LANCE *Ultra* cAMP

Assay Development Guidelines

v. 2

Catalog numbers:

TRF0262: 1,000 assay points
TRF0263: 10,000 assay points
TRF0264: 50,000 assay points

For Laboratory Use Only
Research Chemicals for Research Purposes Only

Table of Contents

1. 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. Precautions

- Upon receiving the kit, store all reagents at 2 – 8°C protected from light.
- Expiration date of the kit is indicated on the box label.
- Small volumes used in the assay may be prone to evaporation. It is recommended that microplates be covered with TopSeal-A™ adhesive sealing film (PerkinElmer cat. Number 6005185) to reduce evaporation during incubation. TopSeal-A film **must be removed** prior to reading signal.

3. Reagents

3.1 Kit description

Three following kit sizes are available:

- **TRF0262:** 1,000 assay points
- **TRF0263:** 10,000 assay points
- **TRF0264:** 50,000 assay points

The amount of reagent provided with each kit format is indicated in the table below.

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 suggested protocols20-µL assay in 384-well microplate format.

** 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.

3.2 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.

3.3 Additional reagents and materials required

The following reagents and materials are required but not supplied with the kit.

Item	Suggested source	Catalog #
HBSS 1X	Invitrogen	14025
HEPES 1 M, pH 7.2-7.5	Invitrogen	15630
PBS 1X, pH 7.4	Invitrogen	10010
Ultrapure water (18.2 MΩ·cm)		
Forskolin	Calbiochem	344270
IBMX*	Sigma	I7018
DMSO	Sigma	D2650
NaOH 0.1N		
White, opaque OptiPlate™-384 well microplates	PerkinElmer	6007290
TopSeal™-A adhesive film	PerkinElmer	6005185

*3-isobutyl-1-methylxanthine

4. 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 and phosphodiesterase. Adenylyl cyclase promotes the synthesis of cAMP from adenosine triphosphate (ATP), while phosphodiesterase degrades cAMP to AMP. The activity of adenylyl cyclase is controlled through various guanosine triphosphate (GTP) binding protein-coupled receptors (GPCRs), *via* their interaction with one of two distinct GTP binding proteins, G_s and G_i. These G proteins are heterotrimeric molecules composed of the subunits G_α (s or i), $G_ß$ 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 $G_{ß\gamma}$. Upon dissociation, $G_{\alpha s}$ is primarily involved in adenylyl cyclase stimulation, whereas $G_{\alpha i}$ is inhibitory. The measurement of intracellular cAMP is thus an ideal method for measuring the effect of test compounds on GPCR-mediated adenylyl cyclase activation or inhibition.

5. 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 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 (Fig. 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 (Fig. 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 (Fig. 1, right panel).

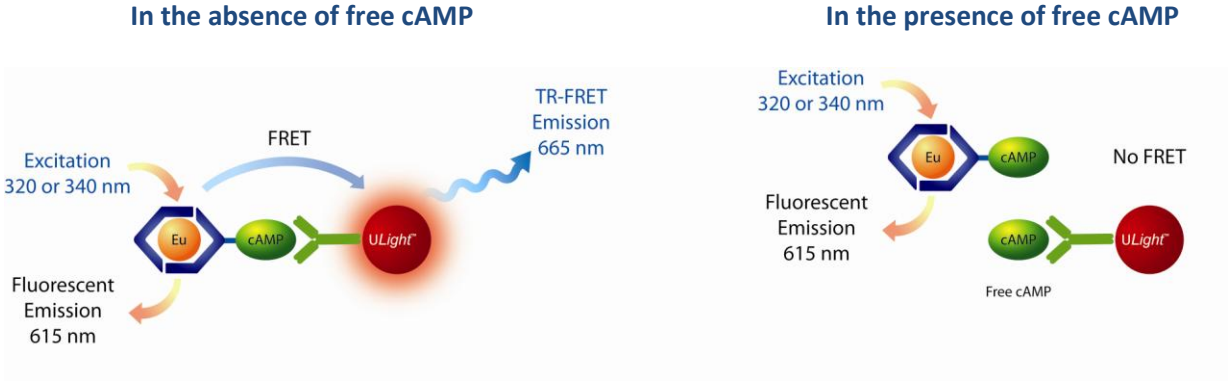


Figure 1. LANCE® *Ultra* cAMP assay principle.

The standard assay procedure for the LANCE *Ultra* cAMP assay is illustrated in Figure 2. Cells (in suspension or attached) are stimulated for 30 min. Following stimulation, the Eu-cAMP tracer and U*Light*-anti-cAMP are added to the wells. Signal is detected after 1 h with a TR-FRET plate reader.

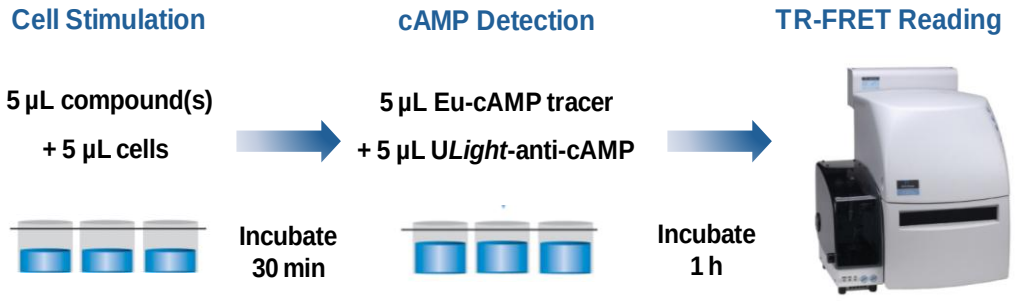


Figure 2. Standard assay procedure for the LANCE *Ultra* cAMP assay.

The LANCE *Ultra* cAMP assay is highly sensitive and robust. Assay reactions are stable for at least 24 hours at room temperature, with no significant loss of sensitivity or assay window. These characteristics make the LANCE *Ultra* cAMP kit ideal for HTS screening campaigns.

The current Assay Development Guidelines describe procedures to perform LANCE *Ultra* cAMP assays in 384-well microplates in a total assay volume of 20 µL. Assays can also be performed in 96- and 1536-well plate formats, providing all assay component concentrations and volumes are scaled up or down proportionally. LANCE *Ultra* cAMP assays can be performed using either cells (attached or in suspension, fresh or frozen) or cell membrane preparations.

6. Reagent preparation

6.1 IBMX

Dissolve 50 mg of IBMX in 900 µL DMSO for a **250 mM** stock solution. Aliquote and store at -20°C.

6.2 Stimulation Buffer

The recommended Stimulation Buffer for cell-based assays is **HBSS 1X, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA; pH 7.4**.

Prepare 15 mL of Stimulation Buffer as indicated in the table below. Adjust pH to 7.4 with NaOH 0.1N and complete to 15 mL with HBSS 1X. Make fresh.

Reagent	Volume	Final concentration
HBSS 1X	14 mL	-
BSA Stabilizer 7.5%	200 µL	0.1%
IBMX 250 mM	30 µL	0.5 mM
HEPES 1M, pH 7.2-7.5	75 µL	5 mM

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, we recommend using IBMX at a concentration of 0.5 mM in Stimulation Buffer. At this concentration, IBMX does not reduce the signal in cAMP standard curve. The IBMX concentration may require optimization when working with different cell lines or membrane preparations. IBMX can be replaced by other phosphodiesterase inhibitors (e.g., 100 µM RO-201724).**
- **Addition of BSA might not be essential for your cellular assay. However, if BSA is added, we strongly recommend the use of the BSA Stabilizer included in the kit, which is a highly purified preparation of BSA, free of europium and heavy metal ion contaminants.**

6.3 Eu-cAMP tracer solution in cAMP Detection Buffer

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

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

6.4 U*Light*-anti-cAMP solution in cAMP Detection Buffer

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

Example: Add 5 µL of the ULight-anti-cAMP stock solution to 745 µL of cAMP Detection Buffer. Mix gently.

NOTES:

- **Eu-cAMP and U*Light*-anti-cAMP 4X working solutions can be stored for up to 24 hours at room temperature or 2 – 8°C.**
- **For optimal assay performance, do not modify the recommended dilutions for both the Eu-cAMP tracer and U*Light*-anti-cAMP.**

7. Protocols for cell preparation

7.1 Frozen cell preparation

1. Thaw frozen cells rapidly at 37°C in a water bath by agitating the vial until its content is completely thawed.
2. Transfer vial content to a 15-mL centrifuge tube containing 4 mL of HBSS 1X.
3. Centrifuge cells for 10 min at 275 $\times g$.
4. Decant supernatant and resuspend pellet in 1.5 mL of HBSS 1X.
5. Determine cell concentration and viability using Trypan Blue stain or an automated cell culture analyzer (ideally, cell viability should be over 90%).
6. Centrifuge cells for 5 min at 275 $\times g$.
7. Resuspend cells in stimulation buffer at the concentration required for the assay.

7.2 Fresh culture cell preparation

1. Cells should be ~ 70% confluent for optimal results.
2. Remove culture medium and briefly rinse the cell layer with PBS without Ca2+ and Mg2+.
3. Add PBS + 5 mM EDTA (Versene solution) or other non-enzymatic cell dissociation solution and incubate at 37°C for about 5 min to detach the cells.
4. Collect cells and centrifuge them for 10 min at 275 $\times$ g.
5. Decant supernatant and resuspend pellet in HBSS 1X.
6. Determine cell concentration and viability using Trypan Blue stain or an automated cell culture analyzer (ideally, cell viability should be over 90%).
7. Centrifuge cells for 5 min at 275 $\times$ g.
8. Resuspend cells in stimulation buffer at the concentration required for the assay.

8. Assay development workflow

LANCE *Ultra* cAMP assays are developed and optimized following the assay development workflow described in the table below.

Step	$G_{\alpha s}$-Receptor Assay	$G_{\alpha i}$-Receptor Assay	Purpose
1	cAMP standard curve	cAMP standard curve	To determine the sensitivity (IC_{50} value) and dynamic range (IC_{10} – IC_{90}) of the cAMP assay.
2	Known full-agonist or forskolin concentration-response experiment at different cell densities.	Forskolin concentration-response experiment at different cell densities.	To define the optimal cell density giving the highest assay window while staying within the assay dynamic range. For $G_{\alpha i}$ assays: define the EC_{90} of forskolin to be used for the agonist assay.
3	Rank order of agonist potency.	Rank order of agonist potency (using EC_{90} forskolin).	To estimate agonist potencies (EC_{50} values) and EC_{90} of selected agonist to be used for the antagonist assay.
4	Rank order of antagonist potency (using EC_{90} agonist).	Rank order of antagonist potency (using EC_{90} forskolin + EC_{90} agonist).	To estimate antagonist potencies (IC_{50} values).

9. Generating a cAMP standard curve

The cAMP standard curve allows determining assay sensitivity (IC_{50} value) and dynamic range (IC_{10} – IC_{90}). It also provides a means to translate the measured TR-FRET signal into actual quantities of cAMP produced in cell- or membrane-based assays.

9.1 Reagent preparation

9.1.1 Stimulation Buffer

Prepare 15 mL, as described in Section 6.2.

9.1.2 cAMP standard serial dilutions (4X) in Stimulation Buffer

Prepare the **4X cAMP standard serial solutions** from the 50 µM cAMP standard supplied with the kit by making half-log dilutions in Stimulation Buffer, as indicated in the table below. Include a "no cAMP" control (dilution #12). Prepare fresh prior to assay.

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

9.1.3 Eu-cAMP tracer solution in cAMP Detection Buffer

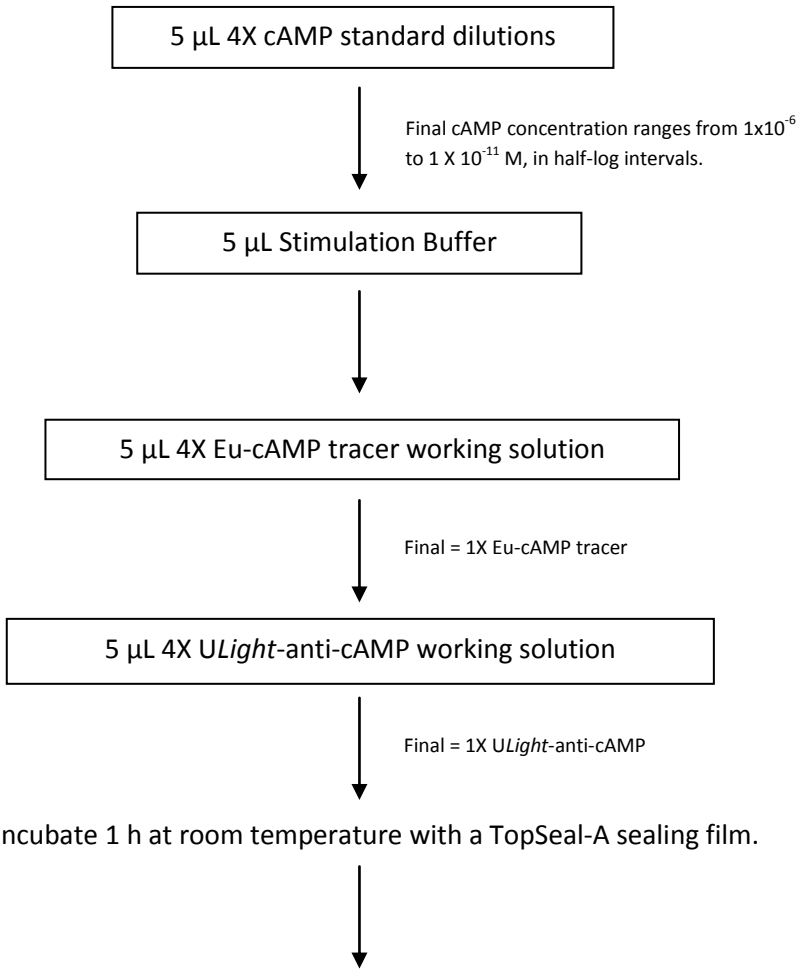
Prepare as described in Section 6.3.

9.1.4 U*Light*-anti-cAMP solution in cAMP Detection Buffer

Prepare as described in Section 6.4.

9.2 Assay flowchart

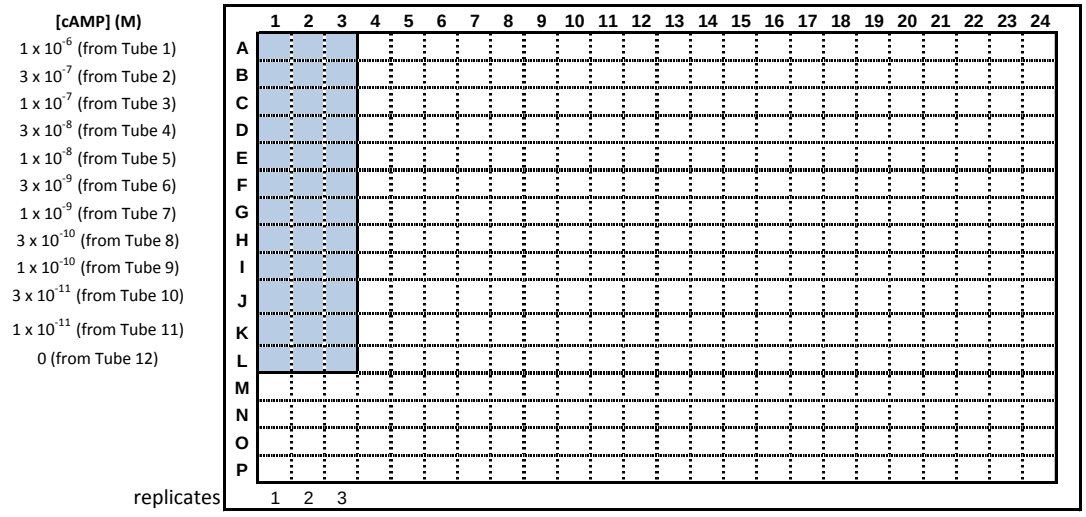
To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



Read on TR-FRET microplate reader. **Remove TopSeal-A film prior to reading.**

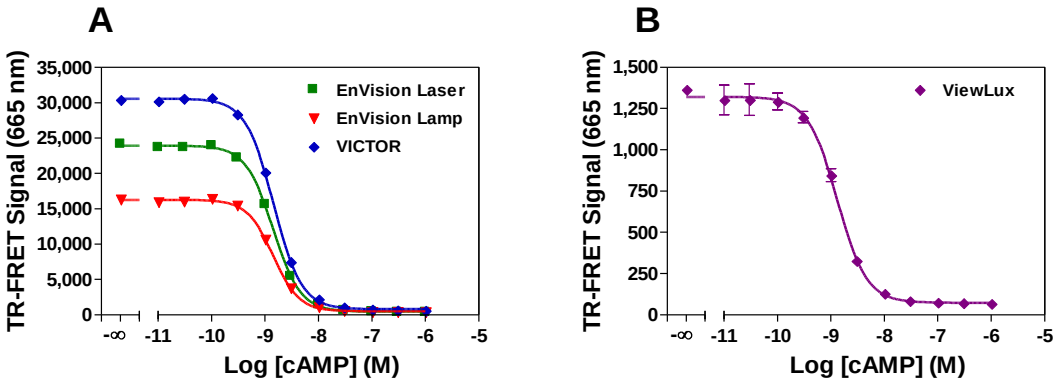
NOTE:
Additional readings can be performed for up to one week without significant decrease in assay sensitivity.

9.3 Plate set-up



9.4 Representative cAMP standard curves

Representative LANCE *Ultra* cAMP standard curves obtained on different instruments using the recommended settings listed in Section 15 are shown in Figure 3.



	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 TR-FRET readers. Assay reagents were incubated in a white opaque OptiPlate™-384 microplate for 1 hour at room temperature. TR-FRET signal was detected with the **(A)** EnVision® Multilabel reader (laser and lamp settings), VICTOR™ reader and **(B)** ViewLux® CCD imager.

OBSERVATIONS:

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

As expected for a competitive immunoassay, the LANCE *Ultra* cAMP standard curve exhibits a sigmoidal relationship between the log of the cAMP concentrations and TR-FRET signal (Figure 4). This type of curve is best fit using a four-parameter logistic equation, which optimizes accuracy and precision over the maximum usable calibration range.

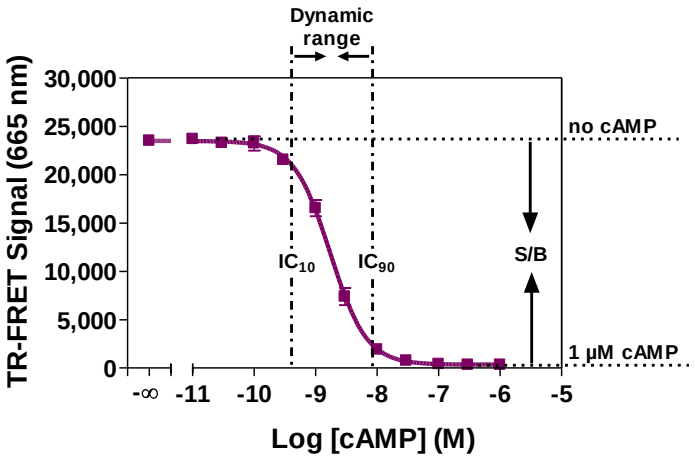


Figure 4. Analysis of a LANCE *Ultra* cAMP standard curve obtained with the EnVision® Multilabel Plate reader set in laser mode. This representative standard curve was analyzed with the GraphPad Prism® software using a sigmoidal dose response model with variable slope. In this example, signal-to-background (S/B) ratio was of of 70, with a ~1.5-log dynamic range (IC_{90}-IC_{10} values) and an IC_{50} value of 1.4 nM..

Since cAMP standard curves are non-linear, it is common among users to consider as dynamic range only the "linear portion" of the cAMP standard curve (section of the standard curve between the IC_{20} to IC_{80}). However, because data are fitted by non-linear regression analysis, there is no need to restrict the assay dynamic range to the linear portion of the standard curve.

In fact, the use of a four-parameter logistic equation allows extending significantly the assay dynamic range. For this reason, we operationally defined the assay dynamic range throughout this document as the section of the standard curve located between the IC_{10} to IC_{90}.

10. Determination of optimal cell density for $G_{\alpha s}$- and $G_{\alpha i}$-coupled receptors

Determination of the optimal cell density is a key step of LANCE *Ultra* cAMP assay development. Selection of an inappropriate cell density can result in:

- Decreased assay window (S/B ratio) due to either high "minimal" signal at high forskolin concentrations (typically due to the use of a very low cell density) or low "maximal" signal (typically due to the use of a very high cell density, which increases basal cAMP levels).
- Development of a cAMP assay outside of the assay dynamic range (typically due to the use of a very high cell density).

Forskolin dose-response curves are generated at different cell densities in order to establish the optimal cell density to be used in cAMP assays. Forskolin acts directly on the adenylyl cyclase to produce cAMP, independently of receptor activation. A **decrease** in LANCE signal is observed following cell stimulation with forskolin. The amount of cAMP produced at a saturating forskolin concentration represents the maximum amount of cAMP that can be produced by the cells.

At the optimal cell density, the forskolin dose-response curve typically gives the highest assay window (S/B ratio) while staying within the assay dynamic range. We recommend performing forskolin dose-response curves using 250 to 5,000 cells per well in a 20-µL assay.

For $G_{\alpha s}$-coupled receptors, the optimal cell density can also be determined by performing dose-response experiments with a full agonist, rather than forskolin.

Assays for $G_{\alpha i}$-coupled receptors require forskolin stimulation in order to be able to measure the decrease in cAMP levels induced by an agonist. The forskolin dose-response curve at the optimal cell density allows determining the forskolin concentration that will be used for stimulating cAMP production in agonist and antagonist assays for $G_{\alpha i}$-coupled receptors (see Section 13 and 14).

10.1 Reagent preparation

10.1.1 IBMX

Prepare as described in Section 6.1.

10.1.2 Forskolin

Dissolve 5 mg forskolin in 244 µL of DMSO to make up the required 50 mM stock solution. Aliquote and store at -20°C.

10.1.3 Stimulation Buffer

Prepare 15 mL, as described in Section 6.2.

10.1.4 Cell dilutions

- See Section 7 for cell preparation.
- Dilute cells in Stimulation Buffer to a concentration of 1,000 cells/µL (1 X 10^6 cells/mL).
- Make additional dilutions in Stimulation Buffer to obtain solutions containing cells at concentrations of 600, 400, 200, 100 and 50 cells/µL, as indicated in the table below. Dilute cells just prior to assay.

Cells/µL	Volume of cell solution (µL)	Volume of stimulation buffer (µL)	Number of cells/well
1,000	-		5,000
600	600 of 1,000 cells/µL	400	3,000
400	500 of 600 cells/µL	250	2,000
200	350 of 400 cells/µL	350	1,000
100	300 of 200 cells/µL	300	500
50	200 of 100 cells/µL	200	250

10.1.5 Forskolin dilutions (2X) in Stimulation Buffer

- Thaw the 50 mM forskolin stock solution (see Section 10.1.2 for stock solution preparation).
- Prepare a 500 µM working dilution by adding 5 µL of the 50 mM stock solution to 495 µL Stimulation Buffer.
- Make serial dilutions from the 500 µM forskolin working dilution in Stimulation Buffer to obtain 2X intermediate solutions ranging from 2×10^{-4} to 2×10^{-9} M in half-log intervals, as indicated in the table below. Include a "no forskolin" control (dilution #12). Prepare fresh prior to assay.

Dilution	[Final] (M)	[2X] (M)	Volume of dilution	Stimulation Buffer
1	1×10^{-4}	2×10^{-4}	160 µL of 500 µM	240 µL
2	3×10^{-5}	6×10^{-5}	120 µL of 1	280 µL
3	1×10^{-5}	2×10^{-5}	120 µL of 2	240 µL
4	3×10^{-6}	6×10^{-6}	120 µL of 3	280 µL
5	1×10^{-6}	2×10^{-6}	120 µL of 4	240 µL
6	3×10^{-7}	6×10^{-7}	120 µL of 5	280 µL
7	1×10^{-7}	2×10^{-7}	120 µL of 6	240 µL
8	3×10^{-8}	6×10^{-8}	120 µL of 7	280 µL
9	1×10^{-8}	2×10^{-8}	120 µL of 8	240 µL
10	3×10^{-9}	6×10^{-9}	120 µL of 9	280 µL
11	1×10^{-9}	2×10^{-9}	120 µL of 10	240 µL
12 (ctrl)	0	0	-	280 µL

10.1.6 Eu-cAMP tracer solution in cAMP Detection Buffer

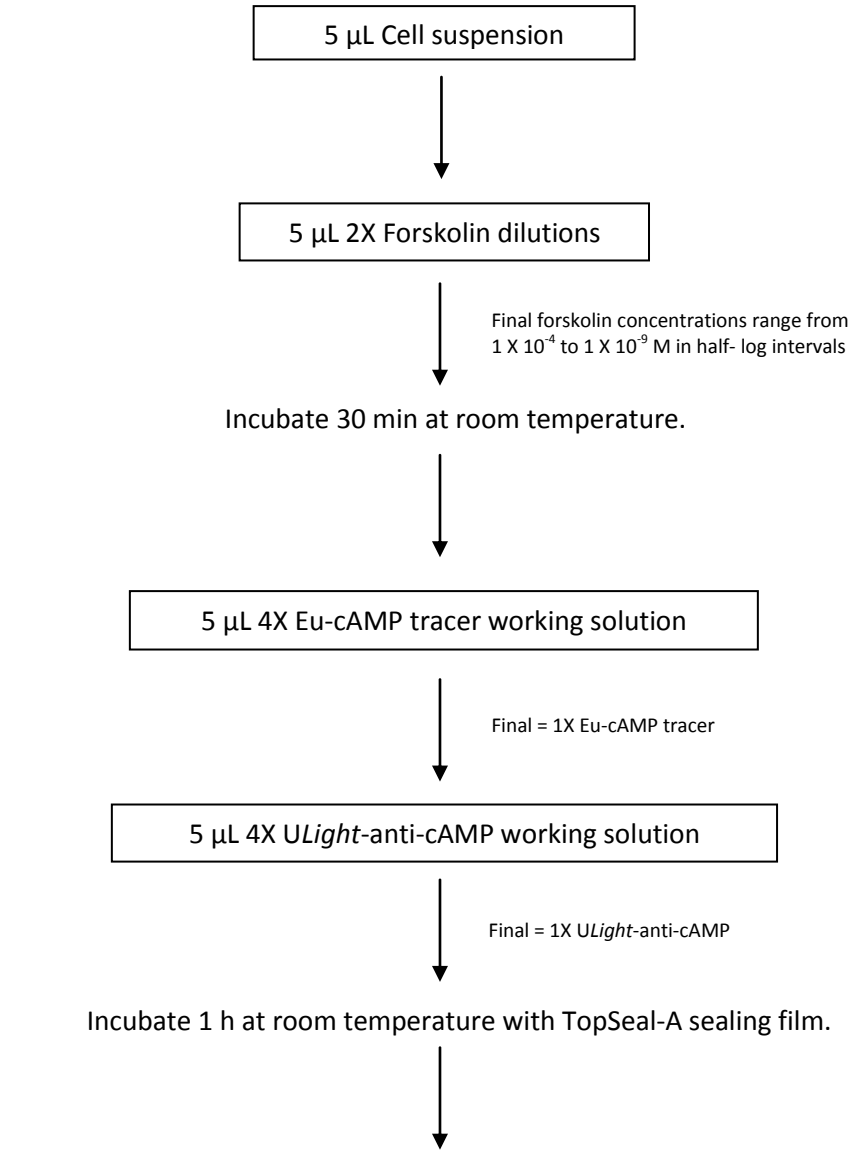
Prepare as indicated in Section 6.3.

10.1.7 U*Light*-anti-cAMP solution in cAMP Detection Buffer

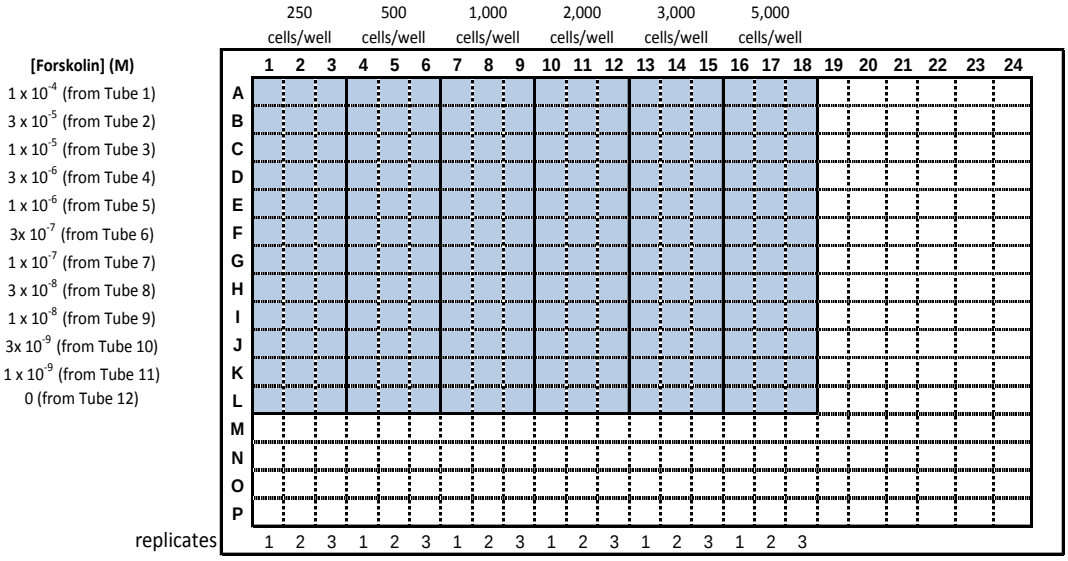
Prepare as indicated in Section 6.4.

10.2 Assay flowchart

To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



10.3 Plate set-up



10.4 Interpreting forskolin and cell cross-titration data

10.4.1 Optimal cell density

The TR-FRET signal is plotted against the logarithmic values of forskolin concentrations for each cell density tested. The forskolin dose-response curves obtained are related to the cAMP standard curve in order to establish which cell number provides a response that covers most of the dynamic range of the cAMP standard curve (operationally defined as IC_{10} – IC_{90}). This typically corresponds to the cell density giving the highest S/B ratio calculated using the maximal signal (untreated cells) and the minimal signal obtained with a saturating concentration of forskolin (fully activated cells), as shown in Figure 5.

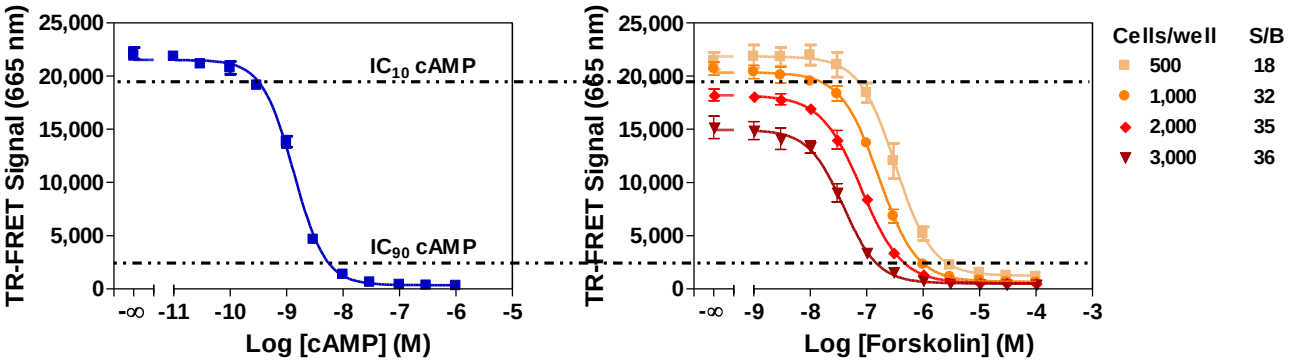


Figure 5. Determination of optimal cell density. Left panel: cAMP standard curve; **right panel:** cell and forskolin cross-titration. TR-FRET signal was measured with the EnVision Multilabel Plate reader set in laser mode.

OBSERVATIONS:

The optimal cell concentration to be used in subsequent experiments (e.g., agonist dose-response curves) would be 1,000 cells per well, since at this cell density the highest S/B ratio is obtained while staying within the IC_{10} – IC_{90} dynamic range of the cAMP standard curve. 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.

It is also recommended to convert TR-FRET data obtained at 665 nm into cAMP production values by interpolating from a standard curve run in parallel. Then, forskolin dose-response curves at different cell densities are re-plotted in terms of cAMP data, as shown in Figure 6.

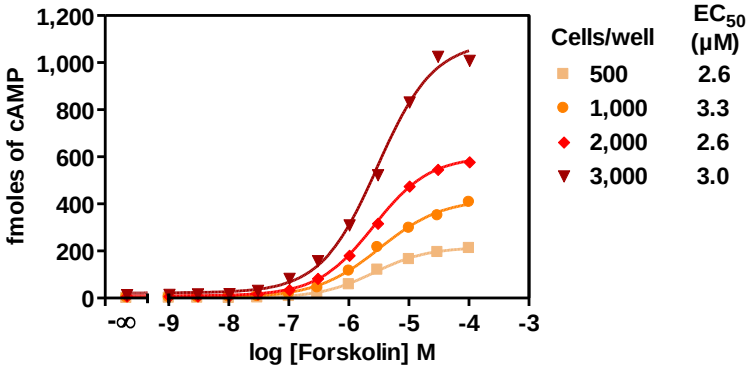


Figure 6. Forskolin dose-response curves at different cell densities. Data from Figure 5 (right panel) were re-plotted as fmoles of cAMP produced by interpolating from a cAMP standard curve run in parallel (Figure 5, left panel).

OBSERVATIONS:

EC_{50} values for forskolin are comparable regardless of the cell density used. This is a clear indication that at all cell densities tested the LANCE *Ultra* cAMP assay is within the assay dynamic range.

10.4.2 Determination of forskolin concentration for $G_{\alpha i}$-GPCRs assays

cAMP assays for $G_{\alpha i}$-coupled receptors require forskolin stimulation. The optimal forskolin concentration to be used in those assays can be determined as follows:

- **In high-throughput screening (HTS) for agonists of $G_{\alpha i}$-GPCRs**, the assay requires the highest signal difference between forskolin-activated cells and cells co-stimulated with forskolin and a fixed concentration of agonist. Such a maximal signal difference is typically obtained using forskolin at the EC_{90} value (concentration that reduces by 90% the maximal TR-FRET response). When screening for antagonist of $G_{\alpha i}$-GPCRs, forskolin is also used at its EC_{90} value.
- **For ligand characterization, the preferred forskolin concentration is its EC_{50} value based on cAMP data.** The EC_{50} value based on cAMP production provides information about the biology of the cellular system, whereas the EC_{50} value based on fluorescence data provides no biologically relevant information. The forskolin EC_{50} value based on cAMP production is typically close to the EC_{90} value based on fluorescence data and as such, it can also be used in HTS assays and will give a very good signal difference between forskolin- and forskolin/agonist-activated cells (agonist screening) or forskolin/agonist- and forskolin/agonist/antagonist-activated cells (antagonist screening). Higher forskolin values

can be used but the higher the forskolin concentration selected for activating the cell system, the lower the sensitivity of the cAMP assay to weak agonists.

- The EC_{50} value based on cAMP data is typically higher than the EC_{50} value obtained with TR-FRET data; why? The main reason behind such a discrepancy is the fact that the relationship between fluorescent signal and cAMP concentration is not linear. Changes in cAMP concentrations below IC_{10} and above IC_{90} of the cAMP standard curve are accompanied by minor changes in fluorescence signal. The use of a very high cell density generating cAMP levels beyond the assay dynamic range will exacerbate differences between fluorescence and cAMP data.

11. $G_{\alpha s}$-coupled receptor stimulation/Agonist dose-response curve

Agonist stimulation of cells or membranes expressing $G_{\alpha s}$-coupled receptors **increases cAMP production**. As a result, a concentration-dependent **decrease** in LANCE signal is observed.

Agonist potency (EC_{50} value) is determined by performing a dose-response curve at the optimal cell density.

11.1 Reagent preparation

11.1.1 Stimulation Buffer

Prepare 15 mL, as described in Section 6.2.

11.1.2 Agonist dilutions (2X) in Stimulation Buffer

Agonist dilutions are prepared in Stimulation Buffer as 2X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected agonist EC_{50} value. Include 11 half-log agonist dilutions and a "no agonist" control.

11.1.3 Eu-cAMP tracer solution in cAMP Detection Buffer

Prepare as indicated in Section 6.3.

11.1.4 U*Light*-anti-cAMP solution in cAMP Detection Buffer

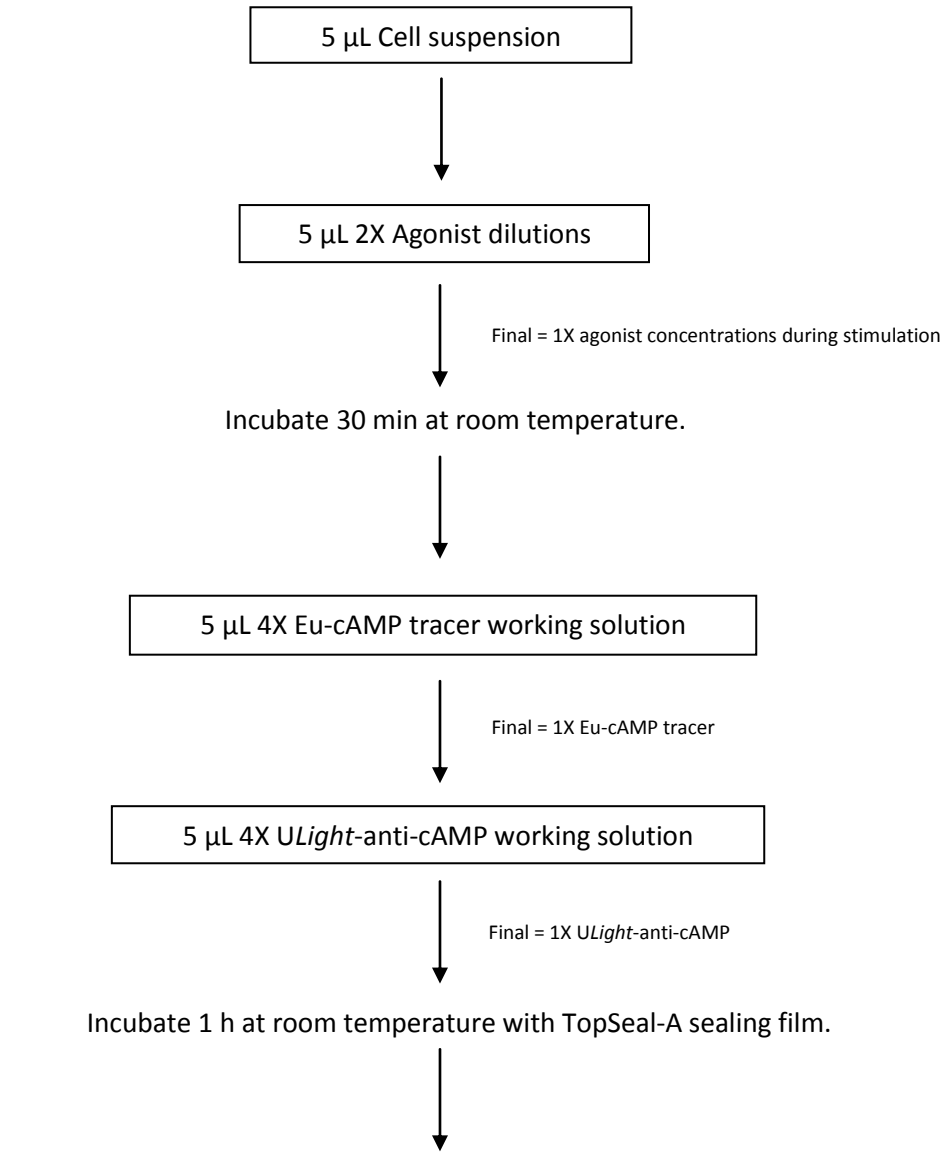
Prepare as indicated in Section 6.4.

11.1.5 Cell dilution

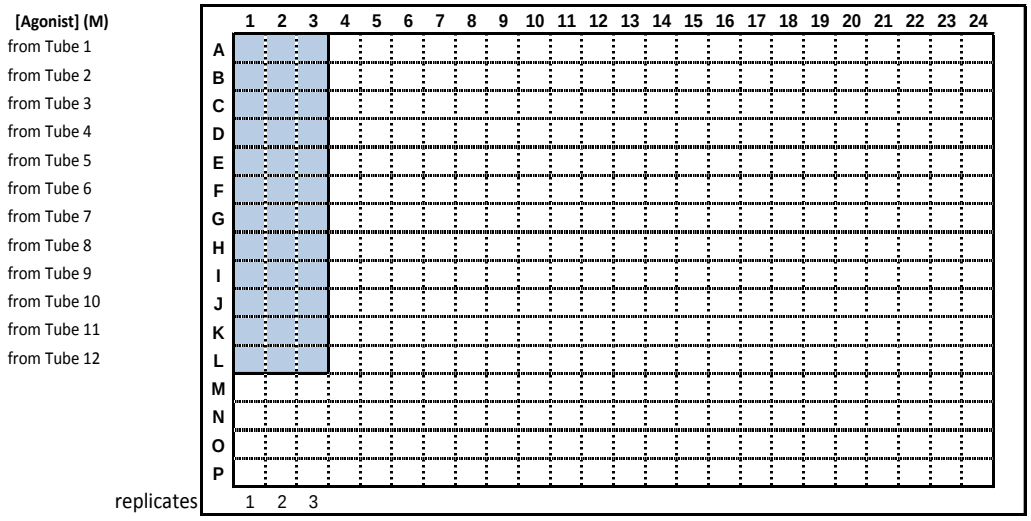
- See Section 7 for cell preparation.
- Dilute cells in Stimulation Buffer to obtain a final cell concentration corresponding to the selected cell density.

11.2 Assay flowchart

To a white, opaque Optiplate-384 microplate, add in triplicate wells:



11.3 Plate set-up



11.4 Interpreting the $G_{\alpha s}$ agonist dose-response data

The TR-FRET signal is plotted against the logarithmic values of agonist concentrations (Figure 7, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel. The amount of cAMP produced can then be re-plotted against the logarithmic values of agonist concentrations (Figure 7, right panel).

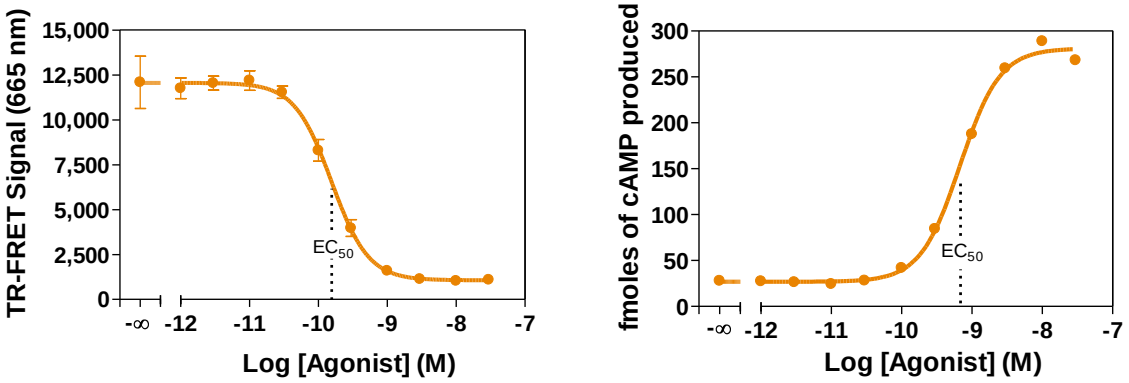


Figure 7. $G_{\alpha s}$ agonist dose-response curve. Left panel: TR-FRET signal plotted against the logarithmic values of agonist concentrations; **right panel:** TR-FRET signal (left panel) re-plotted in terms of cAMP data by interpolating from a cAMP standard curve run in parallel. TR-FRET signal was measured with the EnVision Multilabel Plate reader set in laser mode.

OBSERVATIONS:

The EC_{50} is the concentration of a $G_{\alpha s}$ agonist that reduces the maximal TR-FRET response by 50%. This is observed at the mid-point of the agonist dose-response curve (Figure 7, left panel). Interpolated cAMP values allow determining the EC_{50} for the $G_{\alpha s}$ agonist-induced stimulation of cAMP production (Figure 7, right panel). Minor differences between EC_{50} values based on fluorescence or cAMP production data are expected and explained by the non-linear relationship between fluorescence signal and cAMP concentration.

11.5 Determination of $G_{\alpha s}$ agonist concentration for antagonist assay

The presence of an agonist is required to screen for antagonists of $G_{\alpha s}$-coupled receptors. The optimal concentration for the agonist can be determined as follows:

- **In HTS for antagonist of $G_{\alpha i}$-GPCRs**, the assay requires the highest signal difference between agonist-activated cells and cells co-stimulated with agonist and a fixed concentration of antagonist. Such a maximal signal difference is typically obtained using the **agonist at its EC_{90} value (based on TR-FRET data at 665 nm).**
- **For ligand characterization, the preferred agonist concentration is its EC_{80} value based on cAMP data**. The EC_{80} value based on cAMP production is typically close to the EC_{90} value based on fluorescence data and as such, it can also be used in HTS assays and will give a very good signal difference between agonist- and agonist/antagonist-activated cells.

12. $G_{\alpha s}$-coupled receptor stimulation/Antagonist dose-response curve

Antagonist stimulation of cells or membranes expressing $G_{\alpha s}$-coupled receptors results in a **blockade of the agonist-induced cAMP production**. As a result, less intracellular cAMP is produced and an **increase** in LANCE signal is observed, compared to cells or membranes treated with the agonist alone.

Antagonist potency (IC_{50} value) is determined by performing a dose-response curve at the optimal cell density using an agonist at either its EC_{90} (TR-FRET data at 665 nm) or EC_{80} (cAMP data) concentration.

12.1 Reagent preparation

12.1.1 Stimulation Buffer

Prepare 15 mL, as described in Section 6.2.

12.1.2 Agonist working solution (4X) in Stimulation Buffer

The agonist is prepared in Stimulation Buffer as a 4X working solution. We recommend adding the agonist at a concentration producing 90% (EC_{90}) of the maximum agonist activation, which allows obtaining maximal assay window while minimizing inter-day data variability.

12.1.3 Antagonist dilutions (4X) in Stimulation Buffer

Antagonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected IC_{50} value. Include 11 half-log antagonist dilutions and a "no antagonist" control.

12.1.4 Eu-cAMP tracer solution in cAMP Detection Buffer

Prepare as indicated in Section 6.3.

12.1.5 U*Light*-anti-cAMP solution in cAMP Detection Buffer

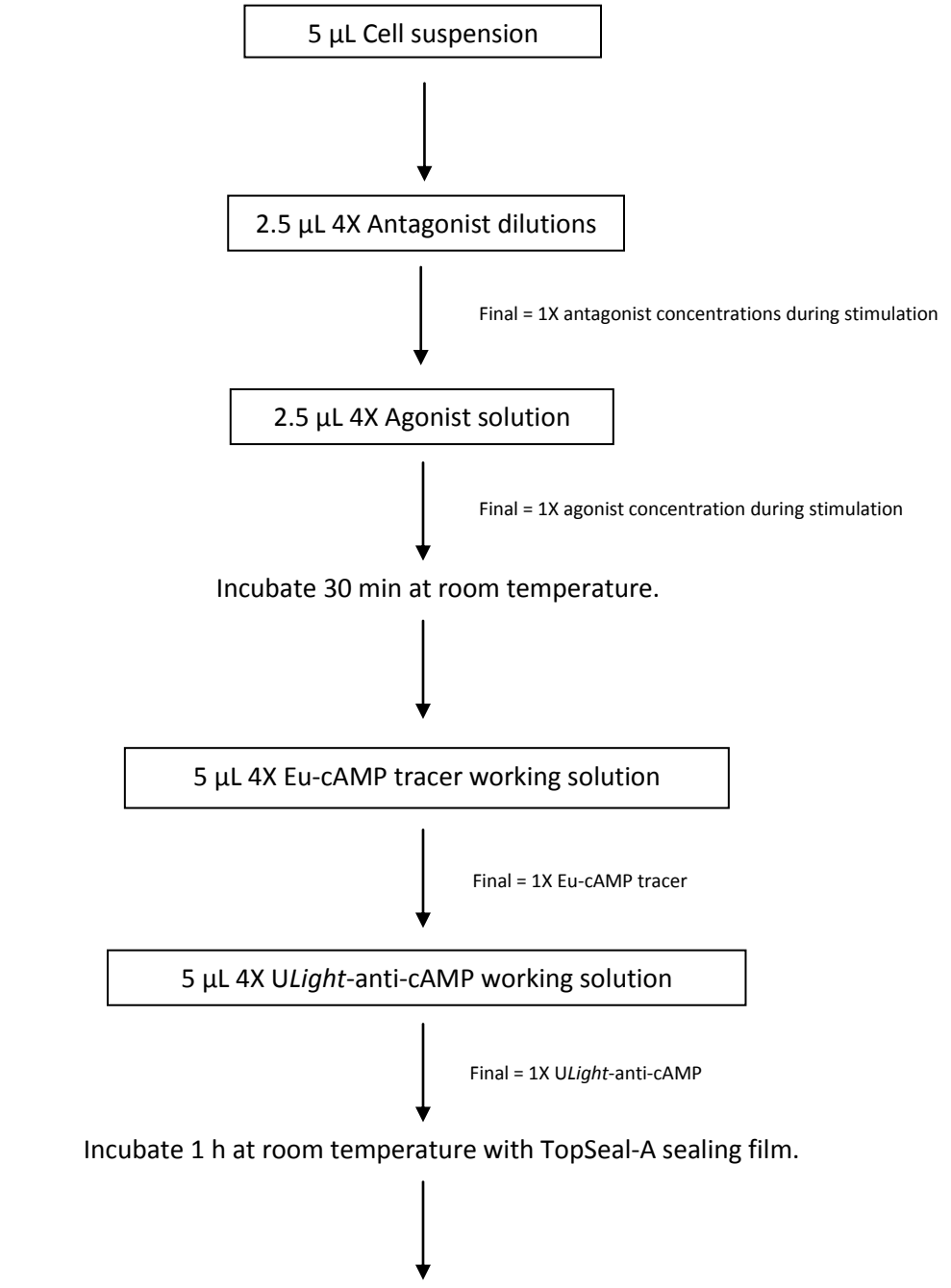
Prepare as indicated in Section 6.4.

12.1.6 Cell dilution

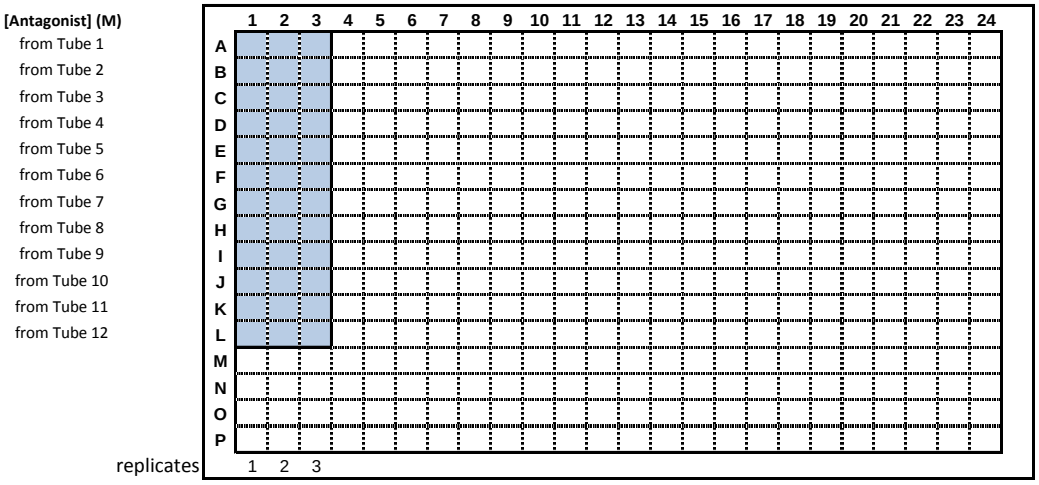
- See Section 7 for cell preparation.
- Dilute cells in Stimulation Buffer to obtain a final cell concentration corresponding to the selected cell density.

12.2 Assay flowchart

To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



12.3 Plate set-up



12.4 Interpreting the $G_{\alpha s}$ antagonist dose-response data

The TR-FRET signal is plotted against the logarithmic values of antagonist concentrations (Figure 8, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of antagonist concentrations (Figure 8, right panel).

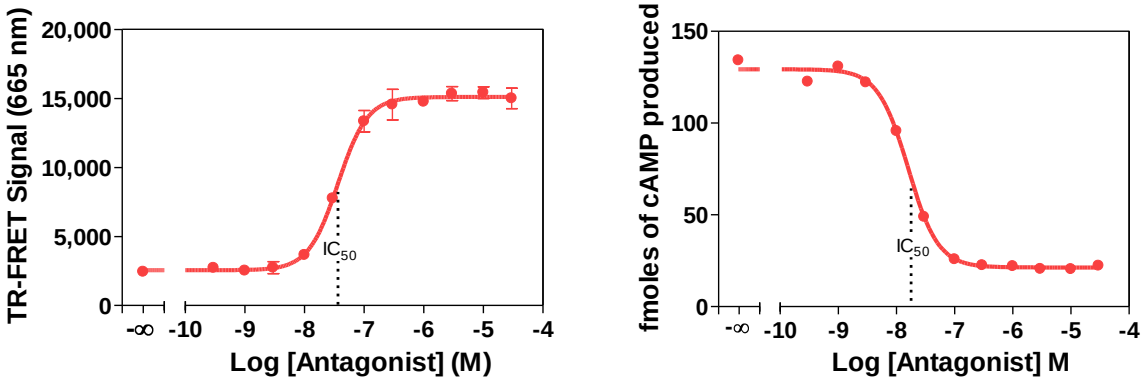


Figure 8. $G_{\alpha s}$ antagonist dose-response curve. Left panel: TR-FRET signal plotted against the logarithmic values of antagonist concentrations; **right panel:** TR-FRET signal (left panel) re-plotted in terms of cAMP data by interpolating from a cAMP standard curve run in parallel (data not shown). TR-FRET signal was measured with the EnVision Multilabel Plate reader set in laser mode.

OBSERVATIONS:

The IC_{50} is the concentration of a $G_{\alpha s}$ antagonist that reduces the maximal TR-FRET response of the $G_{\alpha s}$ agonist by 50%. This is observed at the mid-point of the antagonist dose-response curve (Figure 8, left panel). Interpolated cAMP values allow determining the IC_{50} for the $G_{\alpha s}$ antagonist-induced inhibition of cAMP production (Figure 8, right panel). Minor differences between IC_{50} values based on fluorescence or cAMP production data are expected and explained by the non-linear relationship between fluorescence signal and cAMP concentration.

13. $G_{\alpha i}$-coupled receptor stimulation/Agonist dose-response curve

Agonist stimulation of cells expressing $G_{\alpha i}$-coupled receptors decreases cAMP production. In order to detect this agonist-induced reduction in cAMP levels, cells are stimulated with forskolin to increase the intracellular cAMP concentration. In the presence of an agonist, **the forskolin-induced cAMP production is decreased**. As a result, a concentration-dependent **increase** in LANCE signal is observed compared to cells treated only with forskolin.

Agonist potency (EC_{50} value) is determined by performing a dose-response curve at the optimal cell density in the presence of forskolin at either its EC_{90} (TR-FRET data at 665 nm) or EC_{50} (cAMP data) concentration.

13.1 Reagent preparation

13.1.1 Stimulation Buffer

Prepare 15 mL, as described in Section 6.2.

13.1.2 Forskolin working solution (4X) in Stimulation Buffer

Forskolin is prepared in Stimulation Buffer as a 4X working solution. We recommend adding the forskolin at a concentration producing 90% (EC_{90}) of the maximum forskolin activation, which allows obtaining maximal assay window while minimizing inter-day data variability.

Prepare a 500 µM forskolin working dilution by adding 5 µL of the 50 mM stock solution (see Section 10.1.2 for stock solution preparation) to 495 µL Stimulation Buffer. Further dilute to obtain a solution at 4X the EC_{90} value.

13.1.3 Agonist dilutions (4X) in Stimulation Buffer

Agonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected agonist EC_{50} value. Include 11 half-log agonist dilutions and a "no agonist" control.

13.1.4 Eu-cAMP tracer solution in cAMP Detection Buffer

Prepare as indicated in Section 6.3.

13.1.5 U*Light*-anti-cAMP solution in cAMP Detection Buffer

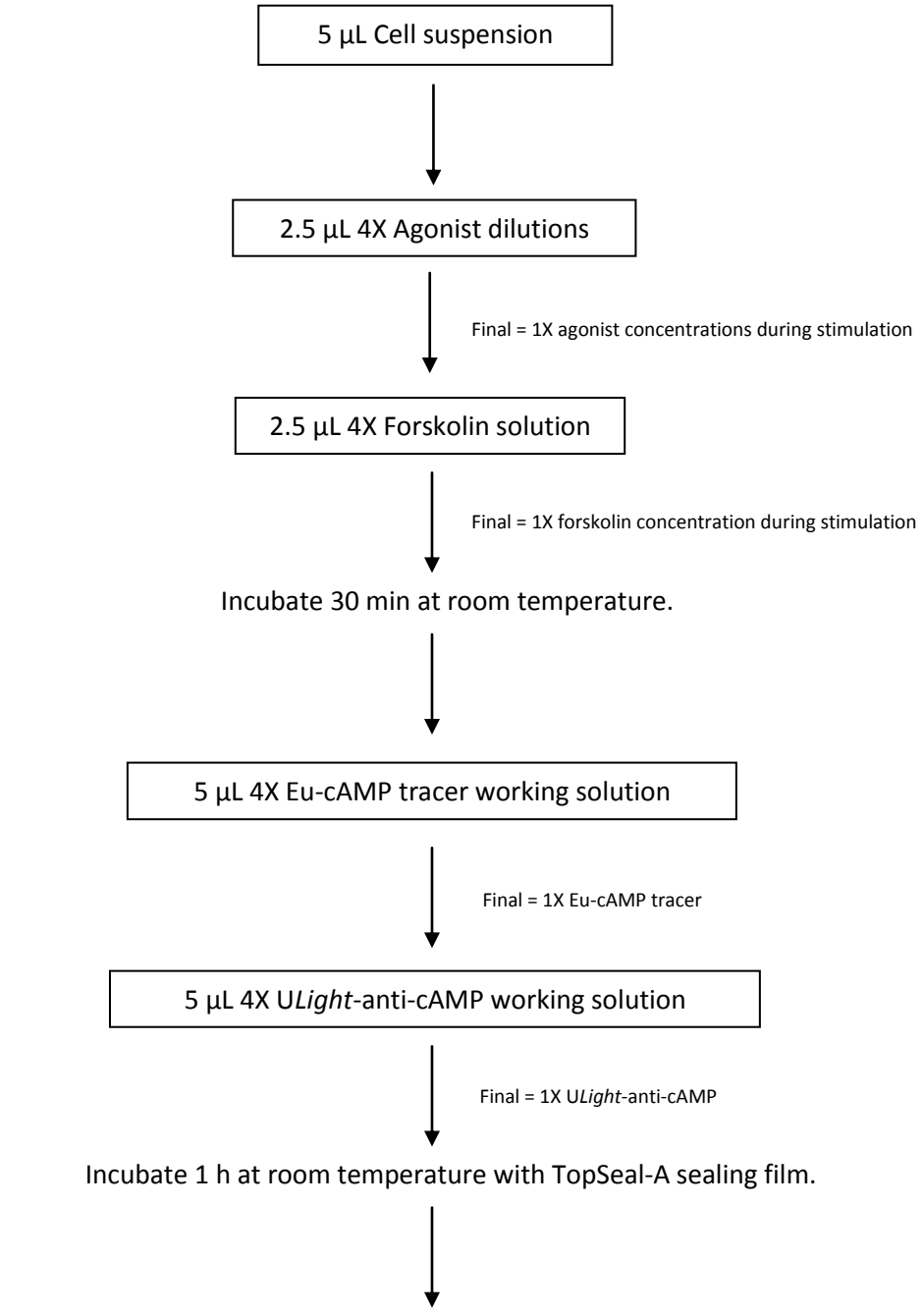
Prepare as indicated in Section 6.4.

13.1.6 Cell dilution

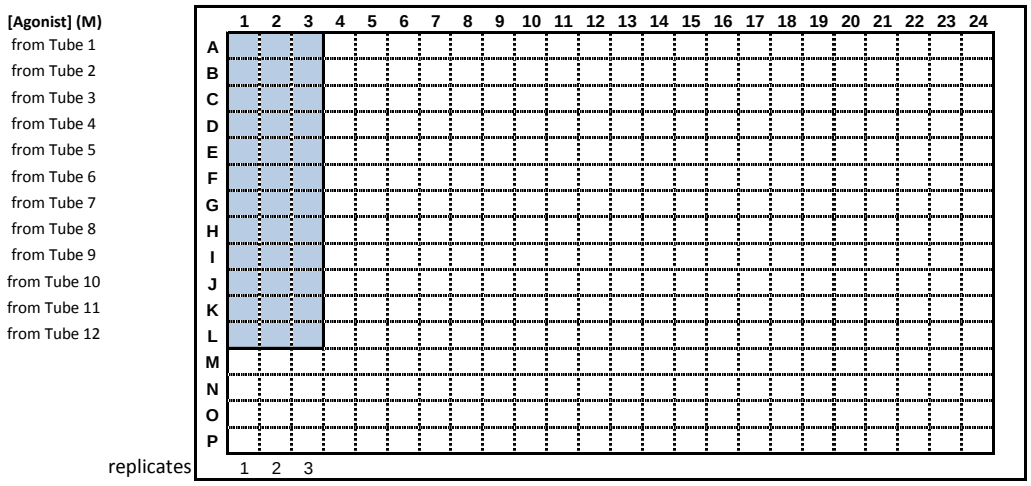
- See Section 7 for cell preparation.
- Dilute cells in Stimulation Buffer to obtain a final cell concentration corresponding to the selected cell density.

13.2 Assay flowchart

To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



13.3 Plate set-up



13.4 Interpreting the $G_{\alpha i}$ agonist dose-response data

The TR-FRET signal is plotted against the logarithmic value of agonist concentrations (Figure 9, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of agonist concentrations (Figure 9, right panel).

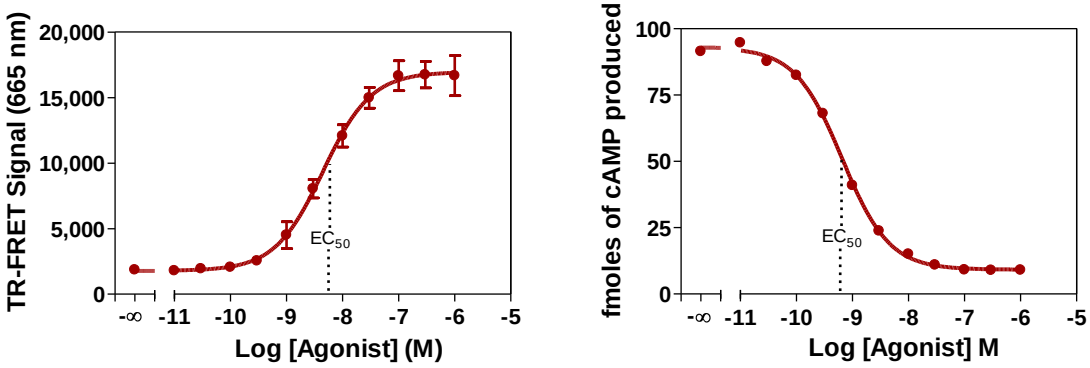


Figure 9. $G_{\alpha i}$ agonist dose-response curve. Left panel: TR-FRET signal plotted against the logarithmic values of agonist concentrations; **right panel:** TR-FRET signal (left panel) re-plotted in terms of cAMP data by interpolating from a cAMP standard curve run in parallel (data not shown). TR-FRET signal was measured with the EnVision Multilabel Plate reader set in laser mode.

OBSERVATIONS:

The EC_{50} is the concentration of a $G_{\alpha i}$ agonist that produces 50% of the maximal TR-FRET response in the presence of forskolin at its EC_{90} concentration. This is observed at the mid-point of the agonist dose-response curve (Figure 9, left panel). Interpolated cAMP values allow determining the EC_{50} for the $G_{\alpha i}$ agonist-induced inhibition of cAMP production (Figure 9, right panel). Minor differences between EC_{50} values based on fluorescence or cAMP production data are expected and explained by the non-linear relationship between fluorescence signal and cAMP concentration.

14. $G_{\alpha i}$-coupled receptor stimulation/Antagonist dose-response curve

Antagonist stimulation of cells expressing $G_{\alpha i}$-coupled receptors results in a **blockade of the agonist-induced cAMP reduction**. As a result, a concentration-dependent **decrease** in LANCE signal is observed compared to cells co-treated with forskolin and agonist.

Antagonist potency (IC_{50} value) is determined by performing a dose-response curve at the optimal cell density using forskolin and a reference agonist at their EC_{90} concentration (TR-FRET data at 665 nm). Alternatively, forskolin (EC_{50} concentration) and reference agonist (EC_{80} concentration) can be used (cAMP data).

14.1 Reagent preparation

14.1.1 Stimulation buffer

Prepare 15 mL, as described in Section 6.2.

14.1.2 Combined agonist/forskolin working solution mix (4X) in Stimulation Buffer

Forskolin and agonist are prepared in Stimulation Buffer as a single 4X working solution. We recommend adding both forskolin and agonist for cell stimulation at concentrations producing 90% of their maximal effect (EC_{90} concentrations) in order to obtain a maximal assay window while minimizing inter-day data variability.

14.1.3 Antagonist dilutions (4X) in Stimulation Buffer

Antagonist dilutions are prepared in Stimulation Buffer as 4X intermediate solutions. Ideally, the dilution series should cover concentrations that are 3 log units higher and lower than the expected antagonist IC_{50} value. Include 11 half-log antagonist dilutions and a "no antagonist" control.

14.1.4 Eu-cAMP tracer solution in cAMP Detection Buffer

Prepare as indicated in Section 6.3.

14.1.5 U*Light*-anti-cAMP solution in cAMP Detection Buffer

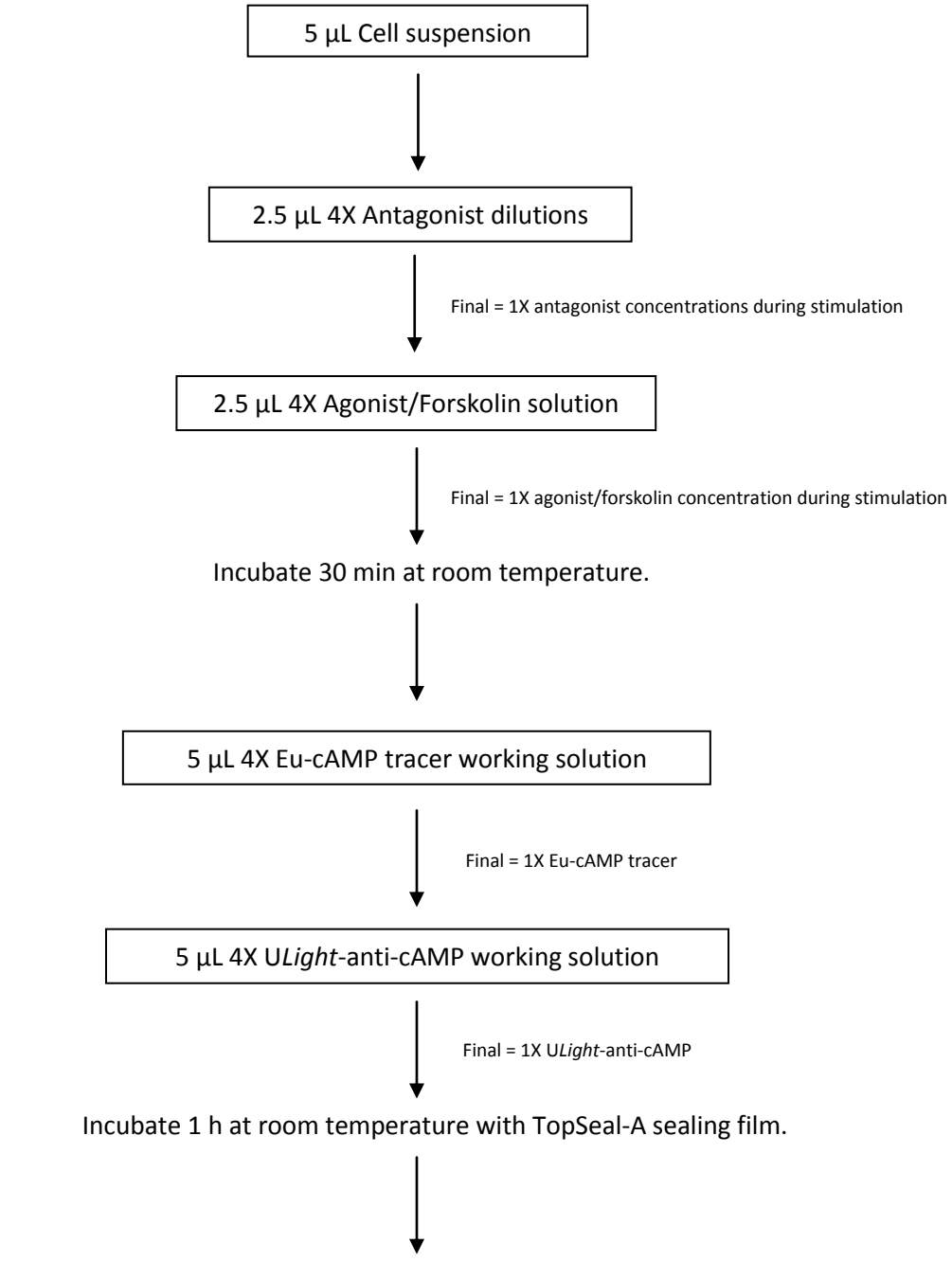
Prepare as indicated in Section 6.4.

14.1.6 Cell dilution

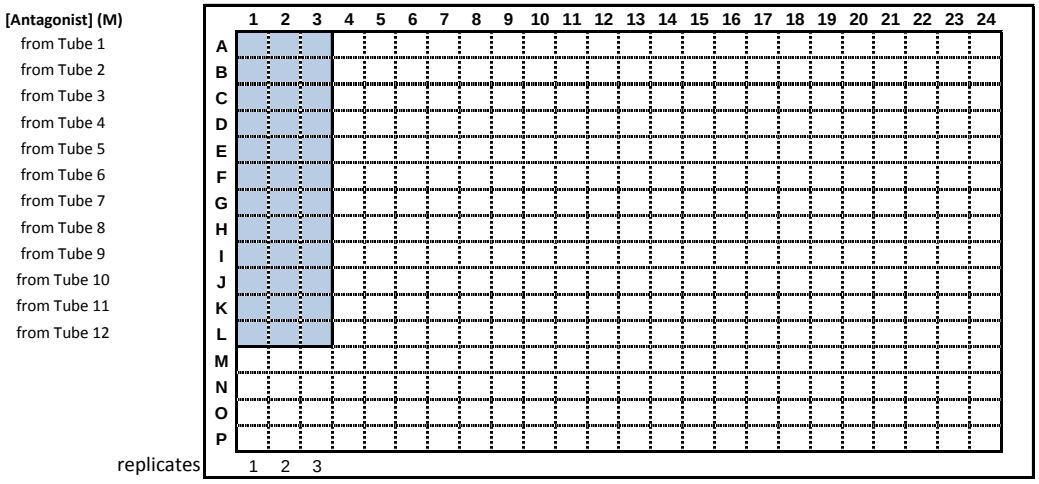
- See Section 7 for cell preparation.
- Dilute cells in Stimulation Buffer to obtain a final cell concentration corresponding to the selected cell density.

14.2 Assay flowchart

To a white, opaque OptiPlate-384 microplate, add in triplicate wells:



14.3 Plate set-up



14.4 Interpreting the $G_{\alpha i}$ antagonist dose-response data

The TR-FRET signal is plotted against the logarithmic values of antagonist concentrations (Figure 10, left panel). Signal can then be transformed to the amount of cAMP produced using a cAMP standard curve run in parallel (not shown). The amount of cAMP produced can then be re-plotted against the logarithmic values of antagonist concentrations (Figure 10, right panel).

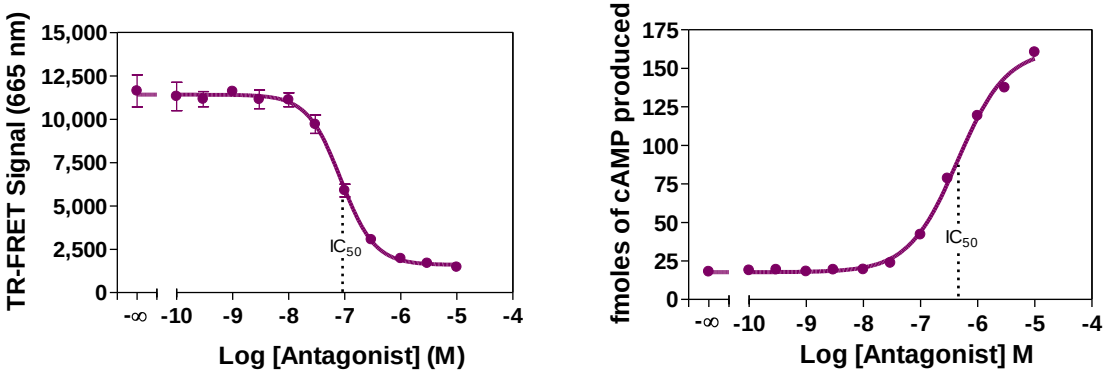


Figure 10. $G_{\alpha i}$ antagonist dose-response curve. Left panel: TR-FRET signal plotted against the logarithmic values of antagonist concentrations; **right panel:** TR-FRET signal (left panel) re-plotted in terms of cAMP data by interpolating from a cAMP standard curve run in parallel (data not shown). TR-FRET signal was measured with the EnVision Multilabel Plate reader set in laser mode.

OBSERVATIONS:

The IC_{50} is the concentration of a $G_{\alpha i}$ antagonist that reduces the maximal TR-FRET response of the $G_{\alpha i}$ agonist by 50% in the presence of both forskolin and agonist at their EC_{90} concentration. This is observed at the mid-point of the antagonist dose-response curve (Figure 10, left panel). Interpolated cAMP values allow determining the IC_{50} for the $G_{\alpha i}$ antagonist-induced stimulation of cAMP production (Figure 10, right panel). Minor differences between IC_{50} values based on fluorescence or cAMP production data are expected and explained by the non-linear relationship between fluorescence signal and cAMP concentration.

15. Instrument settings

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® plate readers, modifications to locked protocols according to the table below are recommended. Modification to locked protocols can be made after copying them under a different name (e.g. Copy of LANCE High Count 615 and 665 labels).

To perform a flatfield calibration on the ViewLux® imager, we recommend using the LANCE Positive Control (LANCE Controls, no. AD0163) diluted 1:5 in water as reference sample, rather than the LANCE *Ultra* cAMP kit reagents. The volume of the calibration sample should be the same as the assay volume. Flatfield calibration is performed using the calibration wizard for both the 615 nm and 665 nm channels. Additional instructions for calibration can be found in the ViewLux Reference Manual.

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.

NOTE:

Signal level and S/B ratio may vary depending on the reader used. This should not affect significantly assay robustness or sensitivity.

16. Data analysis

TR-FRET signal obtained at 665 nm (U*Light* acceptor channel) can be used directly for data analysis of cAMP standard curves, determination of optimal cell density, as well as for agonist and antagonist characterization. Counts at 665 nm obtained in cAMP standard curves allow interpolating the amount of cAMP produced in stimulated cells.

Time-resolved fluorescence signal measured at 615 nm (Eu chelate donor channel) is useful for identifying dispensing malfunctions and some compound artifacts. Indeed, some compounds may quench signal at 615 nm either by absorption of the excitation light at 320 or 340 nm ("inner filter effect"), absorption of the emitted light at 615 nm or by light diffraction (poorly soluble compounds). Therefore, for HTS of compound libraries, a quench correction of the TR-FRET signal is recommended.

Quench correction is performed using a **blank-corrected normalized ratio**. The following equation is applied:

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

The blank value ($F_{665,BL}$) is determined by measuring the background signal at 665 nm of buffer-containing wells. This reading is specific for the plate type, reader and measurement protocol.

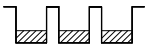
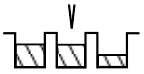
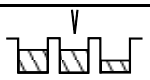
Signals at 665 nm ($F_{665,S}$) and at 615 nm ($F_{615,S}$) are measured from sample wells containing the assay components and test compounds. With the LANCE *Ultra* cAMP kit, the maximal signal at 615 nm ($F_{615,MAX}$) is obtained from wells with **no cAMP**.

Signal from most of the quenched samples can be corrected using this equation. For more information, see Application note 1234-9860 *"Quench Correction for LANCE Time-Resolved Fluorescence Resonance Energy Transfer"*.

It should be noted that some compounds can increase signal at 665 nm without increasing signal at 615 nm. This can be due to diffusion-enhanced FRET, wherein a fluorescent compound having a similar excitation/emission spectra as the acceptor dye and screened at a high concentration (>1 µM) is into close proximity with the Eu chelate donor. Another reason could be compound aggregation.

Regardless of the way data are expressed (TR-FRET signal at 665 nm or blank-corrected ratio), a comprehensive evaluation of compound artifact in TR-FRET assays requires examination of data obtained at both emission channels (615 and 665 nm).

17. 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 15		

18. Additional notes

- A thorough understanding of this manual is recommended for the successful use of the LANCE *Ultra* cAMP kit.
- Reagents supplied with this kit should be used together. Do not mix reagents from kits with different lot numbers in order to maintain assay performance between lots.
- The use of white, opaque OptiPlate-384 microplates is recommended. The use of black microplates will provide reduced signal but acceptable S/B ratios.
- It is critical to **remove the TopSeal-A** film from the plate prior to reading.
- Additional readings can be performed for at least 24 hours after addition of LANCE *Ultra* reagents without significant change in assay sensitivity.
- In order to eliminate one addition step, the U*Light*-anti-cAMP antibody can be premixed with the cells by adding 5 µL of cell suspension in Stimulation Buffer containing 4X U*Light*-anti-cAMP. In this 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-well plate format.
- Addition of BSA to the Stimulation Buffer (0.1%) is recommended. However, it is possible to either reduce the BSA concentration or eliminate BSA in some assays.

- A special grade of BSA (7.5% Stabilizer Solution) specifically designed for LANCE applications is provided with the kit. Do not use other sources of BSA, as you might obtain unreliable data.

19. LANCE *Ultra* cAMP FAQS

- **What is the minimum receptor expression level required?**

There is no minimum in receptor expression levels. Some endogenous receptors were shown to induce the release of high amounts of cAMP. Tight coupling between receptors, G-proteins ($G_{\alpha s}$ or $G_{\alpha i}$) and adenylyl cyclase is the most critical determinant to yield conditions allowing for robust cAMP assays.

The LANCE *Ultra* cAMP assay is extremely sensitive and allows the measure of receptor stimulation in cells producing low levels of cAMP.

- **Should I use transient or stable transfected cell lines?**

Both transient and stable cell lines have been shown to produce good responses upon adenylyl cyclase activation.

- **Can I use frozen cell lines?**

Frozen cell lines, including cAMPZen® cells from PerkinElmer, have been shown to produce excellent responses upon adenylyl cyclase activation.

- **How many cells should I use in my assay?**

Cell number influences cAMP level before (basal level) and following adenylyl cyclase activation (stimulated level). Performing a cell titration allows optimizing the assay window by maximizing the difference between basal and stimulated counts.

- **Is it necessary to perform a time course for cell stimulation?**

The stimulation time is critical for reaching optimal detection of cAMP. When determining the optimal cell density for the assay, we recommend stimulating the cells initially for 30 minutes. Once the optimal cell density has been determined, the experimenter could perform a time course of cell stimulation starting at 15 minutes up to 120 minutes. The stimulation time may vary depending on the cell line, receptor and agonist being studied.

- **Can attached cells be used?**

Both attached cells and cells in suspension can be used. The LANCE *Ultra* cAMP assay is tolerant to culture medium containing 10% FBS and phenol red. If needed, attached cells can be rinsed with 1X HBSS to remove cell culture medium prior to conducting the LANCE *Ultra* cAMP assay.

- **Can membrane preparations be used instead of whole cells?**

The LANCE *Ultra* cAMP assay allows detecting cAMP in cellular membrane preparations when the Stimulation Buffer contains the appropriate supplements (e.g. ATP, GTP and $MgCl_2$). Quantities of 1 to 5 µg membranes are typically used in membrane assays. We recommend titrating membranes and all supplemented components for optimizing the performance of membrane-based assays.

- **Does DMSO interfere in the assay?**

DMSO at concentrations up to 10% does not affect significantly the performance of the LANCE *Ultra* cAMP kit. It is recommended to titrate DMSO with each cell line used.

- **Can I pre-mix the Eu-cAMP tracer and the U*Light*-anti-cAMP in order to eliminate an addition step?**

No! Pre-mixing of Eu-cAMP tracer and U*Light*-anti-cAMP before dispensing will create a pre-kinetic equilibrium between both detection reagents. As a result, higher concentrations of free cAMP will be necessary to compete with the Eu-cAMP tracer for binding to the U*Light*-anti-cAMP. Hence, the IC_{50} value in the cAMP standard curve will be right-shifted.

20. Literature

Application Notes

- LANCE *Ultra* cAMP: A New TR-FRET cAMP Assay for G_s- and G_i-Coupled Receptors.
- Measuring Performance of an Automated and Miniaturized LANCE *Ultra* cAMP Assay for the G_i-coupled 5-HT_{1A} Receptor – a Comparative Study.

Scientific Posters

- LANCE *Ultra* cAMP: A New, Two-Component TR-FRET cAMP Assay for HTS of G_s- and G_i-coupled Receptors. SBS 16th Annual Conference (2010).
- Automated 1,536-well cAMP Assay for the Identification and Characterization of Agonists and Antagonists of a G_i-coupled Receptor Using the New LANCE *Ultra* cAMP Kit. SBS 16th Annual Conference (2010).

For technical/application assistance, please contact PerkinElmer technical support:

PerkinElmer Life and Analytical Sciences
Direct Dial U.S. 800-762-4000
Toll Free Europe 00800-33290000
For Finland dial 999 800 33 29 0000
E-mail: global.techsupport@perkinelmer.com
Please visit www.perkinelmer.com for specific country contact details.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com