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ASSAY PROCESS

We use functional GPCR assays to characterize compounds by measuring second messenger levels as a consequence of GPCR activation or inhibition; receptors coupled to G_s and G_i can be investigated using cAMP assays. Compounds acting at receptors coupled to G_q can be assessed by measuring intracellular calcium. Signalling pathway activation can additionally be monitored by measuring IP1, IP3 and phospho-ERK.

Sample Preparation

For live cell assays, the appropriate cell line endogenously expressing the receptor of interest, or transfected cells, are cultured. Standard culture conditions are to maintain cells in T-150 vented flasks at 37°C, 5% CO₂. Cell culture medium (DMEM containing 4.5g/l glucose, L-Glutamine 4 mM, 10% foetal bovine serum) is changed every 2-3 days and cells are passaged at confluency. Cells are seeded into 24 well or 96 well plates as required, or used directly in assays.

If membrane preparation is required, frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50 mM Tris-HCl, 5 mM MgCl₂, 5 mM EDTA, protease inhibitor cocktail). After a low-speed spin (100 x g for 3 minutes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 17,000 x g for 10 minutes at 4 °C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed for a second time, again at 4 °C. The pellet is then resuspended into buffer (15 ml) containing 10% sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay.

On the day of the assay, membrane preparations or live cells are diluted to the appropriate concentration/cell number in PBS or assay buffer.

cAMP ASSAY

Where a GPCR is coupled to G_s , activation by an agonist leads to adenylyl cyclase stimulation and cAMP production. However, if the GPCR is coupled to G_i , inhibition of cAMP production occurs. The effect of compounds upon GPCR-mediated adenylyl cyclase stimulation can thereby be assessed by measuring intracellular cAMP.

We use a homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay to measure cAMP production (LANCE Ultra cAMP assay, PerkinElmer). A europium labelled cAMP tracer competes with cAMP in the test sample for binding sites on a cAMP-specific ULight™-labelled antibody. Where antibodies are bound to Eu-labelled cAMP tracer, excitation at 320 or 340 nm causes excitation of the Eu molecule and the excitation energy emitted causes excitation of the ULight™ molecule on the antibody by FRET. The resulting emission at 665 nm can be measured. When unlabelled cAMP present in the sample competes with the EU-labelled cAMP tracer for antibody binding sites, a decrease in TR-FRET signal occurs.

cAMP Assay - Optimization

A cAMP standard curve is run on every assay plate (96 well or 384 well plates) to determine the dynamic range of the assay and to allow interpolation of data generated from samples to a concentration of cAMP.

The cAMP standard at 50 µM is prepared at 4X concentrations and serially diluted in fresh assay stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4) for final concentrations in the assay plate of 1×10^{-6} to 1×10^{-12} M. For a 96-well plate assay, 10 µl of 4X cAMP standard is added to the wells in triplicate. 10 µl of stimulation buffer is added, and the plate incubated at RT for 30 mins. Eu-cAMP tracer solution is prepared by diluting 1 in 50 in assay detection buffer and 10 µl is added to each assay well. ULight™ -anti-cAMP solution is prepared by diluting 1 in 150 in detection buffer and 10 µl is added to each assay well. The plate is then sealed with a top seal and incubated at RT for 1 hour before reading at 320 nm excitation and 665 nm emission on a microplate reader (PheraStar, BMG Labtech). An example cAMP curve is shown in Figure 1.

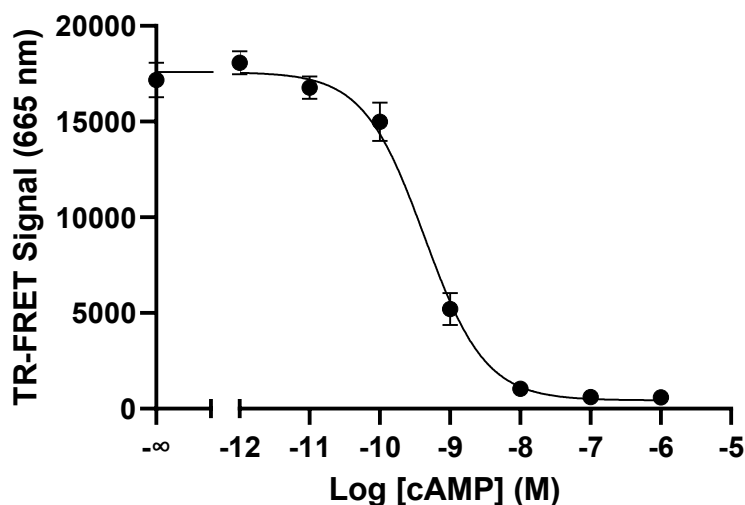


Figure 1: Representative cAMP standard curve. Data generated by incubating LANCE cAMP Ultra kit (PerkinElmer) cAMP standard and reagents for 1 hour at RT in a white 96-well Optiplate. Plate read at 320 nm excitation, 665 nm emission using a PheraStar microplate reader (BMG Labtech).

Typical assay optimization involves stimulating cells with a test compound at a range of cell densities to identify the condition with the highest signal to background ratio within the dynamic range of the cAMP standard curve. This can be assessed by stimulating cells at differing numbers per well with forskolin to directly act on adenylyl cyclase to produce cAMP. The maximum amount of cAMP that the cells are able to produce can be determined by stimulating the cells with a saturating concentration of forskolin.

Forskolin is prepared at 2X concentrations and serially diluted in fresh assay stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4) for final concentrations in the assay plate of 1×10^{-4} to 1×10^{-10} M. For a 96-well plate assay, 10 μ l of 2X forskolin is added to the wells in triplicate. Cells are counted using a haemocytometer, and the required cell number is centrifuged at 100 x g for 5 mins, and the pellet resuspended in stimulation buffer for 10 μ l cell suspension at the target density per well. Upon addition of 10 μ l cell suspension to the forskolin-containing wells, the plate incubated at RT for 30 mins. Eu-cAMP tracer solution is prepared by diluting 1 in 50 in assay detection buffer and 10 μ l is added to each assay well. ULight™ -anti-cAMP solution is prepared by diluting 1 in 150 in detection buffer and 10 μ l is added to each assay well. The plate is then sealed with a top seal and incubated at RT for 1 hour before reading at 320 nm excitation and 665 nm emission on a microplate reader (PheraStar, BMG Labtech). An example forskolin dose-response curve is illustrated in Figure 2.

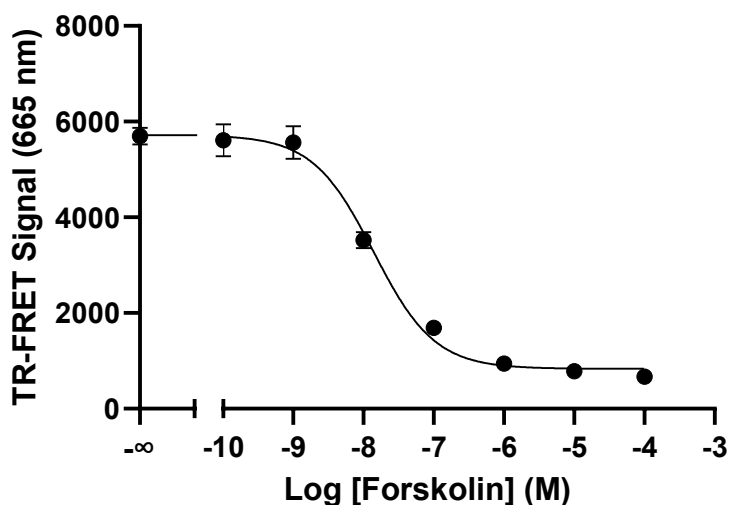


Figure 2: Representative forskolin dose-response curve for HEK293T cells at 3000 cells per well. Data generated by incubating cells with increasing concentrations of forskolin for 30 mins before incubating with LANCE cAMP Ultra kit (PerkinElmer) reagents for 1 hour at RT in a white 96-well Optiplate. Plate read at 320 nm excitation, 665 nm emission using a PheraStar microplate reader (BMG Labtech).

cAMP Assay - Test Compounds

Once the required cell density has been determined, dose-response assays can then be conducted by stimulating cells with the test compounds. A typical assay to determine agonist potency consists of preparing the compounds at 2X concentrations before serially diluting in fresh assay stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4) for final concentrations in the assay plate of 1×10^{-4} to 1×10^{-11} M. For a 96-well plate assay, 10 μ l of 2X compound is added to the wells in triplicate. Cells are counted using a haemocytometer, and the required cell number is centrifuged at 100 x g for 5 mins, and the pellet resuspended in stimulation buffer for 10 μ l cell suspension at the target density per well. Upon addition of 10 μ l cell suspension to the compound-containing wells, the plate incubated at RT for 30 mins. Eu-cAMP tracer solution is prepared by diluting 1 in 50 in assay detection buffer and 10 μ l is added to each assay well. ULight™ -anti-cAMP solution is prepared by diluting 1 in 150 in detection buffer and 10 μ l is added to each assay well. The plate is then sealed with a top seal and incubated at RT for 1 hour before reading at 320 nm excitation and 665 nm emission on a microplate reader (PheraStar, BMG Labtech).

cAMP Assay - Test Compound Data Analysis

Data output for each plate comprises a dose-response curve for a cAMP standard run in parallel and the dose-response curve for the cells stimulated with the test compounds, in addition to a saturating concentration of forskolin to determine the maximum cAMP produced by the cells. Data is analysed using non-linear curve fitting routines, interpolation and normalization transformations using Prism (GraphPad Software Inc). The resulting TR-FRET data for the compounds is interpolated to the cAMP standard curve and can then be transformed to concentrations of cAMP. Alternatively, data can be expressed as a percentage of the maximum amount of cAMP that the cells are capable of producing when treated with a saturating concentration of forskolin, and EC₅₀ or IC₅₀ values can be derived (Figure 3).

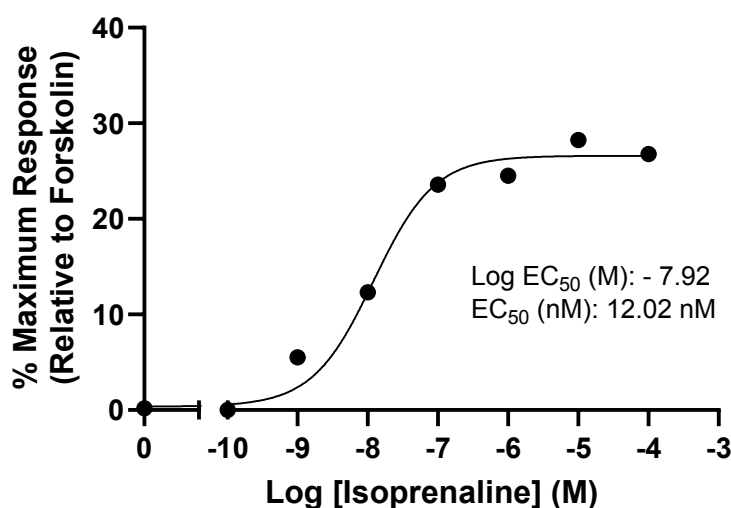


Figure 3: Example dose-response curve for HEK293T cells at 3000 cells per well stimulated by the beta adrenoreceptor agonist isoprenaline. Data generated by incubating cells with increasing concentrations of isoprenaline for 30 mins before incubating with LANCE cAMP Ultra kit (PerkinElmer) reagents for 1 hour at RT in a white 96-well Optiplate. Plate read at 320 nm excitation, 665 nm emission using a PheraStar microplate reader (BMG Labtech). Data was interpolated to a cAMP standard curve run in parallel and normalized relative to forskolin.