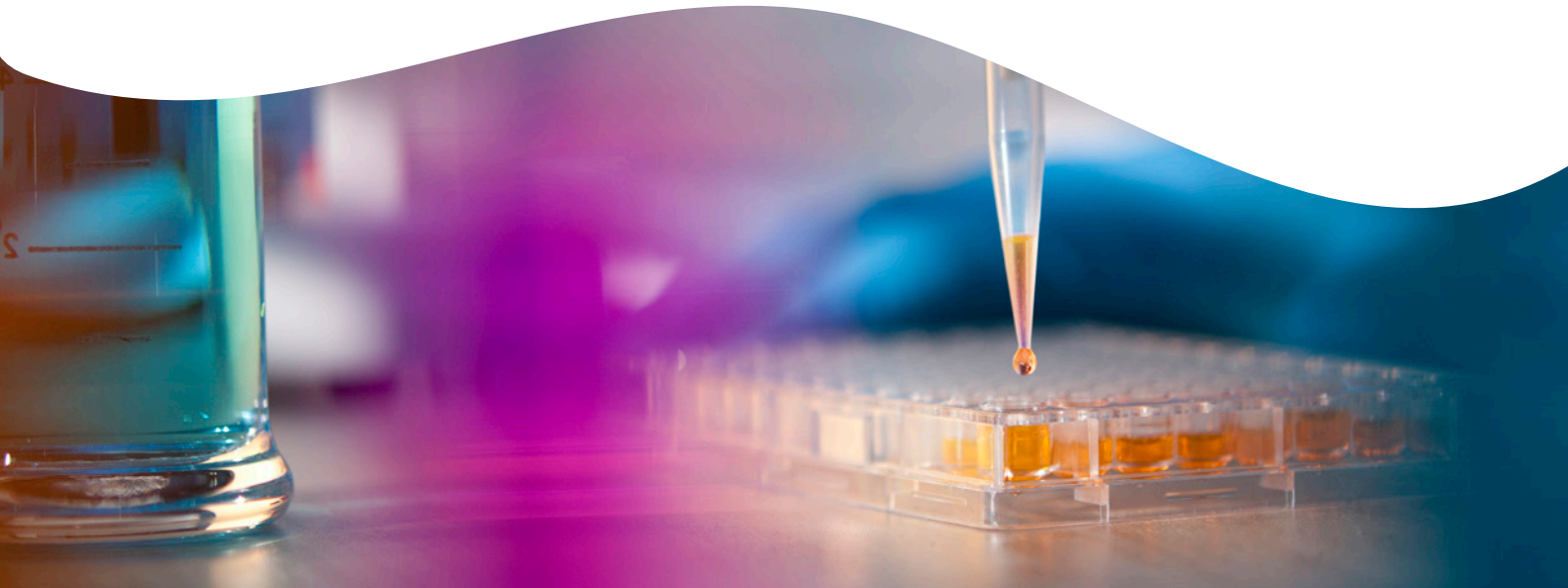
APPLICATION NOTE

Measuring performance of an automated and miniaturized LANCE *Ultra* cAMP assay for the G_i-coupled 5-HT_{1A} receptor – A comparative study.

Introduction

The LANCE® *Ultra* cAMP assay is 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 guanosine triphosphate binding protein-coupled receptors (GPCRs). The homogeneous two-component assay is based on the competition between europium chelate (Eu)-labeled cAMP and cellular cAMP for binding to high-affinity anti-cAMP monoclonal antibodies labeled with the U*Light*™ dye. The assay principle is shown in Figure 1.

Here we describe the miniaturization of a LANCE *Ultra* cAMP assay from 384-to 1,536-well plate format for the identification and characterization of agonists and antagonists of a G_i-coupled GPCR. A G_i-coupled receptor was selected for the current study because this category of receptors is among the most challenging for the development of suitable functional assays for HTS. The performance of the LANCE *Ultra* cAMP assay in both plate formats was compared to that of a commercially available TR-FRET cAMP assay (*dynamic 2* kit from Company C) for assessing the suitability of the two platforms for HTS.



LANCE *Ultra* cAMP assay principle

In the LANCE *Ultra* cAMP assay (Figure 1), the 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, causing a decrease in TR-FRET signal (Figure 1, right panel). The intensity of the signal measured at 665 nM is inversely proportional to the cAMP concentration in the sample (cAMP standard or cell lysate).

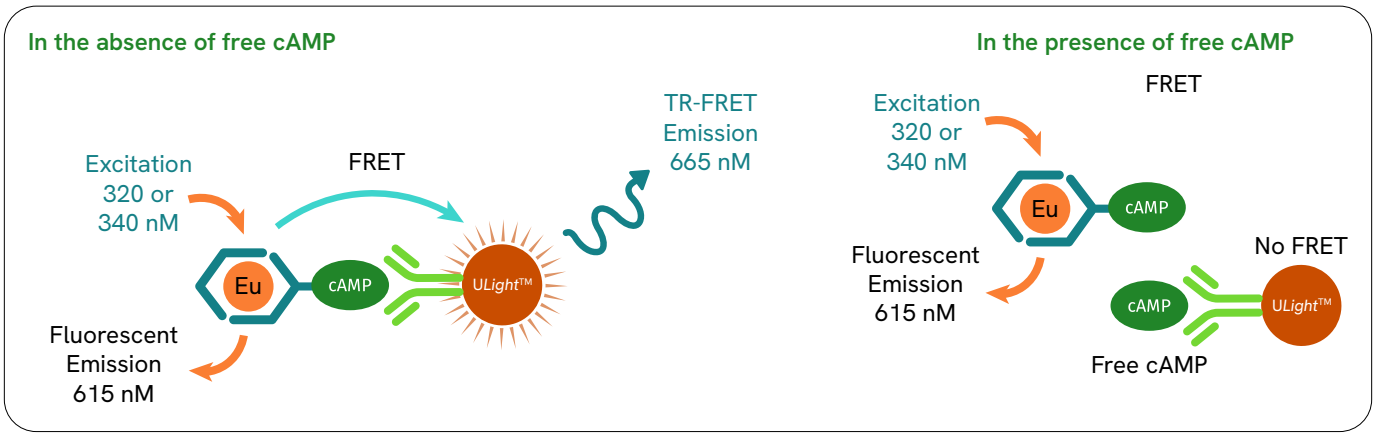


Figure 1: LANCE *Ultra* cAMP assay principle.

Materials and methods

Reagents provided with Revvity's LANCE *Ultra* cAMP kit are listed in Table 1. The TR-FRET *dynamic 2* cAMP kit was purchased through its manufacturer.

Frozen cells were prepared from a stable recombinant human serotonin 5-HT_{1A} cell line (CHO-K1 background; Revvity Cat. No. ES-310-C). Suppliers for forskolin, agonists and antagonists are listed in Table 2. All assays were performed in white, opaque OptiPlate™-384 (Revvity Cat. No. 6007290) and OptiPlate-1,536 (Revvity Cat. No. 6004290) microplates. The stimulation buffer used for all cAMP assays contained 1X HBSS, 5 mM HEPES, 0.5 mM IBMX and 0.1% BSA, pH 7.4.

Table 1: Reagents supplied with the LANCE *Ultra* cAMP kit

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

Table 2: Source of compounds

Compound	Supplier	Catalog #
Forskolin	Calbiochem	344270
(R)-(+)-8-OH-DPAT	Sigma	H140
S 14506	Tocris	1771
5-Carboxamidotryptamine	Sigma	C117
Spiperone	Sigma	S7395
WAY 100135	Tocris	1253
Alprenolol	Sigma	A8676

The standard assay procedure for the LANCE *Ultra* cAMP assay is illustrated in Figure 2. Briefly, the assay is conducted in two steps. In the first step, cells in suspension are stimulated for 30 min with the selected compound(s). Following stimulation, cellular cAMP is detected by the successive additions of Eu-cAMP tracer and U*Light*-anti-cAMP prepared in the cAMP Detection Buffer provided with the kit. Protocols used for assays in 384- and 1,536-well formats are shown in Table 3. Assay miniaturization into the 1,536 format was conducted by decreasing proportionally the volumes of each assay component while keeping constant reagent concentrations. The same reagent volumes and order of addition were used for all cAMP assays. TR-FRET signal at 665 nM was measured at the indicated times on an EnVision® Multilabel Plate Reader (laser mode; 384-well plate format) or ViewLux® ultraHTS CCD Imager (1,536-well plate format). Recommended instrument settings are listed in Table 4.

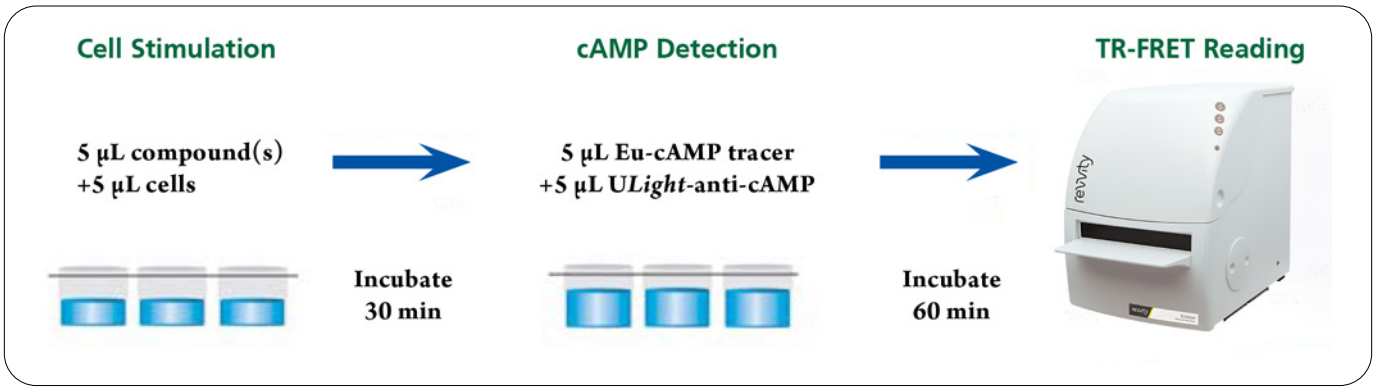


Figure 2: LANCE *Ultra* cAMP assay procedure for 384-well plate format.

Table 3: Standard protocols for the LANCE *Ultra* cAMP assays in 384- and 1,536-well plate format

Step	384-well plate format	1,536-well plate format
Cell stimulation	5 µL cells or cAMP Standard 5 µL compound or buffer alone Incubate for 30 min at RT	2 µL cells or cAMP Standard 2 µL compound or buffer alone Incubate for 30 min at RT
cAMP detection	5 µL Eu-cAMP tracer 5 µL U*Light*-anti-cAMP Incubate for 60 min at RT Read plate on EnVision (laser mode)	2 µL Eu-cAMP tracer 2 µL U*Light*-anti-cAMP Incubate for 60 min at RT Read plate on ViewLux
# Additions	4	4
Total assay volume	20 µL	8 µL
Assay time	90 min	90 min

Table 4: Recommended instrument setting

Parameter	EnVision (laser mode)	ViewLux *
Flash energy area	N/A	N/A
Flash energy level	100%	6,00,000
Excitation filter	N/A	DUG11 (UMB, AMC)
Integrator cap	N/A	N/A
Integrator level	N/A	N/A
Emission filter	1) 203 - Eu 615 2) 205 - APC 665	1) 618/8 (Eu) 2) 671/8 (LANCE)
Delay time	50 µs	50 µs
Readout speed, gain and binning	N/A	Medium, High and 2X
Number of flashes	Laser: 20	N/A
Window	100 µs	354 µs
Mirror module	445 or 446	N/A
Cycle	Laser: 16600 µs	N/A

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

Assay conditions (cell number, forskolin and agonist concentrations) were optimized independently for the two cAMP kits. All reagents were prepared and dispensed according to each manufacturer's recommendations. Experiments with both kits were conducted side-by-side with the same batch of frozen cells and using the same serially diluted solutions, when applicable. Assays in 384-well plate format were conducted manually, whereas assays in 1,536-well plate format were automated using the JANUS® Automated Workstation, except for the cell dispensing step (conducted manually).

Data in figures are presented as mean ± SD of triplicates and are representative of at least two independent experiments. Concentration-response curves were analyzed by fitting data to the four-parameter logistic equation using GraphPad Prism®.

Assay development and optimization in 384-well plate format

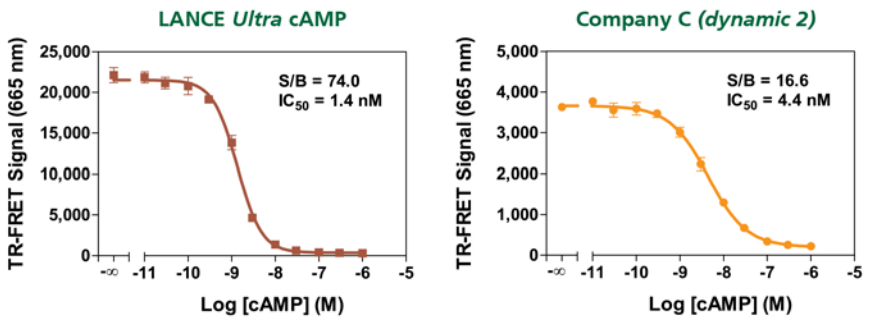
The agonist and antagonist assays for the G_i-coupled 5-HT_{1A} receptor were initially developed in 384-well plate format following the assay development workflow shown in Table 5.

Table 5: Assay development workflow for G_i-coupled receptors

Step	Experiment	Purpose
1	cAMP standard curve	Determine the sensitivity (IC_{50} value) and dynamic range (IC_{10} – IC_{90}) of the cAMP assay
2	Forskolin concentration-response experiment at different cell densities	Define the optimal cell density giving the highest assay window while staying within the assay dynamic range Define the EC_{90} of forskolin to be used for the agonist assay
3	Rank order of agonist potency (using EC_{90} forskolin)	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} forskolin+ EC_{90} agonist)	Estimate antagonist potencies (IC_{50} values)

cAMP standard curves in 384-well plate format

The first step of assay development consisted in running a cAMP standard curve to determine the assay sensitivity and dynamic range provided by each cAMP kit. Figure 3 shows a comparison of typical cAMP standard curves obtained with both cAMP kits in 384-well plate format. Of note, the same cAMP serial dilutions were used for both kits. Data show that the LANCE *Ultra* cAMP kit has both a higher sensitivity and 4.5-fold greater assay window (signal-to-background (S/B) ratio) compared to the alternative TR-FRET kit. The LANCE *Ultra* assay shows an IC_{50} value for cAMP of 1.4 nM, which corresponds to 28 fmoles of cAMP in a 20 µL assay. An approximately three times lower sensitivity was obtained with the alternative kit (88 fmoles). For both cAMP kits the IC_{50} of the assay was stable up to one week of incubation (data not shown). The assay detection range (defined as cAMP concentrations between IC_{10} and IC_{90} of the standard curve) is between 6.6 and 111 fmoles for the LANCE *Ultra* assay and between 9.4 and 836 fmoles for Company C's assay. Overall, these results indicate that small changes in cAMP levels will produce an assay with a superior assay window when the LANCE *Ultra* kit is used.

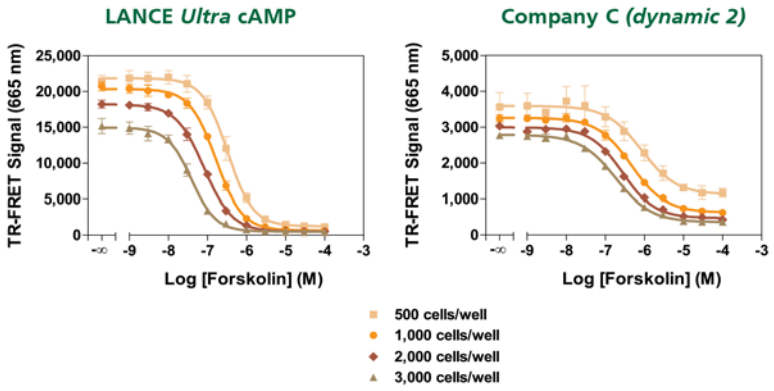


	LANCE *Ultra* cAMP	Company C *(dynamic 2)*
IC_{10} (fmol)	6.6	9.4
IC_{50} (fmol)	28	88
IC_{90} (fmol)	111	836

Figure 3: cAMP standard curves in 384-well plate format. The table indicates the fmoles of cAMP detected per well at the IC_{10}, IC_{50} and IC_{90} values for each kit.

Cell density optimization in 384-well plate format

The next step in cAMP assay development was to identify an optimal cell density for each cAMP kit by performing a forskolin and cell density cross-titration experiment (Figure 4). Data show that the LANCE *Ultra* kit has a higher sensitivity than the alternative TR-FRET kit. While as few as 500 cells/well could have been used for working with the LANCE *Ultra* cAMP kit, a cell density of 2,000 cells/well was selected for both cAMP kits as it gave an optimal assay window while still staying within the assay dynamic range for both kits. A forskolin concentration close to the EC_{90} was selected for agonist and antagonist stimulation (LANCE *Ultra*: 0.5 µM; Company C's kit: 2.5 µM).

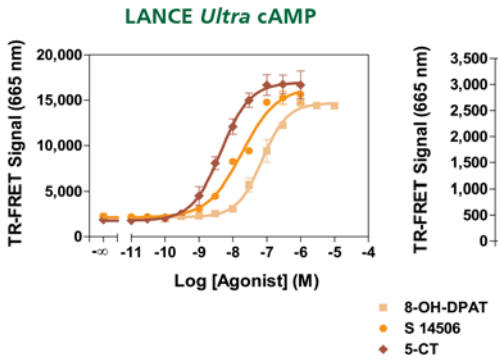


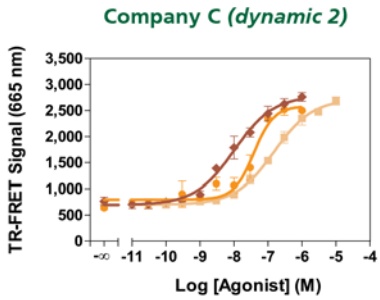
Cells/ well	LANCE *Ultra* cAMP		Company C	
	S/B	IC_{50} (nM)	S/B	IC_{50} (nM)
500	18.3	329	3.0	854
1,000	31.7	166	5.2	459
2,000	34.6	81.7	7.1	275
3,000	35.7	39.1	7.6	186

Figure 4: Cells and forskolin cross-titration in 384-well plate format.

Agonist and antagonist responses in 384-well plate format

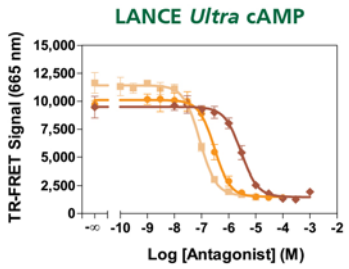
A panel of three known agonists and antagonists of the 5-HT_{1A} receptor was then characterized using each cAMP kit. For agonist assays, cells were co-stimulated with forskolin at the EC_{90} concentration in order to see the agonist-induced decrease in cellular cAMP levels (Figure 5). For antagonist assays, cells were co-stimulated with EC_{90} forskolin and EC_{90} agonist (1 µM 8-OH-DPAT for both cAMP kits) to see the antagonist-induced increase in cellular cAMP levels (Figure 6). Data show that while the rank order of potency and EC_{50} values were comparable between both cAMP kits, the LANCE *Ultra* cAMP kit provided superior S/B ratios for all agonists and antagonists tested. With both cAMP platforms, neither the rank order of potency nor the pharmacology was affected when plates were read following overnight incubation (data not shown).

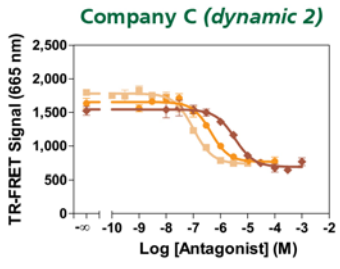


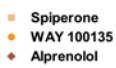


Agonist	LANCE *Ultra* cAMP		Company C *(dynamic 2)*	
	S/B	EC_{50} (nM)	S/B	EC_{50} (nM)
8-OH-DPAT	6.5	73.7	3.9	151
S14506	6.9	19.1	3.9	39
5-CT	9.0	4.5	3.6	10.2

Figure 5: Agonist concentration-response curves in 384-well plate format.







Antagonist	LANCE *Ultra* cAMP		Company C *(dynamic 2)*	
	S/B	IC_{50} (nM)	S/B	IC_{50} (nM)
Spiperone	7.9	86.1	2.4	99.2
WAY 100135	6.8	299	2.1	436
Alprenolol	4.9	2842	2.0	3337

Figure 6: Antagonist concentration-response curves in 384-well plate format.

Assay miniaturization in 1,536-well plate format

The 5-HT_{1A} LANCE *Ultra* cAMP assay was automated and miniaturized to 1,536-well plate format using the Janus Automated Workstation as described in Materials and Methods. The *dynamic 2* TR-FRET cAMP assay was miniaturized in parallel. All 1,536-well assays were read on the ViewLux Imager, which is ideally suited for uHTS as it allows minimal read time per plate. Development of the 1,536-well cAMP assay followed the same assay workflow used for the 384-well assay (see Table 5).

cAMP standard curves in 1,536-well plate format

In order to allow a successful assay miniaturization from 384- to 1,536-well plate format, the miniaturized cAMP assay must maintain the assay sensitivity while providing an acceptable assay window. Figure 7 shows a comparison of cAMP standard curves in 1,536-well plate format for both cAMP kits. As observed in the 384-well plate format, the LANCE *Ultra* cAMP assay shows both a higher assay sensitivity and assay window (S/B ratio) in the 1536-well plate format compared to the *dynamic 2* kit. While assay sensitivity was not affected by miniaturization, both assays show lower S/B ratios compared to those obtained in 384-well plate format. This result was expected based on both the lower assay volume (8 µL in the 1,536 assay versus 20 µL in the 384 assay) and the use of a microplate imager (ViewLux CCD-based Imager versus the photomultiplier-based EnVision Plate Reader). Indeed, higher S/B ratios were obtained when the same plates were read on the EnVision (laser mode): 43 for the LANCE *Ultra* and 10 for *dynamic 2* kit.

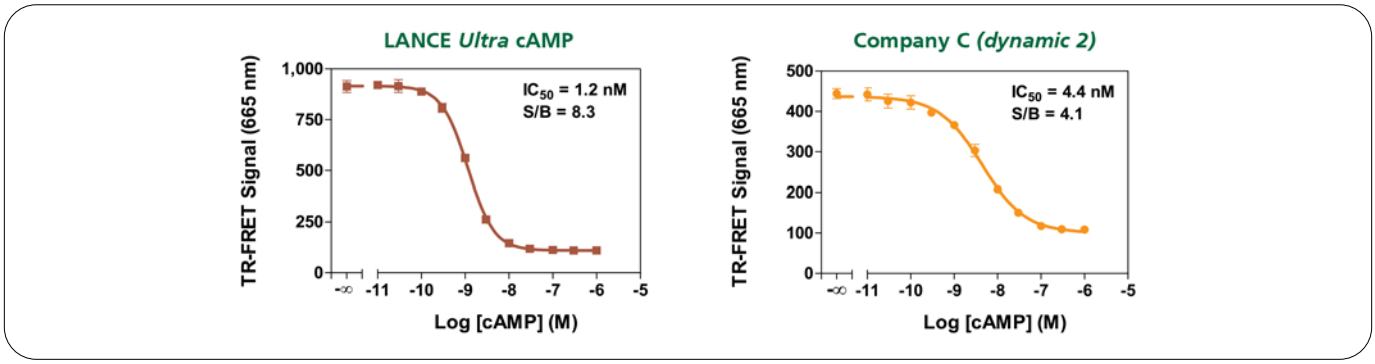
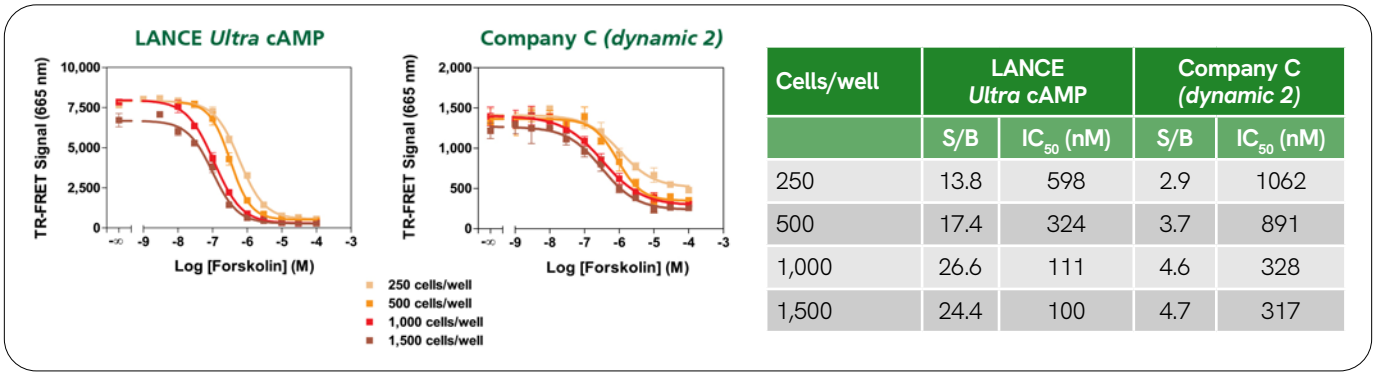


Figure 7: cAMP standard curves in 1,536-well plate format. LANCE *Ultra* cAMP generated 2X signal window in 1536-well format when read on a ViewLux uHTS reader.

Cell density optimization in 1,536-well plate format

Cross-titration of cell number and forskolin is summarized in Figure 8. Data show again a higher sensitivity and S/B ratio for the LANCE *Ultra* kit in the 1,536-well plate format. An optimal cell number of 1,000 cells per well was selected for the two assays to obtain the highest assay window within the assay dynamic range. The EC_{90} forskolin concentrations for subsequent assays were 300 nM and 700 nM for the LANCE *Ultra* and Company C (*dynamic 2*) cAMP assays, respectively.



Cells/well	LANCE *Ultra* cAMP		Company C *(dynamic 2)*	
	S/B	IC_{50} (nM)	S/B	IC_{50} (nM)
250	13.8	598	2.9	1062
500	17.4	324	3.7	891
1,000	26.6	111	4.6	328
1,500	24.4	100	4.7	317

Figure 8: Cell and forskolin cross-titration in 1,536-well plate format. An optimal cell number of 1,000 cells per well was selected for the two assays to obtain the highest assay window within the assay dynamic range. Signal for the cell titration assay was detected on the EnVision plate reader.

Agonist and antagonist responses in 1,536-well plate format

The agonist concentration-response experiments were conducted using 8-OH-DPAT. Cells were stimulated with 8-OH-DPAT in the presence of forskolin at the EC_{90} concentration. Results summarized in Figure 9 indicate that while comparable receptor pharmacology was obtained with both kits, the LANCE *Ultra* cAMP kit clearly outperforms the dynamic 2 kit in terms of assay window.

Spiperone was selected for the antagonist concentration-response experiments. Cells were co-stimulated with forskolin and agonist at their EC_{90} concentration (forskolin as indicated above; 300 nM 8-OH-DPAT for both kits). Data summarized in Figure 10 demonstrate again the superior performance of the LANCE *Ultra* cAMP kit over the Company C's kit in terms of assay window. As for the agonist assays, pharmacology of the two antagonist assays was in agreement and IC_{50} values were comparable to those obtained in the 384-well plate format.

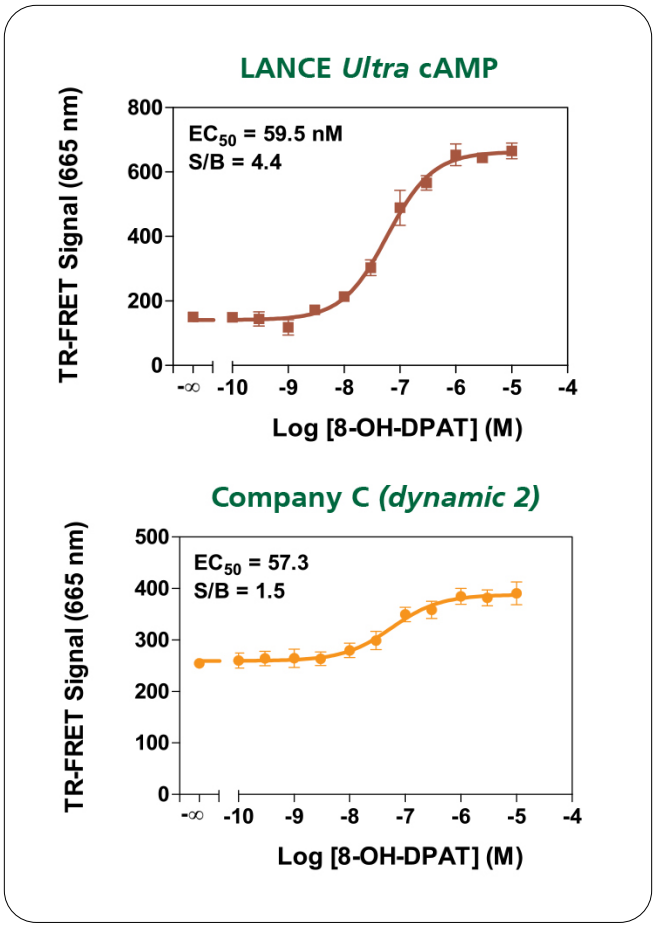


Figure 9: Agonist 8-OH-DPAT concentration-response curves in 1,536-well plate format. Almost 3-fold greater signal window was obtained with LANCE *Ultra* cAMP kit.

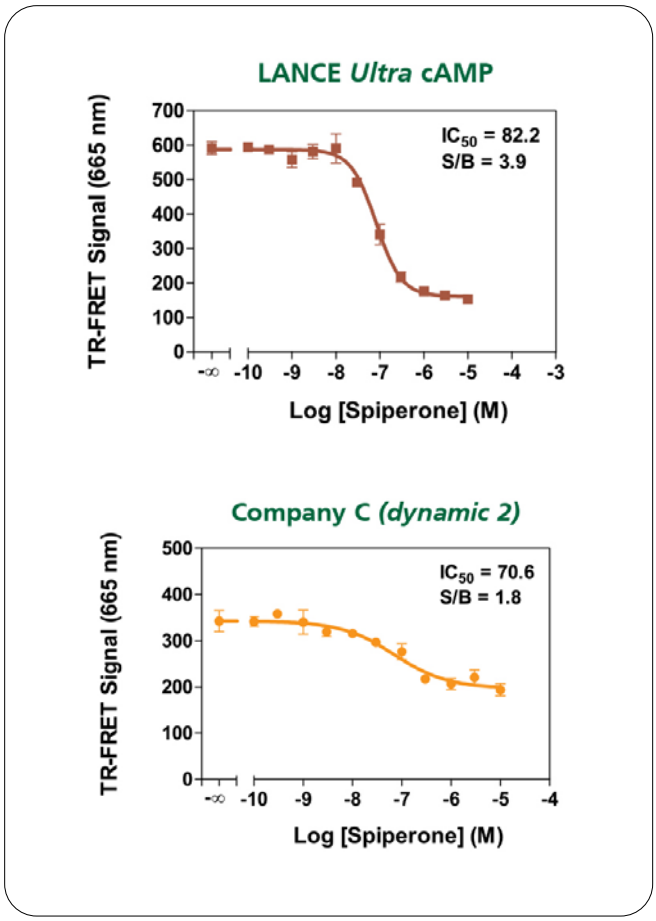
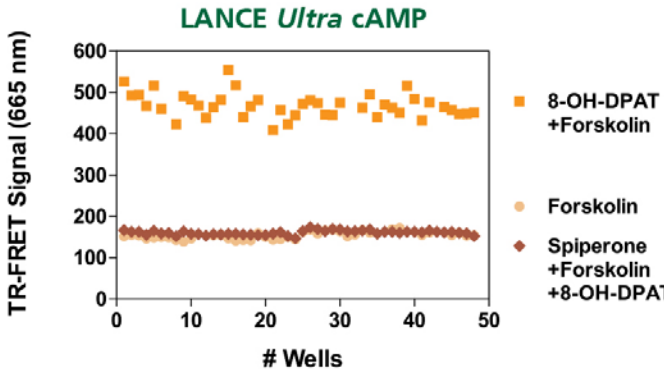
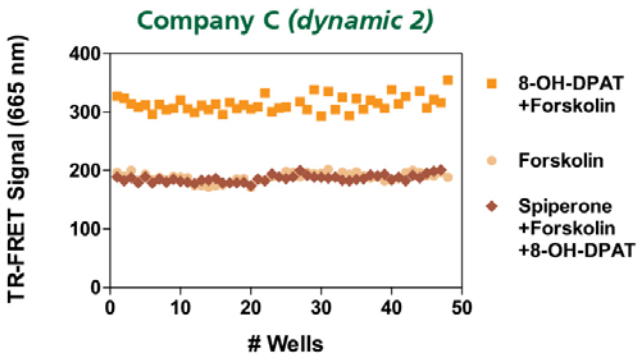


Figure 10: Antagonist spiperone concentration-response curves in 1,536-well plate format. 2.2-fold greater signal window was generated with LANCE *Ultra* cAMP kit.



Detection time	Forskolin			Forskolin + agonist			Forskolin + agonist + antagonist			Agonist assay		Antagonist assay	
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	S/B	Z'	S/B	Z'
1 h	154	9	5.8	469	29	6.2	161	5	3.3	3.0	0.64	2.9	0.66
4 h	146	9	6.5	456	25	5.4	152	6	3.7	3.1	0.67	3.0	0.70
O/N	165	11	6.7	489	24	4.9	172	7	4.1	3.0	0.68	2.9	0.71



Detection time	Forskolin			Forskolin + agonist			Forskolin + agonist + antagonist			Agonist assay		Antagonist assay	
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	S/B	Z'	S/B	Z'
1 h	189	8	4.2	314	13	4.2	186	6	3.2	1.7	0.50	1.7	0.55
4 h	172	7	4.3	283	11	4.0	169	6	3.7	1.6	0.49	1.7	0.54
O/N	173	10	5.6	269	13	4.9	175	12	6.8	1.6	0.28	1.5	0.19

Figure 11: Z′-factor experiments for automated agonist and antagonist assays in 1,536-well plate format. All time points tested with LANCE *Ultra* cAMP produced robust Z′ values whereas values deteriorated after 4 hours and overnight readings with the alternative kit.

Z'-Factor experiments for automated agonist and antagonist assays in 1,536-well plate format

The robustness of the 1,536-well format LANCE *Ultra* cAMP and alternative TR-FRET cAMP assays developed for screening of the G_i-coupled 5-HT_{1A} receptor was assessed by performing Z′-factor analysis.[1] The corresponding optimized conditions for each cAMP assay were used for this evaluation. The agonist 8-OH-DPAT was tested at 300 nM in the presence of forskolin at the EC_{90}, while the antagonist spiperone was tested at 10 µM in the presence of forskolin and agonist at their EC_{90}. The Z′-factor value was calculated from 48 assay points for each assay condition.

As shown in Figure 11, Z′-factor analysis of the automated agonist and antagonist 5-HT_{1A} assays demonstrated the robustness and stability of the LANCE *Ultra* cAMP assay. The Z′-factor calculated in both the agonist and antagonist assays was above 0.6 and slightly improved over time, the assay remaining remarkably stable following overnight incubation. This confirms the suitability of the LANCE *Ultra* cAMP automated 1,536-well assay for both on-line and off-line HTS plate readings, which allows recovery from potential robotic failure during automated HTS. In contrast, Z′-factor values calculated from the Company C's assays were acceptable only after 1 hour (agonist assay; Z′ = 0.5) or up to 4 hours of incubation (antagonist assay; Z′ = 0.54).

Conclusions

We described the miniaturization of the LANCE *Ultra* cAMP assay into 1,536-well plate format for the identification and characterization of agonists and antagonists of the G_i-coupled receptor serotonin $5HT_{1A}$. The performance of the LANCE *Ultra* cAMP assay was compared to that of an alternative TR-FRET cAMP assay available on the market.

In both 384- and 1,536-well plate formats, the LANCE *Ultra* cAMP assay was shown to be more sensitive than the alternative TR-FRET assay based on lower IC_{50} values derived from cAMP standard curves and forskolin concentration-response curves. This enhanced sensitivity allows the use of fewer cells per well in the assay development.

Both cAMP kits generated similar receptor pharmacology values for agonists and antagonists, regardless of the plate format used. In addition, pharmacology obtained in 1,536-well plate format was comparable to that observed in 384-well plate format. However, S/B ratios obtained with the LANCE *Ultra* kit for forskolin, agonist and antagonist responses in both plate formats were consistently higher than those obtained with Company C's kit.

In addition, the LANCE *Ultra* assay showed stable Z′-factor values even after overnight incubation (Z′ = 0.64-0.71). In contrast, Company C's TR-FRET assay showed lower Z′-factor values at all reading times.

Taken together, these results demonstrate that the LANCE *Ultra* cAMP kit is a superior cAMP assay technology for use as a primary and/or secondary cell-based platform for the identification and characterization of agonists and antagonists of G_i-coupled receptors in 1,536-well plate format.

References

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

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