APPLICATION NOTE

Phosphorylated ERK1/2 (T202-Y204) LANCE *Ultra* cellular detection kit performance in CHO-K1 cells expressing a class A GPCR.

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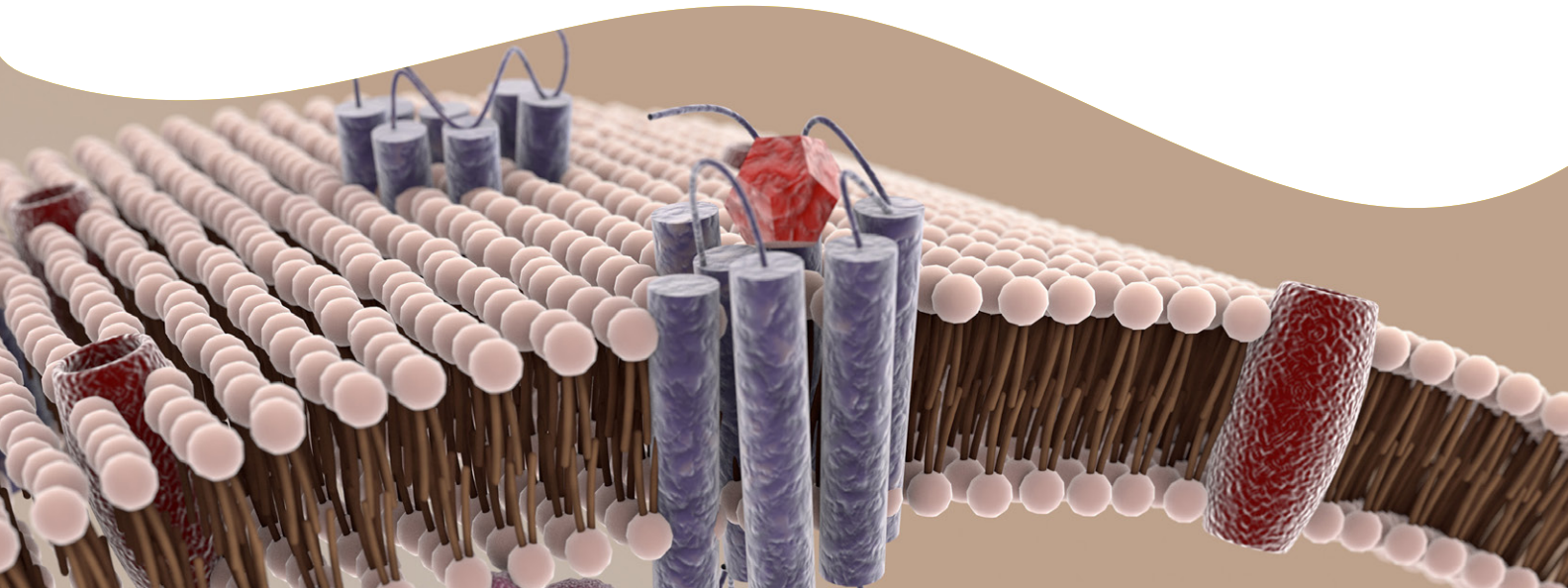
Introduction

G protein-coupled receptors (GPCRs) mediate a plethora of physiological functions and are considered a major therapeutic target. The identification of novel drug candidates targeted to GPCRs depend on the measurement of cellular effectors and secondary messenger generation. Extracellular signal-regulated kinase 1/2 (ERK1/2) is a serine/threonine kinase in the mitogen-activated protein kinase (MAPK) family and a major cellular effector of many GPCRs.[1] ERK1/2 has many known targets (including transcription factors such as activator protein 1 (AP-1), nuclear factor (NF)-κB and Myc) and regulates a number of cellular processes including proliferation, differentiation and meiosis.[2] The measurement of phosphorylated ERK1/2 at position Tyr202 and Tyr204 can be used as a functional outcome of both G protein and β-arrestin mediated pathways whereby pERK is a common endpoint to many GPCR cascades.[3]

The availability of high throughput cell-based screening assays measuring pERK will supplement information acquired from intracellular Ca^{2+} mobilization and cAMP accumulation assays. In this technical note, two pERK1/2 (T202-Y204) cellular detection assays were compared head-to-head: the LANCE® *Ultra* pERK1/2 kit (Revvity, #TRF4000) and a pERK1/2 assay from a competitor company. The adenosine A_1 receptor (A_1R) subtype was chosen as an archetype class A G_i-coupled GPCR, whereby previous reports have confirmed agonists stimulated ERK1/2 phosphorylation.[4]



Description of pERK1/2 assays

The two tested pERK1/2 assays are based on a sandwich format using two specific antibodies: one labelled with a donor fluorophore and the second labelled with an acceptor fluorophore. They are based on homogeneous (no wash) time-resolved fluorescence resonance energy transfer (TR-FRET) technology which combines fluorescence resonance energy transfer (FRET) and time-resolved (TR) measurements. Upon excitation at 320/340 mM, proximity of donor and acceptor (~10 mM) will lead to a TR-FRET signal whereby energy is transferred from the donor to acceptor which in turn fluoresces at 665 mM (Figure 1). The signal can be detected using an appropriate plate reader with TR-FRET capability with an excitation wavelength of 320/340 mM and a detection of both donor and acceptor emissions at 615/620 mM and 665 mM, respectively.

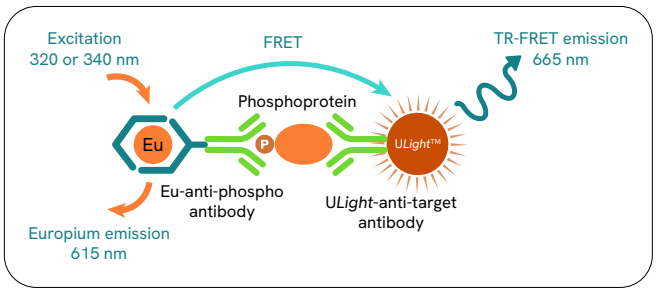


Figure 1: **Assay principle for LANCE *Ultra* TR-FRET pERK1/2** assay. The competitor assay tested has a similar assay principle.

Materials and methods

Cell culture

Chinese hamster ovary (CHO)-K1 cells stably expressing A_1R were maintained in F-12 Nutrient Mixture (Ham) GlutaMAX™ (Thermo Fisher Scientific, #31765035) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, #F9665) in a humidified 5% CO_2 incubator at 37 °C. Cells grown in a 25 cm^2 flask were split at a 1 in 10 every 3 days when 80% confluence was reached.

pERK assay

The pERK assays were used in line with product protocols. Identical temperature and ligand stimulation time were maintained across assays, as determined by optimization experiments for the cell line expressing the GPCR of interest (data not shown). CHO-K1 A_1R cells cultured in a 25 cm^2 flask at approximately 80% confluence were harvested, using 0.05% Trypsin-EDTA and washed before plating in HBS on a white 384-well OptiPlates™ (Revvity, #6007290) at the indicated cell density. Following a 5 minutes agonist stimulation at 37 °C, the cells were lysed in lysis buffer at room temperature on an orbital shaker for 30 minutes. Each condition was set up in duplicate. Cells were then incubated for four or two hours (for the LANCE *Ultra* pERK and competitor assay, respectively) as indicated in the kit protocol at room temperature with premixed donor and acceptor antibody prepared in detection buffer. At all incubation stages, the plate was sealed with a ThermalSeal® film (EXCEL Scientific, #TS2-100) to prevent evaporation of small volumes.

Instrument and data analysis

All assays were measured using a Mithras LB 940 (Berthold technology) plate reader. Data were processed using the calculation featured in the protocol (665/615 mM x 10,000) and, where appropriate, fitted to a three-parameter logistic equation to acquire pEC_{50}, E_{max}, Basal and Span (refers to the range (E_{max} – Basal)) values using Prism® 7.0d (GraphPad software, San Diego, CA). All results were normalized to 5 minutes stimulation with 1 µM Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, # 16561-29-8), a direct protein kinase C (PKC) activator.[5]

Results and discussion

We first set out to investigate the optimal seeding density of CHO-K1 A_1R cells per well to detect a pERK signal using each kit. In order to keep the stimulation and cell lysis conditions consistent between assays, a stimulation time of 5 minutes at the protocol's indicated temperature (23 °C or 37 °C for LANCE *Ultra* pERK and the competitor kit, respectively) and a 30-minutes lysis time was chosen for these initial experiments. The optimal cell number for each kit was determined through stimulation of CHO-K1 A_1R cells seeded at various densities and stimulated with 1 µM PMA or increasing concentrations of A_1R agonist NECA (5′-(N-Ethylcarboxamido) (Figure 2). When assayed using the LANCE *Ultra* pERK assay, increasing the cell seeding density from 10k or 20k/well to 50k/well significantly increased the level of detected pERK following NECA stimulation (Figures 2A and 2C). A further increase in seeding density from 50k to 100k cells did not significantly enhance the level of pERK suggesting the upper limit of the assay window had been attained (Figure 2A).

Despite the protocol for the LANCE *Ultra* pERK assay indicating 100k cells/well to be optimal for most cell lines, these results indicate a cell seeding density of 50k cells/well produces an optimal signal whilst reducing the number of cells required per assay. The level of pERK following NECA stimulation remained similar at the three tested seeding densities when measured using the competitor kit (Figures 2B and 2D). This finding suggests we may be at the upper end of the competitor assaying window at a seeding density of 10k cells and a lower number should be considered. To confirm this, we reduced the cell number to 5k, 2k and 1k cells per well and stimulated with PMA, NECA or adenosine (an A_1R agonist with reduced potency and binding when compared to NECA) (Figure 3). Whereas we appear to be at the upper end of the assay at 5k cells, with the NECA concentration dose-response (ratio) similar to that of higher cell numbers, since the addition of more cells did not enhance the signal (measured as ratio of 665/615 mM x 10, 000) (Figure 3D). Indeed, reducing the number of cells to 2k or 1k showed a significantly reduced 665/615 mM ratio (Figure 3D). However, only when we reached the cell density of 1k did the predicted difference in potency between NECA and adenosine become apparent (Figure 3C). It is important to note that at this low cell number, variability within repeats is enhanced making the reproducibility of the data more difficult with the competitor assay (Figure 4).

We wanted to ensure all experimental conditions were as consistent as possible, yet optimal, for each assay kit including lysis time and temperature. The temperature of 37 °C is recommended for the competitor assay whereas the LANCE *Ultra* pERK kit suggests incubation at 23 °C. A direct comparison between a 5-minutes stimulation at 23 °C and 37 °C for the LANCE *Ultra* kit showed comparable results (Figure 5). With this in mind, and to reduce variables between the LANCE *Ultra* pERK assay the competitor assay, further experiments were conducted with a 5-minutes stimulation at 37 °C. We then wanted to test if a longer or shorter lysis time gave a superior signal. The signal achieved with a 30-minutes lysis step was similar to that obtained following a 10, 15, 45 or 60-minutes lysis time for both the LANCE *Ultra* pERK assay and the competitor assay (Figure 6). As a result, a lysis time of 30 minutes was chosen for all further experiments.

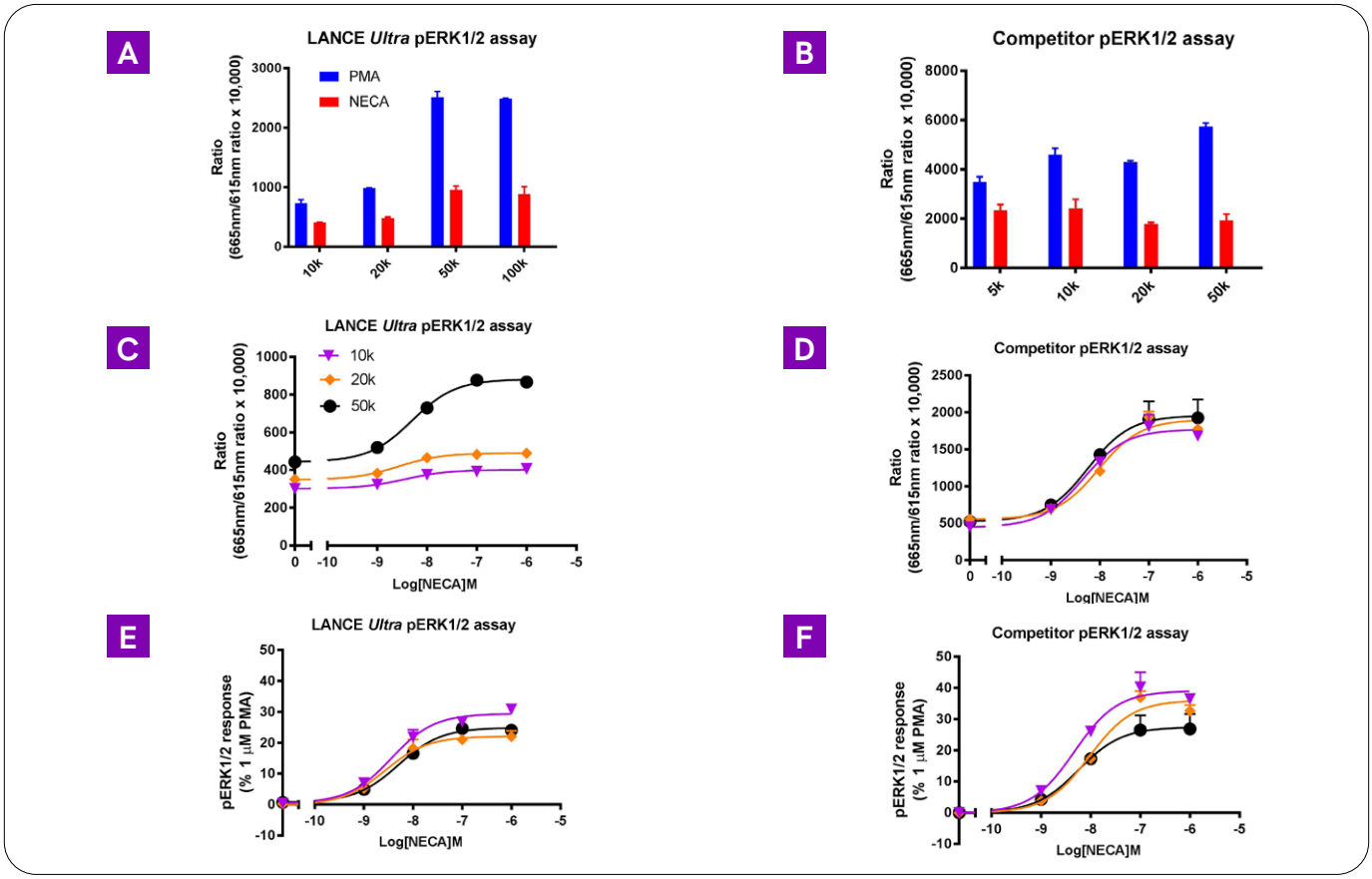


Figure 2: **Cell number optimization.** Detection of pERK with the LANCE *Ultra* pERK1/2 kit **(A, C, E)** or a competitor TR-FRET pERK1/2 assay kit **(B, D, F)** following stimulation of CHO-K1 A1R expressing cells seeded at various densities per well of a 384-well OptiPlate with **(A, B)** a single concentration of PMA or NECA, or **(C,D)** various concentrations of NECA expressed as the ratio or **(E-F)** %PMA response (1 µM).

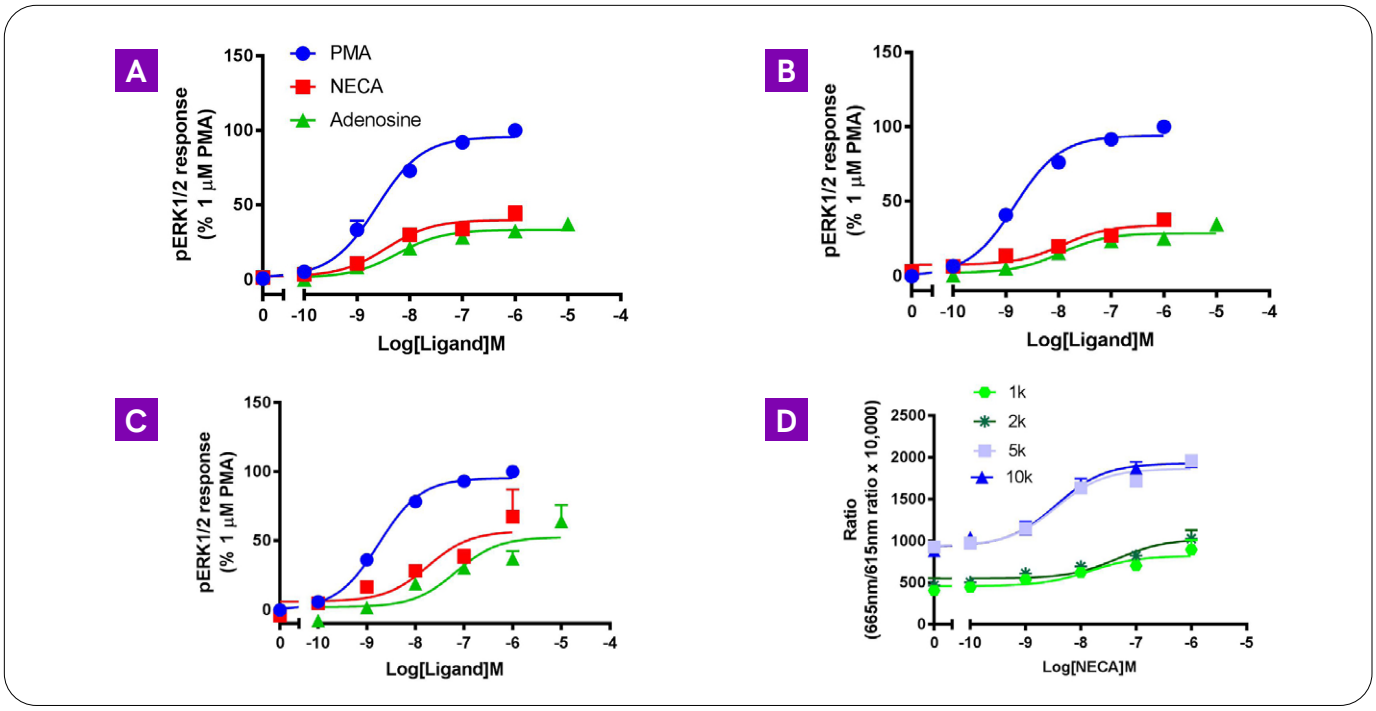


Figure 3: **Cell number optimization for competitor assay**. Detection of pERK with the competitor assay kit following stimulation of CHO-K1 A_1R expressing cells seeded at **A)** 5k, **B)** 2k or **C)** 1k cells per well in a 384-well OptiPlate with various concentrations of PMA, NECA or adenosine expressed as %PMA response (1 µM). **D**) Ratio of NECA concentration dose-response curves at various cell numbers expressed as the ratiometric 665/615 nM x 10,000 signal.

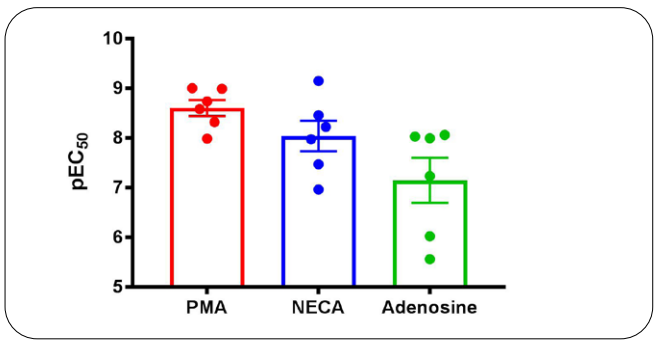


Figure 4: **Assay variability of competitor pERK1/2 assay.** pEC_{50} values as taken from pERK concentration dose response curves for PMA and A_1R agonist (NECA and adenosine) measured in CHO-K1 A_1R cells seeded at 1k cells per well from two experiments conducted in duplicate.

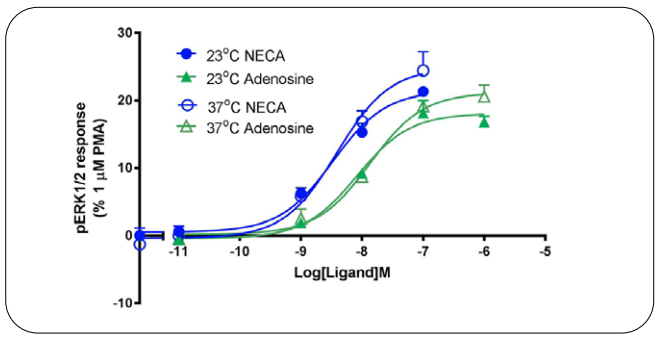


Figure 5: **Comparison of pERK detection using the LANCE *Ultra* pERK1/2 assay at 23 °C or 37°C stimulation.** 50k CHO-K1 A_1R cells/well were stimulated with various concentrations of NECA or adenosine for five minutes.

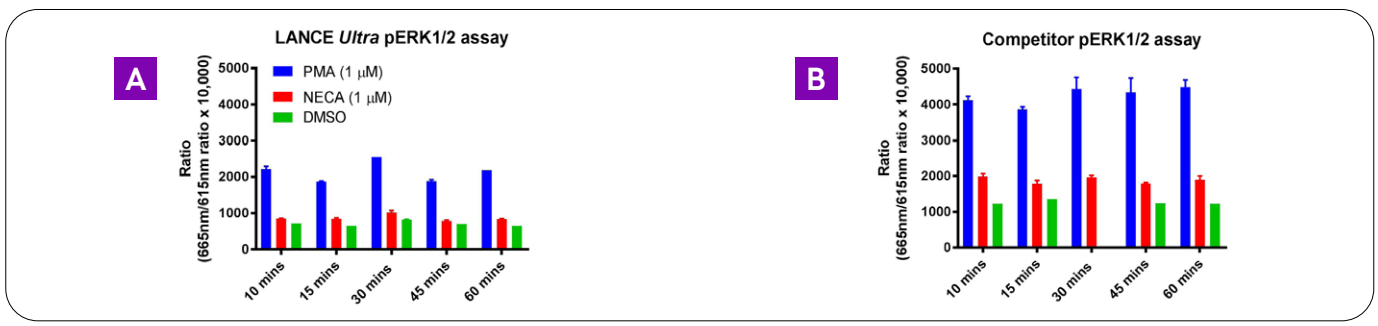


Figure 6: **Lysis time optimization.** CHO-K1 A_1R cells seeded at the optimal cell density (50k cells/well for the LANCE *Ultra* pERK1/2 assay kit in **A** and 10k cells/well for the competitor assay kit in **B**) were stimulated for five minutes with DMSO control, or 1 µM PMA or NECA and incubated in lysis buffer for various times.

Assay sensitivity to pERK was determined by comparing the potency of PMA, NECA and adenosine (pEC_{50}) (Figure 7 and Table 1). Importantly, there were differences in both absolute and relative pEC_{50} values for the compounds between the two assay kits. This finding highlights that experimental conclusions can often heavily rely on the chosen assay kit sensitivity and range. Given that the adenosine has both a reduced A_1R binding and potency for inhibiting forskolin (a potent adenylyl cyclase activator) stimulated cAMP accumulation compared to NECA (Figure 8), and that ERK1/2 phosphorylation following A_1R activation is predominantly G_i-mediated, we would expect adenosine to show a reduced potency in generating pERK when compared to NECA. This prediction was validated by the LANCE *Ultra* pERK assay, but not when using the competitor assay (Table 1). It should be noted that this experiment was conducted at a cell seeding density of 50k and 5k cells for the LANCE *Ultra* pERK assay and competitor assay, respectively.

Table 1: pERK measured in CHO-K1 stably expressing A_1R following stimulation and varying concentrations of PMA, NECA or adenosine.

	pEC_{50} [a]	E_{max} [b]	Basal[c]	Span[d]
LANCE *Ultra* pERK1/2 kit				
PMA	8.24 ±0.1*			
NECA	8.66 ±0.1	21.13 ±1.0	1.32 ±0.8	19.81 ±1.2
Adenosine	8.14 ±0.1*	16.90 ±0.8	1.33 ±0.8	15.57 ±1.2
Competitor pERK1/2 kit				
PMA	8.77 ±0.1*			
NECA	8.41 ±0.1	37.99 ±1.1	2.33 ±1.2	35.66 ±1.5
Adenosine	8.21 ±0.1	35.34 ±1.6	1.43 ±2.1	33.91 ±2.5

[a] Negative logarithm of compound concentration required to produce a half-maximal response.

[b] Maximum pERK in response to compound stimulation; The upper plateau of the fitted sigmoidal dose response curve.

[c] The lower plateau of the fitted sigmoidal dose response curve corresponding to 0% of 1 µM PMA.

[d] The difference between Emin and basal signaling.

Statistical significance *(*, p< 0.1;**, p<0.01;***, p<0.001)* compared to NECA stimulation was determined by one way ANOVA with Dunnett's post-test.

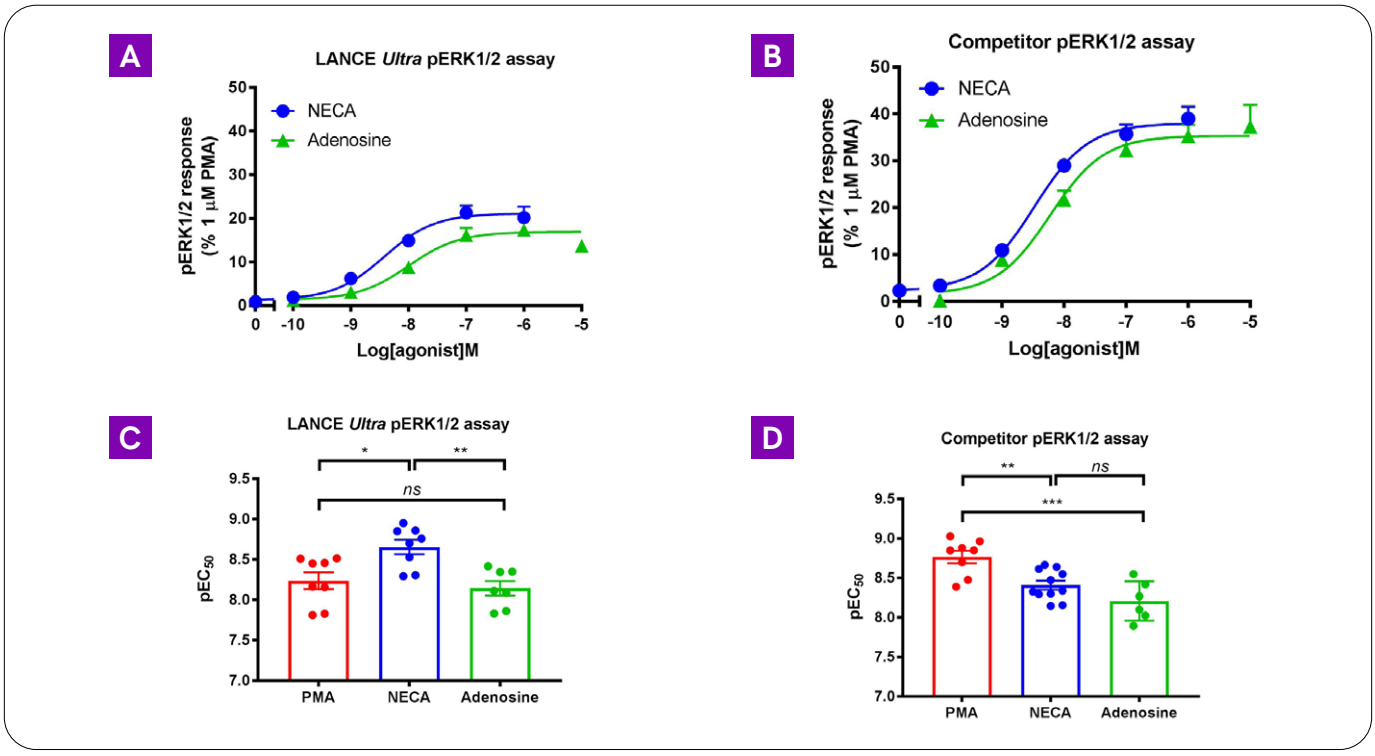


Figure 7: **Assessing assay sensitivity**. pERK concentration dose-response curves for A1R agonist (NECA and adenosine) **(A, B)** and PMA (not shown) were measured in CHO-K1 A_1R cells seeded at the cell density (50k cells/well for the LANCE *Ultra* pERK1/2 kit and 5k cells/well for the competitor assay) following stimulation for five minutes at various agonist concentrations pEC_{50} values, as determined from these dose-response curves, are shown for PMA, NECA and adenosine **(C, D)**. Data were analysed using a three-parameter logistic equation and expressed as % PMA (1 µM). Statistical significance *(*, p< 0.1;**, p<0.01;***, p<0.001)* between pEC_{50} values was determined by one-way ANOVA with Dunnett's post-test.

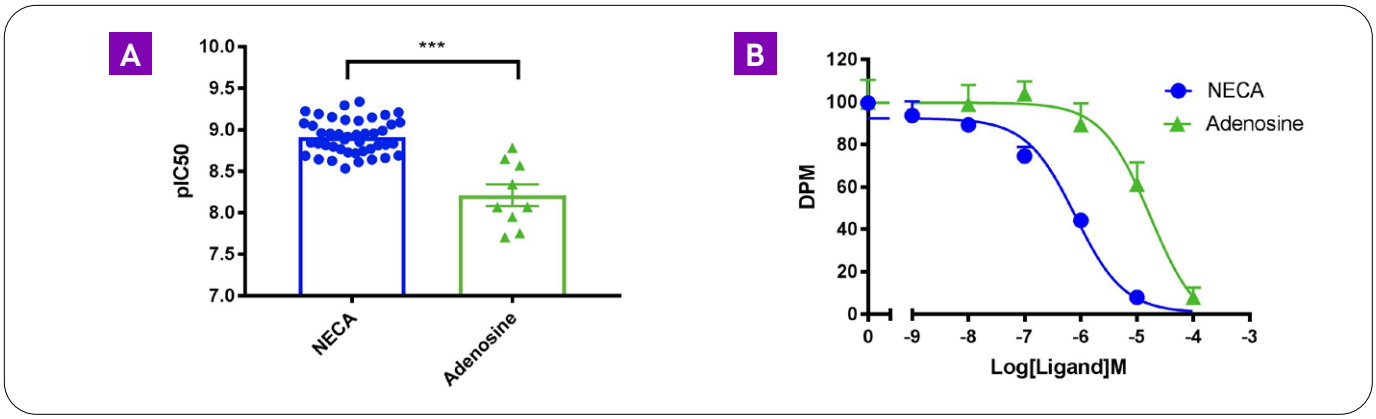


Figure 8: **Adenosine shows reduced potency for cAMP accumulation and reduced binding at the A1R when compared to NECA. A)** pEC_{50} values as taken from cAMP accumulation concentration dose-response curves for A_1R agonist (NECA and adenosine) measured in CHO-K1 A_1R cells seeded at 2k cells per well. B) Radioligand displacement assays conducted using crude membrane preparations (100 µg protein per tube) acquired from homogenization of CHO-K1 cells expressing human A_1 receptors. The ability to displace binding of the A1R selective antagonist radioligand, 1,3-[3H]-dipropyl-8-cyclopentylxanthine ([3H]-DPCPX) at a concentration (1 nM) by increasing concentrations of NECA and adenosine, allowed the binding affinities (K_i) to be determined as 6.36 ±0.1 and 5.02 ±0.1, respectively.

Conclusion

Whilst the overall A_1R-mediated pERK signal detected using the competitor assay appeared greater at lower cell numbers (in our hands at least), the maximum range of the assay was reached at only 5k cells. We find the LANCE *Ultra* pERK kit offers the ability to vary cell number whilst remaining in the dynamic range of the assay and offers increased reproducibility. Indeed, it appears the LANCE *Ultra* pERK kit offers an enhanced sensitivity at determining potency differences between agonists of GPCRs, as exemplified by our studies using NECA and adenosine. This conclusion may of course depend on the cell line and the receptor under investigation.

References

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