APPLICATION NOTE

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Phosphodiesterase 4 assay using the new LANCE® *Ultra* cAMP kit

Introduction

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of 3',5'-cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to their corresponding 5'-nucleotide monophosphates, AMP and GMP. Both cAMP and cGMP are important second messengers that are coupled to the ligand activation of G-protein coupled receptors (GPCRs). Through this coupling, these two cyclic nucleotides mediate diverse physiological responses to a wide variety of neurotransmitters and hormones.

The PDE superfamily is composed of 21 genes categorized into 11 families (PDE1-PDE11) based on sequence homology, enzymatic properties, and sensitivity to inhibitors. Because PDEs regulate a variety of cellular functions, they have become important drug targets for the treatment of numerous diseases. The cAMP-specific PDE4 isoenzymes are the most widely characterized PDEs and are therapeutic targets for the treatment of several inflammatory disorders.

Here we present the development of a simple, sensitive and robust PDE4A1A assay in 384-well plate format using the new LANCE® *Ultra* cAMP kit.

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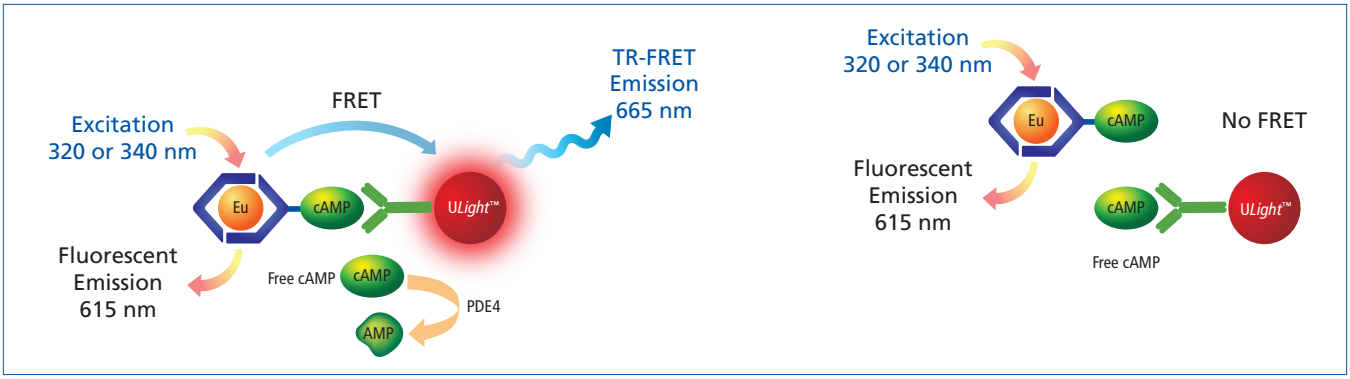


Figure 1. **PDE4 assay principle using the LANCE *Ultra* cAMP kit.**

PDE4 Assay Principle Using LANCE *Ultra* cAMP Kit

The LANCE *Ultra* cAMP assay is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay that was originally developed to measure endogenous levels of cAMP in cell-based assays. 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 (mAb) labeled with the U*Light*™ dye (Figure 1).

When U*Light*-mAb is bound to the Eu-labeled cAMP tracer, light pulse at 320 or 340 nm excites the Eu chelate molecule of the cAMP 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 presence of PDE4, the cAMP is degraded into AMP, which is not recognized by the U*Light*-mAb. This leads to an increase in TR-FRET signal proportional to the concentration of cAMP degraded (Figure 1, left panel). In the presence of a PDE4 inhibitor, cAMP remains intact and competes with the Eu-cAMP tracer for the binding to the U*Light*-mAb, resulting in a decrease in TR-FRET signal (Figure 1, right panel).

Materials and Methods

The LANCE *Ultra* cAMP PDE4A1A assay was optimized using PerkinElmer's LANCE *Ultra* cAMP kit (Cat. no. TRF0262, TRF20263 and TRF0264). Kit components are listed in Table 1. Suppliers for enzyme and inhibitors are indicated in Table 2. All assays were performed at room temperature (RT) in white, opaque OptiPlate™-384 well microplates (PerkinElmer Cat. no. 6007290) and read on an EnVision® Multilabel Plate Reader (lamp mode).

Table 1. **LANCE *Ultra* cAMP kit components**

cAMP Standard
Eu-cAMP tracer
U*Light*-anti-cAMP
cAMP Detection Buffer
BSA Stabilizer (7.5% solution)

Table 2. **Additional reagents required for the assay**

Reagent	Supplier	Catalog #
PDE4A1A	BPS Bioscience	60040
IBMX[a]	Sigma	I7018
Ro 20-1724	Sigma	B8279
Rolipram	Sigma	R6520

[a](3-isobutyl-1-methylxanthine)

Assay development and optimization included the following experiments: cAMP standard curve, PDE4A1A reaction progress curves, enzyme inhibitory profile with known PDE inhibitors, and Z'-factor determination. Standard assay protocols are presented in Table 3. The cAMP standard provided with the LANCE *Ultra* cAMP kit was used for the cAMP standard curve and as substrate for the enzymatic reactions. Substrate, enzyme and inhibitors were diluted in **Enzyme**

Table 3. **Assay protocols**

cAMP standard curve	PDE4A1A reaction progress curves	Inhibition of PDE4A1A activity	Z' factor determination
5 µL cAMP dilutions	5 µL cAMP substrate (final 3 nM[a])	5 µL cAMP substrate (final 3 nM[a])	5 µL cAMP substrate (final 3 nM[a])
5 µL Reaction buffer	5 µL PDE4A1A	2.5 µL inhibitor dilutions	2.5 µL Reaction buffer or inhibitor
—	—	2.5 µL PDE4A1A	2.5 µL PDE4A1A
Incubate 60 min at RT[b]	Incubate different times at RT[b]	Incubate 75 min at RT[b]	Incubate 75 min at RT[b]
5 µL Eu-cAMP tracer working solution in Stop/Detection Buffer			
5 µL U*Light*-anti-cAMP working solution in Stop/Detection Buffer			
Incubate 1 h at RT[b]			
Read on a TR-FRET microplate reader (Remove microplate seal prior to reading)			

[a] In the 20-µL Assay.
[b] Cover plate with a TopSeal™-A film (PerkinElmer, Inc. Cat. no. 6005250) to prevent evaporation.

Reaction Buffer (1X HBSS, 5 mM HEPES, 3 mM MgCl2, 0.1% BSA, pH 7.4). Final DMSO concentrations in enzymatic reactions including IBMX and Ro 20-1724 were 0.1 and 0.3% (v/v), respectively. A combined **Stop/Detection Buffer** (cAMP Detection Buffer supplemented with 1 mM IBMX) was used for the preparation of both the Eu-cAMP tracer and U*Light*-anti-cAMP working solutions according to the kit instructions. Enzymatic reactions were conducted in a volume of 10 µL. Total assay volume, including the Stop/Detection Buffer, was of 20 µL. Inhibitor concentrations are expressed as concentrations in the 10-µL enzymatic reaction while cAMP concentrations are expressed as concentrations in the 20-µL final assay volume. Data are presented as mean ± SD of triplicates. Concentration-response curves were plotted as sigmoidal dose-response curves with variable slope by fitting data using a four-parameter logistic equation (GraphPad Prism® 4).

Results and Discussion

Determination of the PDE4A1A assay working range

A cAMP standard curve was run to determine assay sensitivity to cAMP and working range (Figure 2). The LANCE *Ultra* cAMP kit is highly sensitive, with an IC_{50} value for cAMP of 1.3 nM, which corresponds to 26 femtomoles of cAMP in a 20-µL assay volume. The standard curve maximal signal-to-background (S/B) ratio was of 37 when TR-FRET signal was measured with the EnVision Multilabel plate reader set in lamp excitation mode. Maximal and minimal signals were obtained within a dynamic range of approximately 1.5 log units. This narrow range, coupled with the kit high sensitivity, allows detecting small changes in cAMP concentration while providing an assay with a superior S/B ratio. This makes the LANCE *Ultra* cAMP kit ideal for developing a PDE4 assay. Its high sensitivity might also allow using a lower enzyme concentration than what is currently used with other HTS platforms.

The LANCE *Ultra* cAMP standard curve exhibits a non-linear relationship between the log of the cAMP concentrations and measured TR-FRET signal. As a result, conversion of TR-FRET data into cAMP concentration values by interpolation from a cAMP standard curve is necessary to obtain reliable cAMP concentration data. However, to eliminate the need of data conversion and allow direct data interpretation from the TR-FRET signal, one can work strictly within the linear range of the assay (defined here as the IC_{20} – IC_{80} portion of the cAMP standard curve), which corresponds to 0.5 to 3 nM cAMP or 10 to 60 fmoles of cAMP in a 20-µL assay volume. In order to work within that linear range, PDE4A1A assays were thus conducted with the cAMP substrate at a final concentration of 3 nM, which corresponds to 6 nM in the 10-µL enzymatic assay. Under these conditions, a maximal S/B ratio around 4 can be obtained.

PDE4A1A reaction progress curves

The first optimization step consisted in defining assay conditions under which the TR-FRET signal correlated linearly with time. To this end, reaction progress curves were carried out at two enzyme concentrations (0.1 and 0.25 ng/well) using 6 nM of cAMP as substrate in 10-µL assays. The enzymatic reactions were efficiently stopped at different time points by the addition of IBMX present in the combined **Stop/Detection Buffer**. As shown in Figure 3, signal increased linearly as a function of time for up to 120 minutes with 0.1 ng/well of PDE4A1A, whereas signal reached a plateau after only 30 minutes when using 0.25 ng/well of enzyme due to substrate depletion. In order to develop a sensitive assay and ensure that the cAMP concentration stays within the linear range of the cAMP standard curve, a concentration of 0.1 ng/well PDE4A1A and an enzymatic reaction time of 75 minutes were selected for subsequent experiments. This PDE4A1A concentration corresponds to 100 pM in the enzymatic reaction.

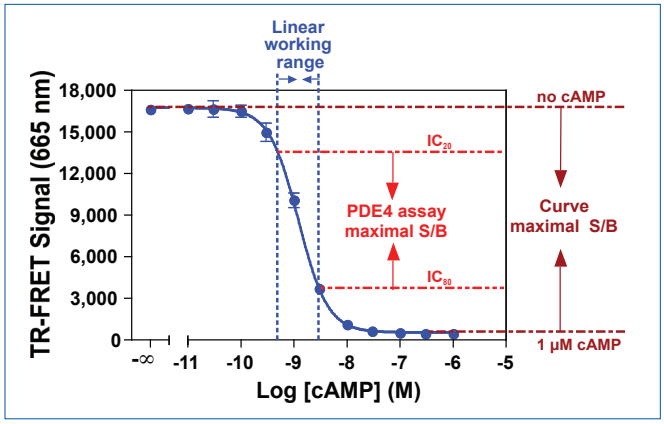


Figure 2. **LANCE *Ultra* cAMP standard curve.** Maximal S/B ratio is calculated by dividing the TR-FRET signal of wells with no cAMP by the signal of wells containing 1 µM cAMP. The IC_{20} and IC_{80} values correspond to the limits of the linear portion of the curve, which are used to define the working range of the PDE4 assay. TR-FRET signal was measured with the EnVision (lamp excitation mode).

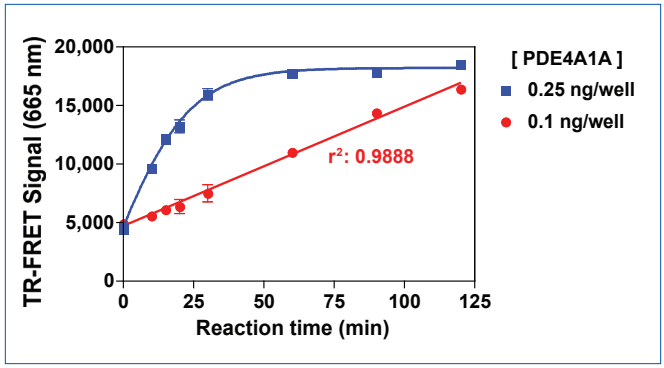


Figure 3. **PDE4A1A reaction progress curves of cAMP substrate degradation as a function of enzymatic reaction time.**

Inhibition of PDE4A1A enzymatic activity

The enzyme inhibitory profile was assessed using three well-known PDE inhibitors, namely the selective PDE4 inhibitor rolipram and the two non-selective PDE inhibitors IBMX and Ro 20-1724. Assays were performed using the optimal conditions selected previously (PDE4A1A at 0.1 ng/well and incubation time of 75 minutes) with detection times of 1 hour (Figure 4A and Table 4) and O/N (Table 4) to demonstrate the assay's stability over time. Data summarized in Figure 4 show that all three compounds tested inhibited PDE4A1A activity in a concentration-dependent manner. In addition, both the IC_{50} values and rank order of potency are in agreement with data reported in the literature and obtained with radioactive assays (Table 4). Of note, no significant changes in compound potencies were observed after O/N incubation, confirming the stability of the PDE4A1A assay.

TR-FRET data were converted to cAMP values by interpolation from a cAMP standard curve run in parallel. Interpolated cAMP values were then plotted against the logarithmic values of inhibitor concentrations (Figure 4B). As summarized in Table 4, IC_{50} values for the interpolated cAMP data are very similar (within 2 to 3-fold) to those obtained with fluorescence data, thereby demonstrating that the PDE4A1A assays were conducted under conditions generating data within the linear range of the cAMP standard curve.

Z'-factor determination

The robustness of the PDE4A1A assay in 384-well plate format was assessed by conducting a Z'-factor analysis[4]. This experiment was performed manually with two populations of 48 replicates in the absence (maximal signal) or presence of 30 µM rolipram (minimal signal). Assays were conducted using the optimal conditions determined previously and signal was read following 1 hour and O/N incubation. Data are summarized in Figure 5. After 1 hour, excellent S/B (4.4) and Z'-factor (0.79) were obtained, together with low percent coefficients of variation (CVs). Of note, both the S/B ratio and Z'-factor were maintained following O/N incubation

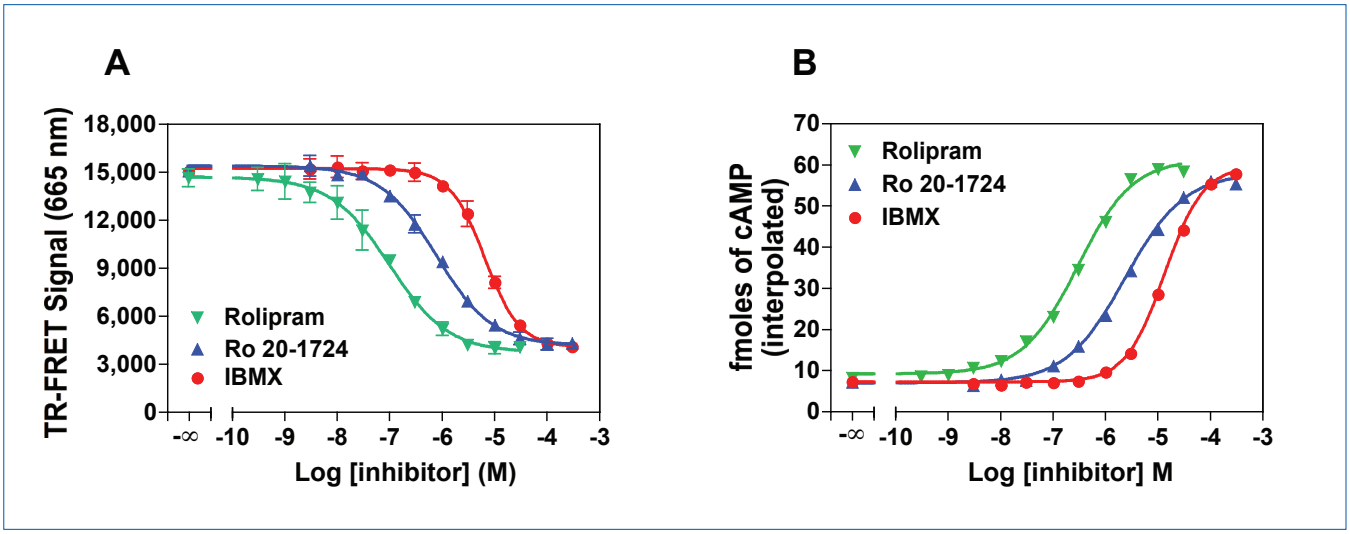


Figure 4. **Inhibition of PDE4A1A enzymatic activity by three PDE inhibitors.** Data was plotted as TR-FRET signal (A). The amount of cAMP remaining in each well after the enzymatic reaction was interpolated from a cAMP standard curve run in parallel (B). TR-FRET signal was read 1 hour after the addition of the Stop/Detection buffer using the EnVision (lamp excitation mode).

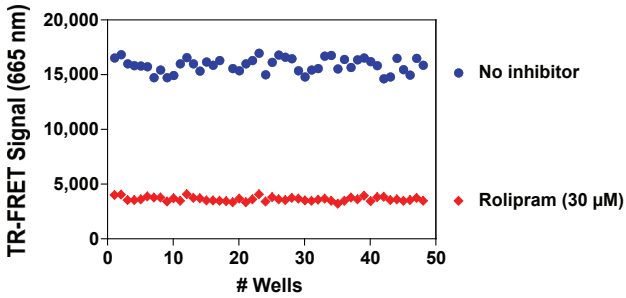
Table 4. **Summary of S/B and IC_{50} values obtained for PDE4A1A inhibitors**

Inhibitor	S/B value TR-FRET data		IC_{50} value (µM) TR-FRET data		IC_{50} value (µM) cAMP interpolated data	Published IC_{50} (µM)
	1 h	O/N	1 h	O/N	1 h	
Rolipram	3.6	3.2	0.10	0.12	0.30	0.2-2.6[1-3]
Ro 20-1724	3.5	3.1	0.81	0.79	2.4	0.9-13[1-3]
IBMX	3.7	3.3	6.7	6.9	13.8	2-50[1-3]

of the plates, confirming assay stability. These data clearly show that the PDE4A1A assay is robust and suitable for off-line HTS plate readings.

Conclusion

The current data demonstrate the suitability of the new LANCE *Ultra* cAMP kit for measuring PDE4A1A enzymatic activity in a simple, rapid and homogeneous assay. The outstanding sensitivity and signal window of the LANCE *Ultra* cAMP kit allowed the development of a sensitive and robust PDE4A1A assay with an S/B ratio around 4 and a Z' factor of 0.8 in a 384-well plate format. The optimized PDE4A1A assay is also cost-effective, requiring little enzyme (0.1 ng/well). These key advantages, combined with stable assay signal, wide assay window, and simple protocol, makes the LANCE *Ultra* cAMP kit the platform of choice for measuring PDE enzymatic activity in HTS format.



Detection time	No inhibitor			Rolipram (30 µM)			Data analysis	
	Mean	SD	%CV	Mean	SD	%CV	S/B	Z' factor
1 h	15902	647	4.1	3655	199	5.4	4.4	0.79
O/N	15267	578	3.8	3758	208	5.5	4.1	0.80

***Figure 5.* Z' factor study for the PDE4A1A assay.** This figure presents data obtained 1 hour after the addition of the Stop/Detection buffer.

References

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