3673561, US 5,032,677, EP 0341298,
DE 3850385, CA 1339746, US 5,055,578,
US 5,106,957, US 5,116,989, US 5,245,038,
US 5,252,740, US 5,202,423, US 5,324,825,
EP 0403593, DE 68927886, AT 150461,
US 5,457,186, US 5,571,897, EP 0626068,
DE 69131206.

The Alexa Fluor® 647 dye used to label the anti cAMP antibodies is licensed from Molecular Probes, Inc.

LANCE™ cAMP 384 Kit
Summary Protocol Sheet
General cell-based assay protocols for 96, 384 and 1536 formats

		Opti-Plate-96	OptiPlate-384 & ProxiPlate-384 Plus		Opti-Plate-1536
Total Assay Volume		40 µL	20 µL	24 µL	8 µL
Add cAMP Standards or forskolin and agonist/antagonist dilutions		10 µL	5 µL	6 µL	2 µL
Add cell suspension containing the Alexa Fluor® 647-anti cAMP antibodies		10 µL	5 µL	6 µL	2 µL
Incubate		30–60 min at RT			
Add Detection Mix (containing the Eu-labeled streptavidin and the Biotin-cAMP)		20 µL	10 µL	12 µL	4 µL
Incubate		1–20 h at RT			
Measure the TR-FRET signal		See Instrument settings on page 9			

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