

**Multimode Detection****Authors:**

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## Characterization of Compound Effects on PDE Activity Using the LANCE *Ultra* cAMP Kit and VICTOR Nivo Multimode Plate Reader

**Introduction**

In this application note, we describe the characterization of compound effects on phosphodiesterase (PDE)

activity, using the LANCE® *Ultra* cAMP assay and the VICTOR Nivo™ multimode plate reader.

cAMP is a key second messenger for intracellular signal transduction of a variety of activated G-protein coupled receptors. It plays a role in numerous diseases and biological responses including apoptosis, inflammation and cancer<sup>1,2</sup>. The intracellular concentration of cAMP is regulated by adenylyl cyclase-mediated synthesis and PDE-dependent degradation<sup>3,4</sup>.

In this assay, we used both purified PDE and HeLa cells to examine the sensitivity, dynamic range, reaction kinetics, inhibitor profiles, assay quality and robustness of the LANCE assay in both biochemical and cell-based assay formats, and to demonstrate the performance characteristics of a typical LANCE TRF assay when using the VICTOR Nivo multimode plate reader.

## Material and Methods

The LANCE *Ultra* cAMP Detection Kit (#TRF0262, PerkinElmer) is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay for detection and quantification of cAMP in cell lysates<sup>5</sup>. It is a competition assay between a europium (Eu) chelate-labeled cAMP tracer and cellular or sample cAMP for binding to a cAMP-specific monoclonal antibody (mAb) labeled with the *ULight*<sup>™</sup> dye. In the absence of cellular or sample cAMP, Eu chelate-labeled cAMP binds to the *ULight*<sup>™</sup> labeled cAMP-specific monoclonal antibody. Following a light pulse at 320 or 340 nm, energy is transferred by FRET from the Eu chelate to the *ULight*<sup>™</sup> molecule, which emits light at 665 nm. In the presence of free cAMP, the TR-FRET signal is reduced as a result of competition between free and labeled cAMP for binding to the *ULight*<sup>™</sup> labeled cAMP-specific monoclonal antibody (Fig. 1). Residual energy from the Eu-chelate is emitted as light at 615 nm.

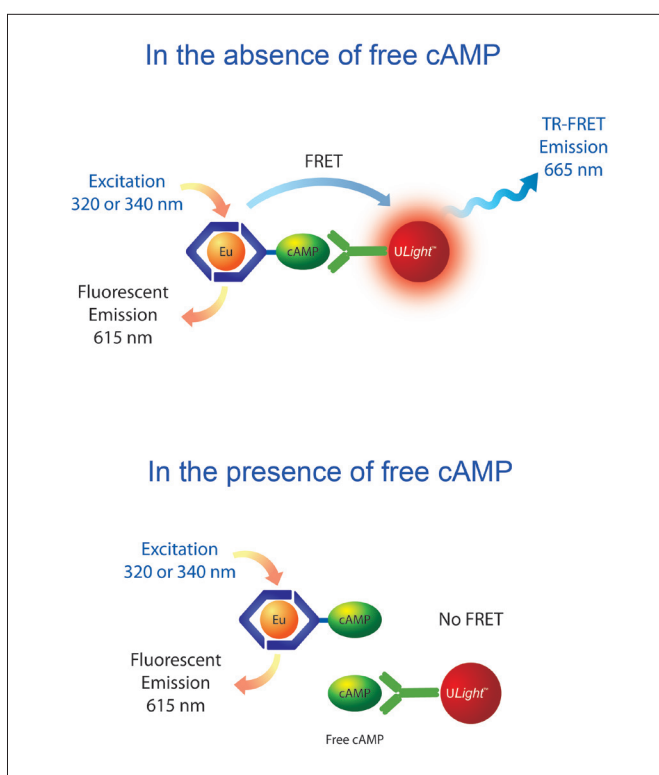


Figure 1. LANCE® *Ultra* cAMP assay principle.

### Biochemical Assay

The biochemical assay was optimized using PDE4D2 enzyme (PDE4D2 active human, recombinant, #SRP0432, Sigma-Aldrich). The assay protocol was performed in accordance with the LANCE® *Ultra* cAMP Assay Development Guide<sup>5</sup> and the application note *Phosphodiesterase 4 assay using the new LANCE® Ultra cAMP kit*<sup>6</sup>. PDE4D2 and cAMP (cAMP standard supplied in the kit) were diluted to the assay concentration in reaction buffer containing 1X HBSS, 5 mM HEPES, 3 mM MgCl<sub>2</sub>, 0.1% BSA (BSA stabilizer 7.5% solution supplied in the kit), pH 7.4. Compounds were transferred to white, opaque 384-well microplates (OptiPlate-384, #6007290, PerkinElmer) using the Echo® Liquid Handling System 550 (Labcyte). The maximum DMSO concentration used was 0.5% v/v. 5 µL of PDE4D2 was added to compounds, spun down and incubated at room temperature (RT) for 15 minutes followed by the addition of 5 µL cAMP and incubation at RT for one hour.

At the end of the incubation time, 5 µL of *ULight*<sup>™</sup> labeled cAMP-specific monoclonal antibody (1:150) and 5 µL Eu chelate-labeled cAMP tracer (1:50) were diluted in the stop/detection buffer supplied in the kit and supplemented with 1 mM IBMX (#BML-PD140-0200, Enzo Life Sciences) then added to the reaction mix. After one hour incubation at RT, signal was read on the VICTOR Nivo multimode plate reader at 616 and 665 nm.

The enzyme reaction volume was taken as 10 µL including compounds, PDE4D2 and cAMP. All compound concentrations were based on the enzyme reaction volume. The cAMP concentration was based on a total assay volume of 20 µL after addition of *ULight*<sup>™</sup> labeled cAMP-specific monoclonal antibody and Eu chelate-labeled cAMP tracer. Data analysis was performed using GraphPad Prism® software.

### Cell Based Assay

For the cell-based assay, the human cervical cancer cell line, HeLa (HeLa Cell Line human, #93021013-1VL, Sigma-Aldrich), was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L Glucose, L-Glutamine, #DMEM-HA, Capricorn) supplemented with 10 % fetal bovine serum (#FBS-12A, Capricorn) 1 % Penicillin (100 U/mL)/Streptomycin (100 µg/mL) (#PS-B, Capricorn). To perform the LANCE® *Ultra* cAMP assay, cells were washed in PBS (Dulbecco's PBS (1x), without Ca and Mg, without Phenol Red, #PBS-1A, Capricorn) harvested using Trypsin/EDTA (#Try-1B, Capricorn) and pelleted for three minutes at 100xg. Cells were then resuspended in reaction buffer containing 1X HBSS, 5 mM HEPES, 3 mM MgCl<sub>2</sub>, 0.1% BSA (BSA Stabilizer 7.5% solution supplied in the kit), pH 7.4. Next, 10 µL of cell suspension were transferred into white, opaque 384-well microplates (OptiPlate-384, #6007290, PerkinElmer) followed by addition of PDE inhibitors using the Echo® Liquid Handling System 550 (Labcyte). After 15 minutes incubation at RT, forskolin was added to increase intracellular cAMP levels. Cells were incubated for an additional 30 minutes at RT. At the end of the incubation time, 5 µL of *ULight*<sup>™</sup> labeled cAMP-specific monoclonal antibody (1:150) and 5 µL Eu chelate-labeled cAMP tracer (1:50) were diluted in the stop/detection buffer supplied in the kit and supplemented with 1 mM IBMX (#BML-PD140-0200, Enzo Life Sciences) and added to the reaction mix. After one hour incubation at RT, signal was read on the VICTOR Nivo multimode plate reader at 616 and 665 nm using the pre-set LANCE protocol included in the system's software.

The assay reaction volume was taken as 10 µL including compounds and cell suspension. All compound concentrations were based on this assay reaction volume. Data analysis was performed using GraphPad Prism® software.

### Instrumentation

The assays were detected using the VICTOR Nivo multimode plate reader, a compact, sensitive and user-friendly benchtop system for detection of a wide variety of assays, ideal for a multi-user environment.

The system's software includes a pre-set protocol for assays based on LANCE technology, and is easily adapted to the requirements of individual assays.

In addition to TRF assays, the VICTOR Nivo system is equipped with all the major detection modalities - absorbance, luminescence, fluorescence intensity, time-resolved fluorescence and fluorescence polarization. All technologies can be read from the top or bottom of the plate, with bottom-reading often preferred for cell-based assays, such as the assay using HeLa cells described in this study.

## Results

### Titration of cAMP Standard

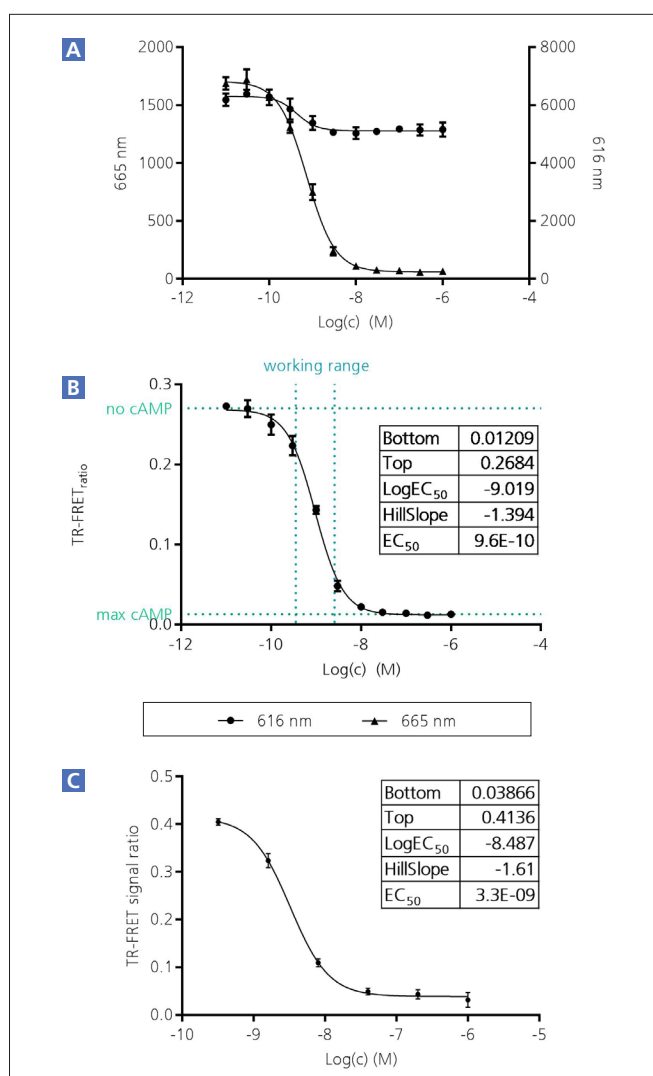
To determine the most sensitive range for the assay, the concentration of the cAMP standard supplied in the kit was titrated. The TR-FRET signal at 665 nm was normalized to the signal of the donor-channel at 616 nm to correct for well-to-well variability of the signal. Nevertheless, changes in the donor channel should always be analyzed separately to avoid interference with the donor channel. According to the results, a linear working range was obtained between 0.5 and 5 nM with an  $EC_{50}$  of 0.95 nM giving a dynamic range of approximately one log unit. Similar values were measured for the 96-well plate (Fig. 2). The maximal signal to background ratio (S/B) within the dynamic range was  $> 10$ , showing high assay sensitivity for small changes in the cAMP concentration within this range (Fig. 2). Therefore the working concentration of 3 nM was used for subsequent assays, which corresponds to 6 nM cAMP in the enzyme reaction volume of 10  $\mu$ L.

### Enzyme Titration and Kinetics

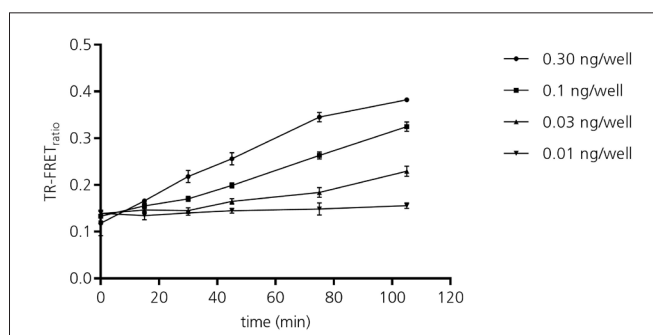
In order to optimize assay conditions, the effect of incubation time and enzyme concentration on signal linearity was examined. PDE4D2 was incubated at different concentrations between 0.01 and 0.3 ng/well with 6 nM cAMP. The reaction was stopped after 0 - 1.75 hours of incubation at RT by the addition of stop/detection buffer containing 1 mM IBMX. The results show a linear correlation between time and TR-FRET signal ratio when using 0.03 to 0.3 ng PDE4D2/well for an incubation time of 0.5 to 1.25 hours. Longer incubation times with 0.3 ng/well PDE4D2 show signal saturation, therefore indicating depletion of cAMP (Fig. 3). No signal increase was observed for 0.01 ng PDE4D2/well showing that an extended incubation time might be needed when using this concentration. Consequently, 0.3 ng PDE4D2/well and an incubation time of one hour were selected for further experiments.

### Selection of Standard PDE Inhibitors

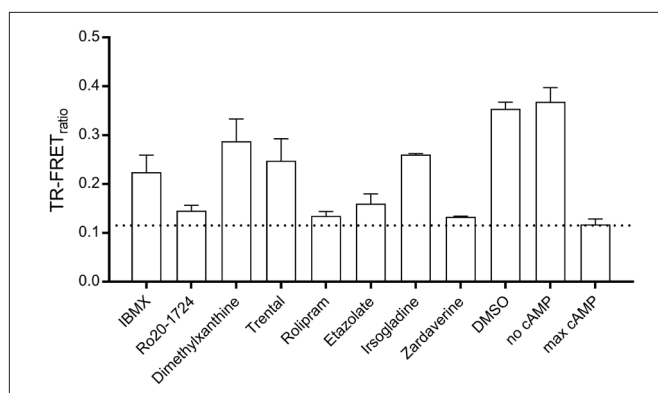
Using the optimized assay conditions determined previously, eight standard inhibitors were selected to characterize the inhibitory profile of PDE4D2. Known PDE inhibitors were tested at 50  $\mu$ M. Four inhibitors showed a reduction in TR-FRET signal ratio comparable to the no-enzyme control (max cAMP) (Fig. 4). Therefore Ro20-1724, rolipram, etazolate and zardaverine were used for determination of the  $IC_{50}$  values.



**Figure 2.** Titration of the LANCE Ultra cAMP standard. A: Detected RFU of the donor (616 nm) and acceptor (665 nm) channels. B: Calculated TR-FRET signal ratio values ( $RFU_{665\text{ nm}}/RFU_{616\text{ nm}}$ ). C: Titration of the LANCE Ultra cAMP standard in a 96-well OptiPlate. Signal to background ratio was calculated according to sample signal without cAMP addition and samples with 1  $\mu$ M cAMP. Linear working range is represented by calculated  $EC_{20}$  and  $EC_{80}$  values. Values represent the mean of four replicates  $\pm$  SD.

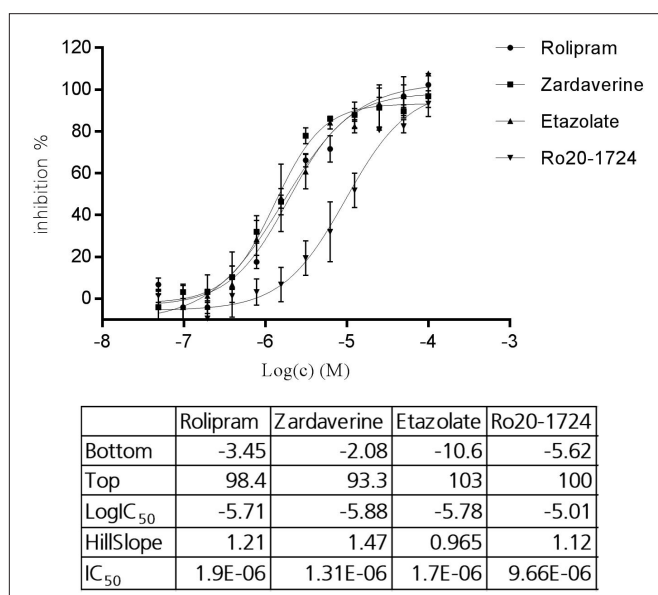


**Figure 3.** Progression of PDE4D2-signal over time. Increasing concentrations of PDE4D2 were incubated for 0-115 minutes with 6 nM of cAMP (in 10  $\mu$ L assay volume). The reaction was stopped by addition of Detection Reagent containing 1 mM IBMX. After one hour of incubation, TR-FRET signal ratio ( $RFU_{665\text{ nm}}/RFU_{616\text{ nm}}$ ) was measured. Values represent the mean of four replicates  $\pm$  SD.



**Figure 4.** Selection of standard PDE inhibitors for characterisation of PDE4D2 inhibitory profile. PDE4D2 was incubated for 15 minutes with inhibitors at 50  $\mu$ M, followed by 60 minutes incubation with cAMP at RT. The reaction was stopped by addition of Detection Reagent containing 1 mM IBMX. After one hour of incubation TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured. Values represent the mean of four replicates  $\pm$  SD.

For all compounds, dose-dependent activity was confirmed (Fig. 5). Calculated IC<sub>50</sub> values were in agreement with literature data (Table 1), although slightly increased for rolipram and Ro20-1724<sup>7</sup>.



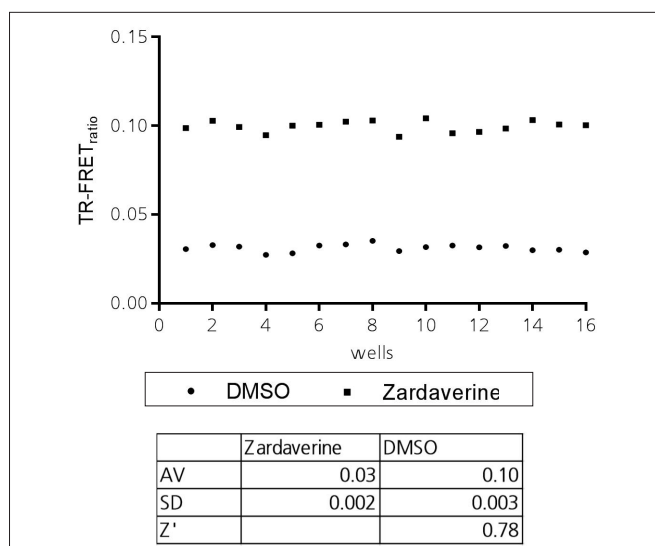
**Figure 5.** Dose dependent inhibition of PDE4D2 by four selected standard PDE inhibitors. PDE4D2 was incubated for 15 minutes with increasing concentrations of inhibitors followed by 60 minutes incubation with cAMP at RT. The reaction was stopped by addition of the Detection Reagent containing 1 mM IBMX. After one hour of incubation TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured. Values represent the mean of four replicates  $\pm$  SD.

### Assay Quality and Robustness

In order to evaluate the quality and robustness of the assay, Z' analysis was performed. Zardaverine at 50  $\mu$ M was used as the positive control for PDE4D2 inhibition. 0.1 v/v % DMSO represents the negative control. The calculated Z' of 0.79 (Fig. 6) reveals a robust assay suitable for multiple compound tests.

**Table 1.** Inhibition of PDE4D2 – experimental and literature data.

IC <sub>50</sub> and SD in $\mu$ M	Rolipram	Zardaverine	Etazolate	Ro20-1724
LANCE <i>Ultra</i> cAMP Detection Kit	1.93 $\pm$ 0.2	1.31 $\pm$ 0.1	1.66 $\pm$ 0.3	9.66 $\pm$ 0.8
Literature Data <sup>7</sup>	0.55 $\pm$ 0.05	0.95 $\pm$ 0.15	1.1 $\pm$ 0.1	2.9 $\pm$ 0.1



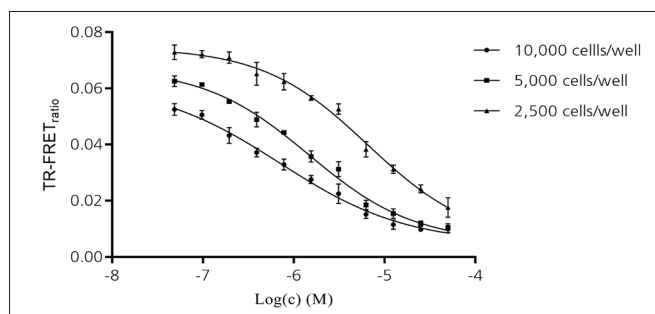
**Figure 6.** Calculation of assay robustness using Z'. PDE4D2 was incubated for 15 minutes with zardaverine (50  $\mu$ M) or DMSO (0.1 % v/v), 16 wells each, followed by 60 minutes incubation with cAMP at RT. The reaction was stopped by addition of Detection Reagent containing 1 mM IBMX. After one hour of incubation TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured.

### Application of the LANCE *Ultra* cAMP Assay for Monitoring PDE Activity in a Cell Based Format

To characterize PDE activity in a cell-based assay, HeLa cells were selected based on the reported endogenous expression level of PDE4D in these cells<sup>8</sup>. The endogenous level of cAMP in cells is highly controlled<sup>3</sup>. To increase the intracellular cAMP level, HeLa cells were incubated with forskolin, an activator of adenylyl cyclase.

#### Titration of Cell Number

In order to optimize assay conditions and achieve best signal linearity, cell numbers of 10,000, 5,000 and 2,500 HeLa cells/well were incubated with increasing forskolin concentrations in the presence of 0.5 mM IBMX to inhibit the intracellular PDE activity (Fig. 7). The results showed that 2,500 cells/well incubated with 10  $\mu$ M forskolin give a 2.5-fold signal window within the dynamic range of the assay (Fig. 2). Using this data standard PDE inhibitors were retested in the cell-based assay.

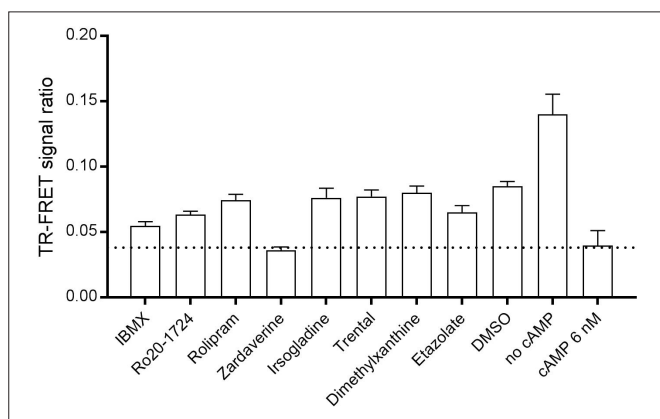


**Figure 7.** Dose response curve of forskolin in HeLa cells. HeLa cells were incubated for 30 minutes with increasing concentrations of Forskolin in the presence of 0.5 mM IBMX. The reaction was stopped by the addition of Detection Reagent containing 0.5 mM IBMX. TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured after one hour of incubation. Values represent the mean of three replicates  $\pm$  SD.



## Selection of Standard PDE Inhibitors

The results showed that only zardaverine was able to reduce the TR-FRET signal ratio to the level of the positive control containing 6 nM cAMP (Fig. 8). IBMX and etazolate had a limited effect on the reduction of PDE activity in HeLa cells. Therefore, IBMX was used as a nonselective PDE inhibitor and zardaverine as a known inhibitor of PDE4 for determination of the  $IC_{50}$  values.



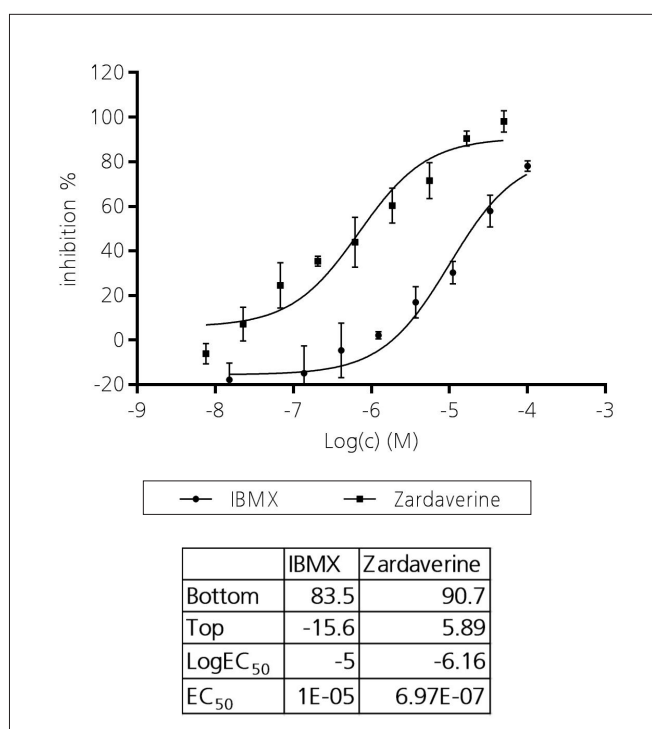
**Figure 8.** Selection of standard PDE inhibitors for characterisation of PDE4D inhibitory profile in HeLa cells. HeLa cells were incubated for 15 minutes with inhibitors followed by 30 minutes incubation with 10  $\mu$ M forskolin. The reaction was stopped by the addition of Detection Reagent containing 0.5 mM IBMX. After one hour of incubation TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured. Values represent the mean of three replicates  $\pm$  SD.

The calculated  $EC_{50}$  value of zardaverine for inhibition of PDE activity in HeLa cells was in the range of the value measured in the biochemical assay using PDE4D2. Also the  $EC_{50}$  of IBMX was in line with reported data for non-specific inhibition of human PDEs<sup>3</sup> (Table 2).

## Conclusion

This study describes the application of the LANCE® *Ultra* cAMP assay together with the VICTOR Nivo multimode plate reader for analysis of PDE inhibition in biochemical and cell-based assay formats. The assay is robust with a  $Z' > 0.75$  and  $S/B$  ratio  $> 10$ . It is highly sensitive, enabling the use of low enzyme concentrations with short incubation times, therefore reducing time and costs required for the assay. The pre-set LANCE protocol included in the VICTOR Nivo system's software was easily applied to the requirements of the assay kit, with adjustments easily made for incubation time, plate format or readout parameters.

The data obtained for the biochemical readout of the PDE4D2 inhibition were in line with reported data shown in Tables 1 and 2. Differences in observed potencies may be caused by the enzyme constructs tested (full length or catalytic domain) and



**Figure 9.** Dose dependent inhibition of PDE in HeLa cells using two selected standard PDE inhibitors. HeLa cells were incubated for 15 minutes with increasing concentrations of inhibitors followed by 30 minutes incubation with 10  $\mu$ M forskolin. The reaction was stopped by the addition of the Detection Reagent containing 0.5 mM IBMX. After one hour of incubation TR-FRET signal ratio (RFU<sub>665 nm</sub>/RFU<sub>616 nm</sub>) was measured. Values represent the mean of three replicates  $\pm$  SD.

**Table 2.** Inhibition of PDE activity in cell based assays – experimental and literature data.

IC <sub>50</sub> in $\mu$ M	IBMX	Zardaverine
LANCE <i>Ultra</i> cAMP Detection Kit (biochemical data in brackets)	10.0 (n.m)*	0.7 (1.31 $\pm$ 0.1)
Literature Data <sup>3,9**</sup>	2 - 50	0.8 - 1.7

\*n.m.- not measured

\*\* - cell based or *in vivo* data

buffer composition, as PDE enzymes are highly dependent on the concentration of divalent ions. For the cell based assay, HeLa cells were chosen for characterization of PDE inhibition based on the reported endogenous expression level of PDE4D in these cells<sup>8</sup>. The results showed that non-selective inhibitors like IBMX inhibited the reduction of intracellular cAMP. Surprisingly, the PDE4D specific inhibitor rolipram did not show an effect in HeLa cells, whereby zardaverine (a PDE III/IV inhibitor) fully inhibited the reduction of intracellular cAMP. The results indicate a low endogenous level of PDE4 in HeLa cells. To improve the sensitivity of the assay to PDE inhibitors, recombinant cell systems could be used.

## References

1. Schett, G., Sloan, V. S., Stevens, R. M., Schafer, P. Apremilast: a novel PDE4 inhibitor in the treatment of autoimmune and inflammatory diseases. *Therapeutic Advances in Musculoskeletal Disease*. 2010 (2). 271-278.
2. Yan, K., Gao, L.-N., Cui, Y.-L., Zhang, Y., Zhou, X. The cyclic AMP signaling pathway: Exploring targets for successful drug discovery. *Molecular Medicine Reports*. 2016. 3715-3723.
3. Zhu, W.-H., Majluf-Ctuz, A., Omburo, G., A. Cyclic AMP-specific phosphodiesterase inhibitor rolipram and Ro20-1724 promoted apoptosis in HL60 promyelocytic leukemic cells via cyclic AMP-independent mechanism. *Life Science*, 1998 (63). 265-274.
4. Omori, K., Kotera, J. Overview of PDEs and their regulation. *Circulation Research*. 2007.
5. LANCE® *Ultra* cAMP Assay Development Guide. PerkinElmer.
6. Gauthier, N., Beaudet L., Padrós, J. Phosphodiesterase 4 assay using the new LANCE® *Ultra* cAMP kit. PerkinElmer.
7. Wang, H., Liu, Y., Chen, Y., Robinson, H., Ke, H. Multiple elements jointly determine inhibitor selectivity of cyclic nucleotide phosphodiesterases 4 and 7. *The Journal of Biochemical Chemistry*. 2005 (280). 30949–30955.
8. <http://www.proteinatlas.org/ENSG00000113448-PDE4D/cell> (16/02/2017).
9. Schudt, C., Winder, S., Mueller, B. and Ukena, D. Zardaverine as a selective inhibitor of phosphodiesterase isozymes. 1991. *Biochemical Pharmacology*, Vol. 42, No. 1, pp. 153-162.