APPLICATION NOTE

LANCE *Ultra* cAMP: a new TR-FRET cAMP assay for G_s- and G_i-coupled receptors.

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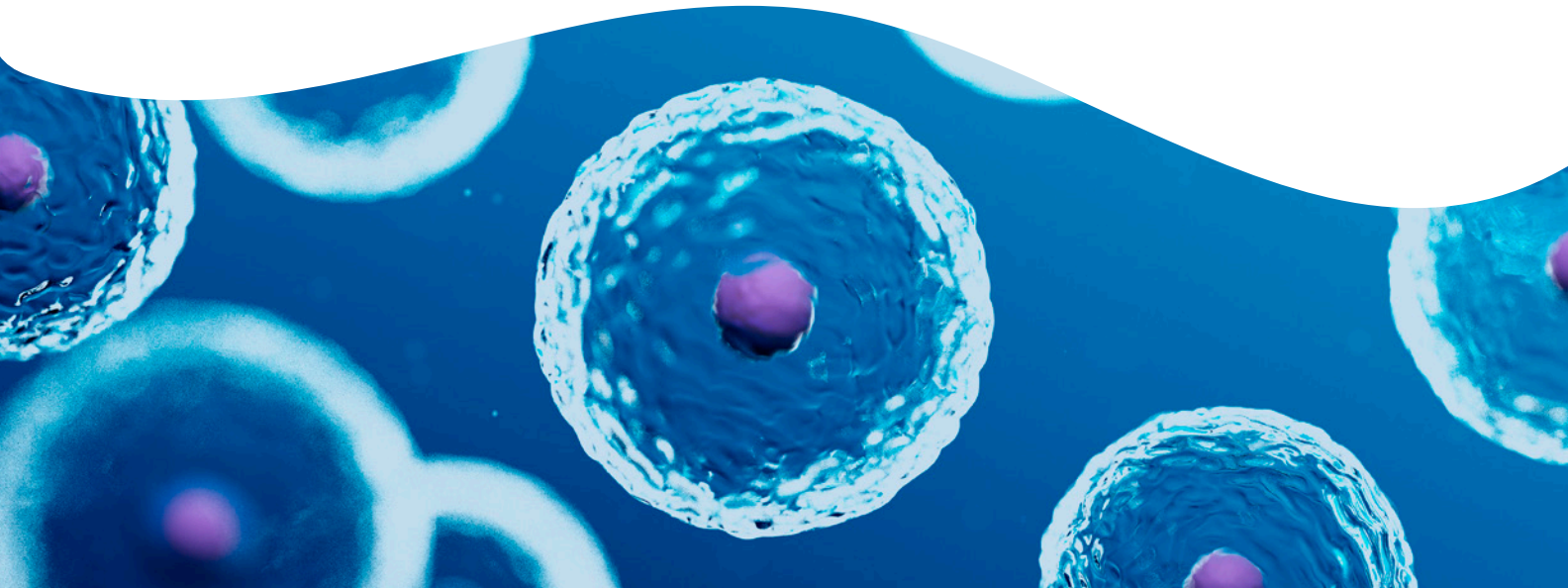
Introduction

Guanosine triphosphate binding protein-coupled receptors (GPCRs) represent one of the largest and most important classes of pharmaceutical drug targets. Approximately 48% of all GPCRs couple through adenylate cyclase making the need for a robust cAMP detection method critical. We have developed a second-generation LANCE® time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay designed to measure cAMP produced upon modulation of adenylyl cyclase activity by activated GPCRs. Here we present data comparing the performance in 384-well plate format of the new LANCE *Ultra* cAMP kit with that of two other commercially available cAMP kits, namely an alternative TR-FRET assay *(dynamic 2)* from Company C and two Enzyme Fragment Complementation (EFC) assays (XS+ and HS+) from Company D. These three cAMP assay technologies were evaluated for their ability to detect agonist- or antagonist-induced cAMP responses in suspension cells expressing either endogenous (G_s-β-adrenergic) or recombinant receptors (G_s-MC4; G_i-CXCR3; G_i-CB_1). The assay principle of the LANCE *Ultra* cAMP kit is shown in Figure 1.



LANCE *Ultra* cAMP assay principle

In the LANCE *Ultra* cAMP assay (Figure 1), the europium chelate (Eu)-cAMP tracer molecule is captured by a U*Light*-labeled anti-cAMP monoclonal antibody (mAb), which brings donor and acceptor dye molecules into close proximity. Following irradiation of the samples at 320 or 340 nm, the excited energy of the Eu chelate donor is transferred by FRET to the U*Light* acceptor dye. U*Light* molecules in turn emit a signal detectable at 665 nm in TR-FRET mode. Residual energy from the Eu chelate will produce light at 615 nm. In the absence of free cAMP (Figure 1, left panel), maximal TR-FRET signal is achieved. Free cAMP produced by stimulated cells competes with the Eu-cAMP tracer for the binding to the U*Light* mAb (Figure 1, right panel), causing a decrease in TR-FRET signal proportional to the concentration of cAMP produced.

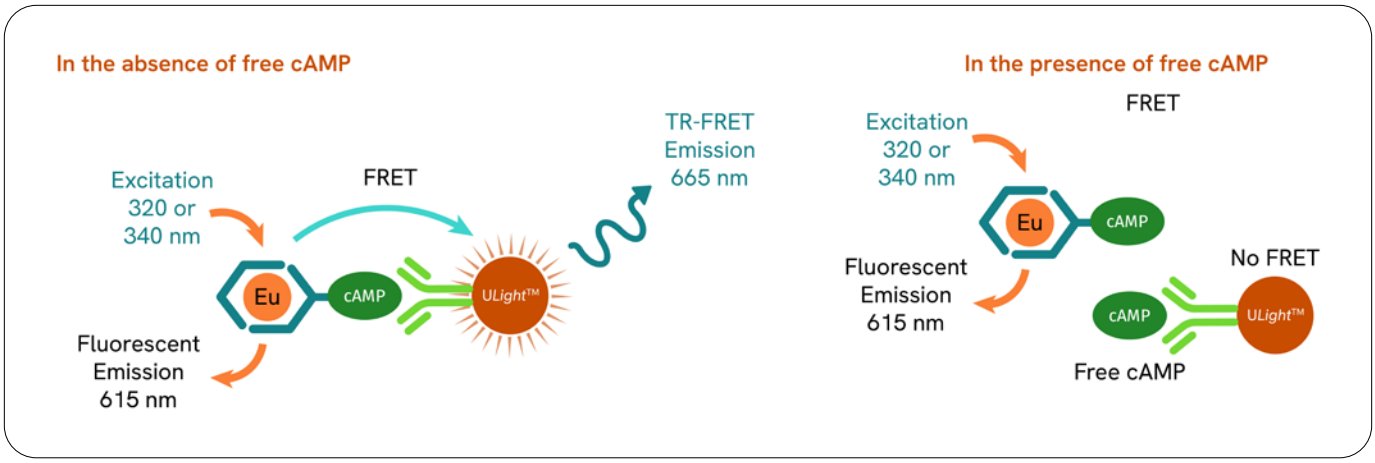


Figure 1: LANCE *Ultra* cAMP assay principle.

Materials and methods

Reagents provided with Revvity's LANCE *Ultra* cAMP kit are listed in Table 1.

Table 1: Reagents Supplied with the LANCE *Ultra* cAMP Kit

Kit contents
cAMP standard
Eu-cAMP tracer
U*Light*-anti-cAMP
cAMP detection buffer
BSA stabilizer (7.5% solution)

Comparative kits were purchased through each manufacturer. Cell lines used for this study are listed in Table 2.

Table 2: GPCRs and Cell Lines used in cAMP Detection Assays

Receptor	Cellular background	Supplier	Catalog #
Human MC4	CHO-K1	Revvity	ES-191-CF (cAMPZen®)
Human CB_1	CHO-K1	Revvity	ES-110-C
Human CXCR3	CHO-K1	Revvity	ES-142-C
Human β-adrenergic (endogenous)	SK-N-MC	ATCC®	HTB-10

Suppliers for forskolin, agonists and antagonists are listed in Table 3. All assays were performed in OptiPlate™-384 white opaque microplates following protocols provided with each kit. The stimulation buffer used for all assays contained 1X HBSS, 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA.

Table 3: Source of compounds

Compound	Supplier	Catalog #	Compound	Supplier	Catalog #
α-MSH	Bachem	H-1075	Isoproterenol	Sigma	I6504
AM 251	Tocris	1117	LY 320135	Tocris	2387
BRL 37344	Tocris	0948	Melanotan II	Sigma	M8693
CCL20/MIP-3 α	R&D Systems	360-MP-025/CF	NDP-α-MSH	Bachem	H-1100
CXCL9	Peprotech	300-26	Norepinephrine	Sigma	A0937
CXCL10	Peprotech	300-12	Salmeterol	Tocris	1660
CXCL11	Peprotech	300-46	SHU9119	Bachem	H-3952
Formoterol	Tocris	1448	SR141716 (Rimonabant)	Cayman	9000484
Forskolin	Calbiochem	344270	WIN 55, 212-2	Sigma	W102

All reagents were prepared and dispensed according to each manufacturer's recommendations. Experiments with the four cAMP kits were conducted side-by-side with the same batch of frozen cells and using the same serially diluted solutions when applicable. All assays were conducted manually in 384-well format.

The standard assay procedure for the LANCE *Ultra* cAMP assay is illustrated in Figure 2. Briefly, cells were stimulated for 30 min with compound(s). Following stimulation, cells were lysed by successive additions of the Eu-cAMP tracer and the *ULight*-cAMP mAb prepared in the cAMP Detection Buffer provided with the kit. Total assay volume was 20 µL. TR-FRET signal at 665 nm was measured at the indicated times on an EnVision® Multilabel Plate Reader in TR-FRET (laser mode). The same instrument settings were used for Company C's TR-FRET assay. Signal from the two EFC assays was measured with the EnVision Multilabel Plate Reader set in luminescence mode. Recommended TR-FRET settings for the EnVision instrument are listed in Table 4.

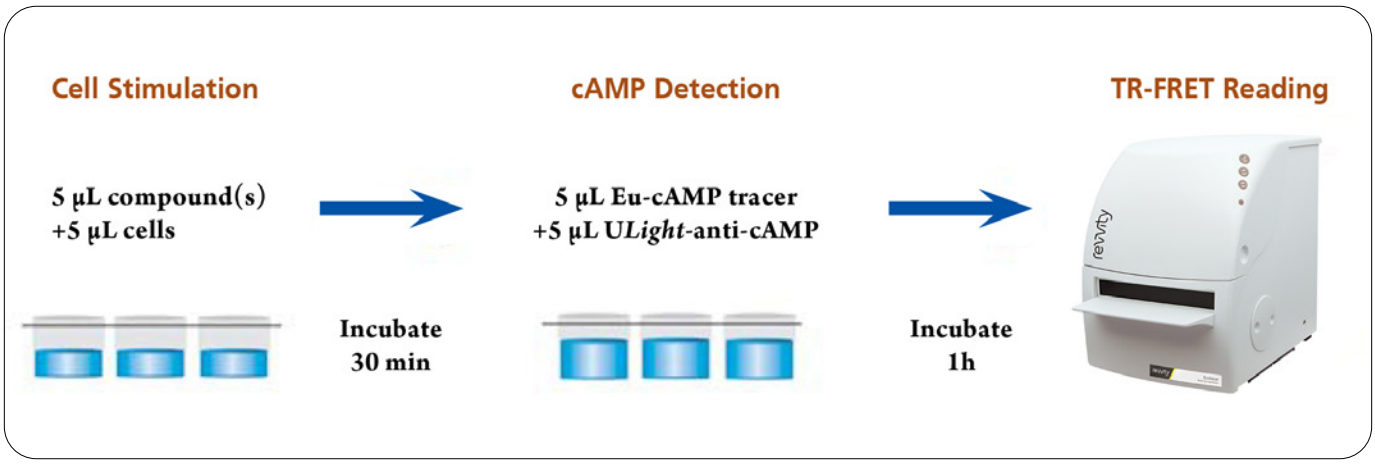


Figure 2: Standard assay procedure for the LANCE *Ultra* cAMP assay.

Data are presented as mean ± SD of triplicates. Concentration-response curves were analyzed by fitting data to the four-parameter logistic equation using GraphPad Prism®.

Table 4: Recommended TR-FRET settings for the EnVision multilabel microplate reader

Parameter	Recommended setting (laser mode)	Parameter	Recommended setting (laser mode)
Flash energy area	N/A	Delay time	50 µs
Flash energy level	100%	Readout speed, gain and binning	N/A
Excitation filter	N/A	Number of flashes	Laser: 20
Integrator cap	N/A	Window	100 µs
Integrator level	N/A	Cycle	Laser: 16600 µs
Emission filter	1) 203 - Eu 615 2) 205 - APC 665	Mirror module	445 or 446

All cAMP assays were fully optimized following the assay development workflow described in Table 5.

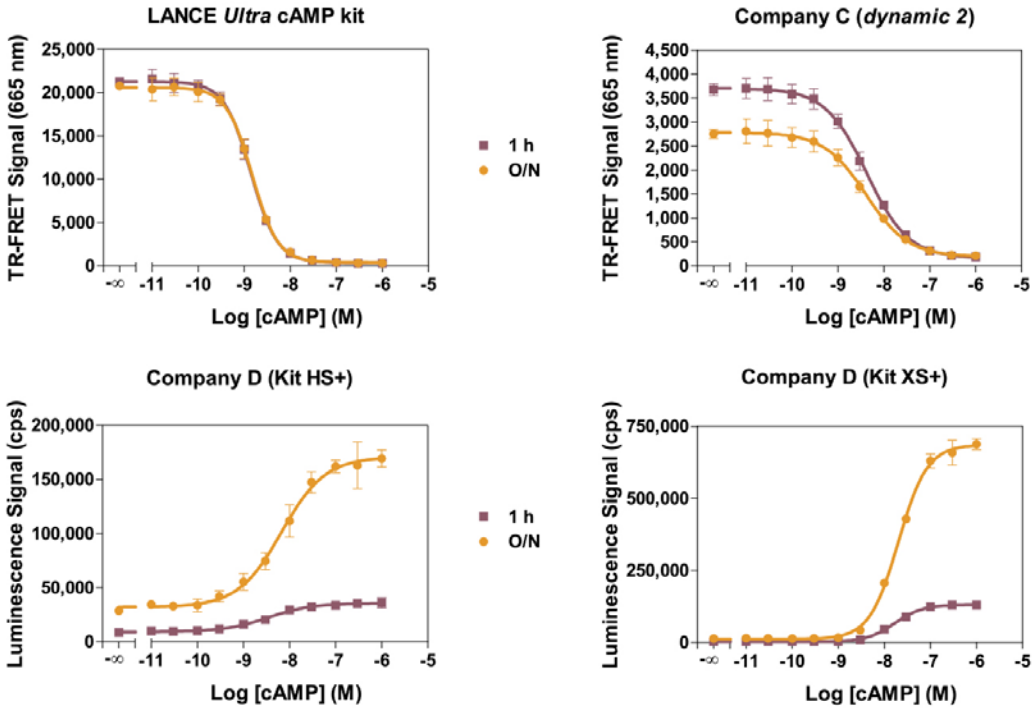
Table 5: cAMP assay development workflow

Step	G_s-receptor assay	G_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_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)

Results and discussion

cAMP standard curves

Running a cAMP standard curve for each kit allows determining sensitivity to cAMP (IC_{50} value) and assay dynamic range, as well as stability over time of the signal and pharmacology (Figure 3). The same cAMP serial dilutions were used for all kits. The LANCE *Ultra* cAMP showed the highest sensitivity to cAMP (IC_{50} value of 1.4 nM; equivalent to 28 femtomoles in 20-µL assay) and the largest assay window (signal-to-background (S/B) ratio close to 70). This superior S/B ratio was obtained within a dynamic range of approximately 1.5 log units. This result, coupled with its higher sensitivity, indicates that the LANCE *Ultra* cAMP kit will allow detection of smaller changes in cAMP concentrations while providing an assay with a superior S/B ratio. Our data also demonstrated that the LANCE *Ultra* cAMP assay was the only one in equilibrium following overnight (O/N) incubation, as overlapping cAMP standard curves are obtained following 1 hour and O/N incubation. Maximal signal and S/B ratio obtained with the TR-FRET kit from Company C decreased overnight. LANCE *Ultra* and the alternative TR-FRET kit showed stable IC_{50} for cAMP following O/N incubation. Luminescence signal significantly increased over time, while S/B ratio and EC_{50} for cAMP showed either a slight increase or remained relatively constant, respectively, with the two EFC kits from Company D (HS+ and XS+).



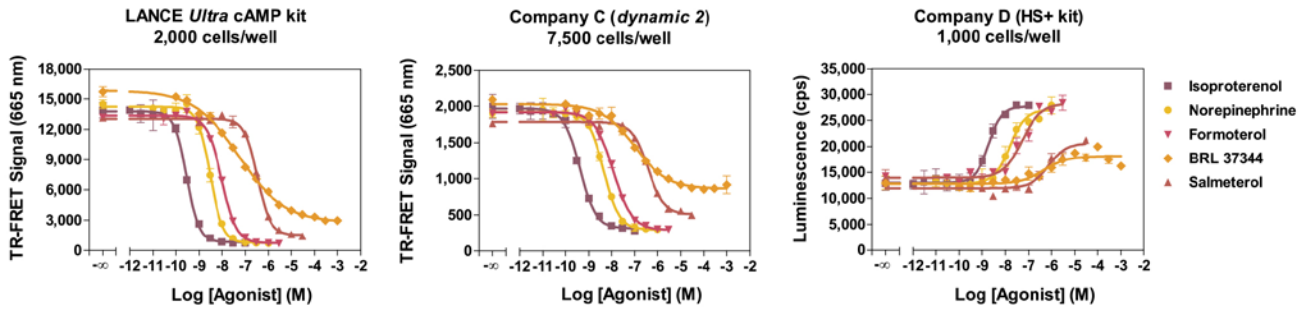
	LANCE *Ultra* cAMP		Company C *(dynamic 2)*		Company D (HS+)		Company D (XS+)	
	1 h	O/N	1 h	O/N	1 h	O/N	1 h	O/N
S/B	68.1	68.1	19.5	12.7	4.2	5.9	41.9	49.5
IC_{50} (nM)	1.4	1.5	4.3	4.1	3.5	6.8	16.9	20.3

Figure 3: cAMP standard curves for the four cAMP kits evaluated in this study. LANCE *Ultra* cAMP kit allows detection of the smallest changes in cAMP while providing an assay with superior S/B ratio; IC_{50} values were stable following overnight incubation.

Agonist responses in SK-N-MC cells expressing endogenous β-adrenergic receptors

Three cAMP kits were evaluated in parallel for characterizing the agonist response in SK-N-MC cells expressing endogenous G_s-coupled β-adrenergic receptors: the LANCE *Ultra* cAMP kit, the TR-FRET *dynamic 2* kit and the EFC HS+ kit. A cross-titration of the full agonist isoproterenol and cell density was initially performed to determine the cell number giving the highest assay window within the assay dynamic range for each kit (data not shown). The selected cell number for each kit is indicated in Figure 4. Agonist concentration-response curves were then performed with the three kits using a panel of five well-known agonists of β-adrenergic receptors (Figure 4). Data show that the assay window obtained with the LANCE *Ultra* kit for each agonist was 2- to 3-fold higher than with *dynamic 2* kit, and this, using almost 4-fold fewer cells per well. The HS+ kit gave poor assay windows with all agonist tested. While EC_{50} values are very similar for the different agonists tested with the two different TR-FRET assays, the HS+ kit appears to generate slightly right-shifted EC_{50} values. The same rank order of potency among the five agonists was obtained regardless of the kit used.

These results demonstrate that the LANCE *Ultra* cAMP kit is far superior to both the TR-FRET *dynamic 2* kit and EFC HS+ kit for assessing agonist response in cells expressing G_s-coupled endogenous receptors.



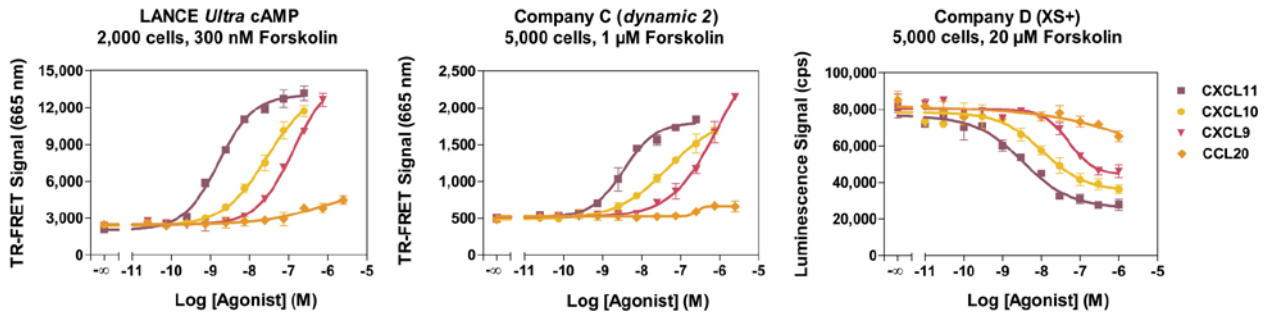
	LANCE *Ultra* cAMP		Company C (*dynamic 2*)		Company D (HS+ kit)	
	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
Isoproterenol	18.8	0.3	7.1	0.4	2.2	1.9
Norepinephrine	20.7	3.0	6.4	4.6	2.1	15.4
Formoterol	19.4	9.7	6.3	11.9	2.0	53.3
BRL 37344	5.3	54.9	2.3	101	1.3	464
Salmeterol	9.2	335	3.6	375	1.5	752

Figure 4: Agonist concentration-response curves in SK-N-MC cells expressing endogenous G_s-coupled β-adrenergic receptors. Almost 4-fold fewer cells were used with the LANCE *Ultra* kit compared to the other TR-FRET kit while still maintaining superior performance. The HS+ kit gave poor assay windows with all agonists tested.

Agonist responses in CHO cells expressing the G_i-coupled human CXCR3 receptor

The LANCE *Ultra* cAMP, *dynamic 2* and EFC XS+ kits were evaluated for measuring the agonist response in CHO-hCXCR3 cells. The EFC XS+ kit had been described as providing the largest assay window with recombinant receptors.

Assay conditions were optimized for the three kits by performing a cross-titration of cell density and forskolin, which allowed determination of the optimal cell density and EC_{90} forskolin concentration for achieving optimal assay window (data not shown). Agonist concentration-response curves were performed using a panel of three known CXCR3 agonists; CCL20 was included as a negative control (Figure 5). In this assay, the largest window for each agonist tested is achieved by the LANCE *Ultra* cAMP kit, using 2.5-fold fewer cells per well than with the alternative cAMP kits. Rank order of potency among the three agonists is the same for the three kits.

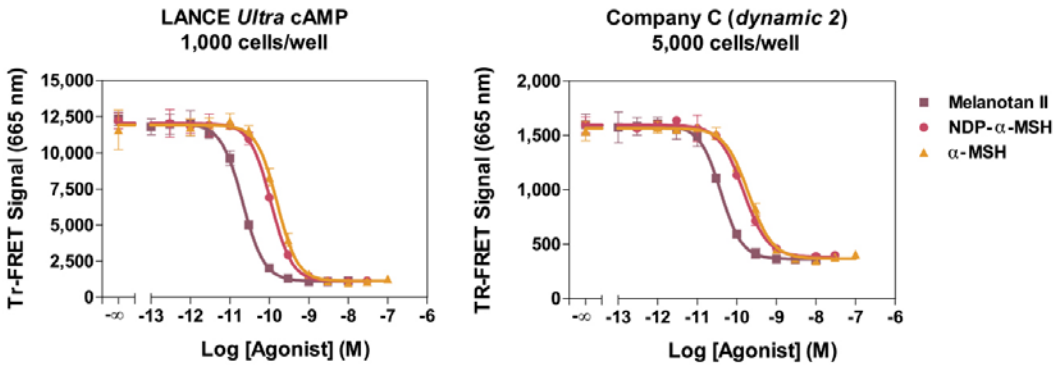


	LANCE *Ultra* cAMP		Company C *(dynamic 2)*		Company D (kit XS+)	
	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
CXCL11	6.3	1.7	3.7	3.6	2.9	3.4
CXCL10	4.7	27.3	3.5	47.7	2.3	11.1
CXCL9	5.3	130	4.3	924	1.8	51.8

Figure 5: Agonist concentration-response curves in CHO cells expressing the G_i-coupled hCXCR3 receptor. LANCE *Ultra* cAMP used 2.5-fold fewer cells and gave a superior S/B regardless of the agonist tested.

Agonist responses in CHO cells expressing the G_s-coupled human MC4 receptor

The LANCE *Ultra* cAMP kit and the TR-FRET *dynamic* 2 kit were used to characterize agonist responses in CHO-hMC4 cells. First, cross-titration of cell density and forskolin was performed to determine the optimal cell density for each kit (indicated in Figure 6). Agonist concentration-response curves were performed using a set of three well-known agonists (Figure 6). Rank order of potency and EC_{50} values were identical for the two TR-FRET assays. Once again, larger assay windows were obtained with the LANCE *Ultra* cAMP kit while using 5-fold fewer cells per well.

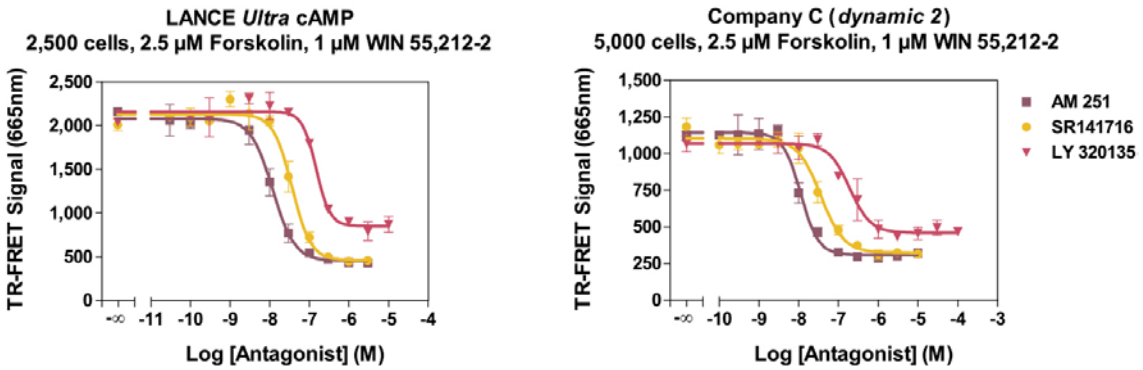


	LANCE *Ultra* cAMP		Company C *(dynamic 2)*	
	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
Melanotan II	10.9	0.02	4.4	0.04
NDP-α-MSH	10.9	0.11	4.0	0.15
α-MSH	9.0	0.16	3.8	0.20

Figure 6: Agonist concentration-response curves in CHO cells expressing the G_s-coupled human MC4 receptor. LANCE *Ultra* cAMP generated S/B ranging from 2.4 to 2.7-fold greater while using 5-fold fewer cells per well with the hMC4 cAMPZen frozen cell line.

Antagonist responses in CHO cells expressing the G_i-coupled human CB_1 receptor

The LANCE *Ultra* cAMP kit and the TR-FRET *dynamic 2* kit were used to characterize antagonist responses in CHO-hCB_1 cells. First, cross-titration of cell density and forskolin was performed to determine the optimal cell density and EC_{90} forskolin for each kit (data not shown). Concentration-response curves using the agonist WIN 55,212-2 were conducted next to determine the EC_{90} agonist concentration to be used for antagonist assays (data not shown). Selected cell density, EC_{90} forskolin and EC_{90} agonist used for each kit in the antagonist assays are indicated in Figure 7. Antagonist concentration-response curves were then performed with a set of three well-known CB_1 antagonists (Figure 7). Data show comparable or superior assay windows for the LANCE *Ultra* cAMP kit depending on the antagonist potency, with almost identical IC_{50} values for the two kits.

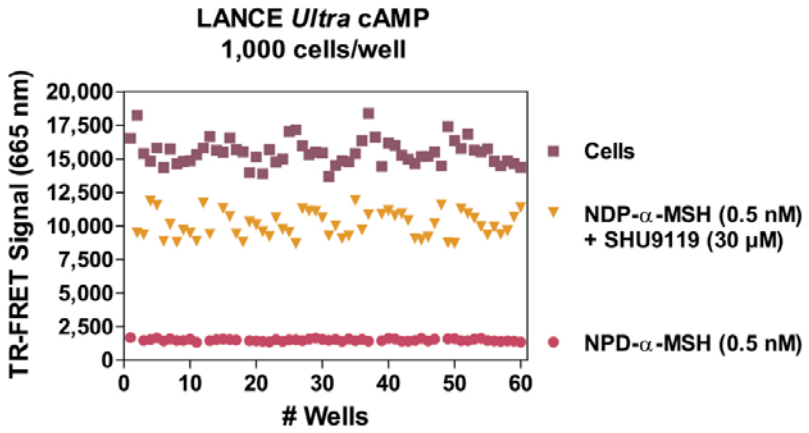


	LANCE *Ultra* cAMP		Company C *(dynamic 2)*	
	S/B	IC_{50} (nM)	S/B	IC_{50} (nM)
AM 251	5.0	11.9	3.5	11.2
SR141716	4.4	36.6	3.7	35.9
LY 320135	2.4	149	2.3	191

Figure 7: Antagonist concentration-response curves in CHO cells expressing the G_i-coupled human CB_1 receptor. 2-fold fewer cells per well were used with LANCE *Ultra* cAMP while providing comparable or superior S/B ratios.

Z′-Factor determination for agonist and antagonist assays for the human MC4 receptor

The robustness of the LANCE *Ultra* cAMP assay in 384-well plate format was assessed by performing Z′-factor analysis[1] using cAMPZen frozen CHO-hMC4 cells (Figure 8). Z′-factor was determined for both the agonist and antagonist assays using the optimized assay conditions described previously. This experiment was performed manually and signal was read following 1 hour and O/N incubation. After 1 hour of incubation, high S/B ratios and Z′-factor values were obtained for both the agonist (S/B around 10 and Z′ = 0.78) and antagonist assays (S/B around 7 and Z′ = 0.64). Percent CV values were below 10 in all conditions tested, which is acceptable for cell-based assays conducted manually. Of note, both the S/B ratios and Z′-factor values were maintained following O/N incubation. These data clearly show that the LANCE *Ultra* cAMP hMC4 assay is robust and well-suited for HTS. In addition, its O/N stability makes it ideal for off-line plate reading of large screens.



Detection time	Cells only			Cells + agonist			Cells + agonist + antagonist			Agonist assay		Antagonist assay	
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	S/B	Z′	S/B	Z′
1 h	15518	952	6.1	1505	83	5.5	10080	945	9.4	10.3	0.78	6.7	0.64
O/N	12412	746	6.0	1363	111	8.1	8506	709	8.3	9.1	0.77	6.2	0.66

Figure 8: Z′-factor determination of the agonist and antagonist hMC4 LANCE *Ultra* cAMP assays. This experiment was performed manually, measuring TR-FRET read following 1 hour and O/N incubation. High Z′-factor values demonstrate that the agonist and antagonist assays are robust and well-suited for both on-line and off-line plate reading of large high throughput screens.

Conclusions

The LANCE *Ultra* kit yields a cAMP assay with the highest sensitivity and signal window available on the market: an IC_{50} value of 28 fmoles and S/B ratio close to 70 in a cAMP standard curve. These features allow the use of fewer cells per well compared to other commercially available cAMP kits.

Results of cell-based assays indicated that all four cAMP kits tested provide comparable assay pharmacology with the expected rank order of agonist or antagonist potency. However, in every application, the LANCE *Ultra* technology outperformed the other cAMP kits in terms of S/B ratio and signal stability. Of note, transformation of raw data (counts obtained at 665 nm) into ratiometric data using a blank-corrected ratio 665 nm/615 nm (for more information, please see Application Note 1234-9860 "Quench Correction for LANCE Time-Resolved Fluorescence Resonance Energy Transfer") did not change the results (pharmacology and S/B ratio). The LANCE *Ultra* technology was also shown to be highly robust, as indicated by the high and stable Z′ values obtained after 1 hour and overnight incubation.

These key advantages, combined with a stable assay pharmacology, simple assay protocol and a single unique kit format for any application, makes the LANCE *Ultra* cAMP kit the best assay technology for primary and secondary screening of endogenous and recombinant GPCRs.

Reference

1. Zhang J., Chung T.D., Oldenburg K.J. Biomol. Screen. 1999; 4:67-73.

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