cAMP Accumulation Assays Using the AlphaScreen® Kit (PerkinElmer)

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[Abstract] Cyclic adenosine monophosphate (cAMP) is an intracellular signaling messenger derived from the catalytic conversion of ATP, and is a major product of activated G_s protein-coupled receptors. Conversely, formation of cAMP is inhibited by G_i protein-coupled receptors. This protocol has been optimized for the detection of ligand-mediated cAMP accumulation in adherent immortal cell lines expressing G_s-coupled receptors.

Materials and Reagents

1. Sterile 96-well clear flat bottom plates (BD Biosciences, Falcon®, catalog number: 353072)
2. Phenol Red free Dulbecco's modified eagle medium (DMEM) (Life Technologies, Gibco®, catalog number: 21063-029)
3. Bovine Serum Albumin (BSA) (Sigma-Aldrich, catalog number: A7906)
4. 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, catalog number: I5879)
5. Tween-20 (Sigma-Aldrich, catalog number: P2287)
6. HEPES (Life Technologies, Gibco®, catalog number: 11344-041)
7. 100% Ethanol
8. Milli-Q H_2O
9. Alphascreen® cAMP Assay Kit (PerkinElmer, catalog number: 6760625)
10. White OptiPlate-384 well microplate (PerkinElmer, catalog number: 6007290)
11. TopSeal (PerkinElmer, catalog number: 6005250)
12. Stimulation buffer (see Recipes)
13. Lysis buffer (see Recipes)
14. Acceptor buffer (see Recipes)
15. Donor buffer (see Recipes)

Equipment

1. Fusion-α plate reader or Envision plate reader with appropriate Alphascreen modules (PerkinElmer)

2. Humidified incubator
3. Multichannel pipettes
4. Micropipettes
5. Benchtop centrifuge
6. Oven
7. Orbital shaker

Procedure

Notes:

1. *Cells can either be stably or transiently expressing receptor of interest.*
2. *All incubations are in a humidified environment at 37 °C, 5% CO_2 unless otherwise indicated.*

A. Cell preparation

Seed cells in suitable nutrient media (*e.g.,* DMEM, 10% FBS, no antibiotics) into a sterile 96-well plate and incubate in a humidified environment at 37 °C, 5% CO_2 to be ~90% confluent the following day (~24 h).

Note: Optimization for cell number depending on the cell line used will be necessary (we suggest a starting range of 10,000-50,000 cells/well). Recommended density for CHO FlpIN cells is 30,000 cells/well.

B. Stimulation

1. The day following seeding, aspirate nutrient media and replace with 90 µl pre-warmed Stimulation buffer.
2. Incubate in a humidified environment at 37 °C, 5% CO_2 for 30 min.
   Note: Do not leave in cells in Stimulation buffer for longer than 2 h prior to stimulation.
3. Prepare serial dilutions of ligands at 10x final concentration in Stimulation buffer, enough for 10 µl/well, to be performed in duplicate (minimum).
   Note: Concentration range to use will depend on ligand affinity for receptor. For initial tests, select a top concentration 100x K_d of ligand, a buffer only control, and several concentrations between these. The range can then be refined in subsequent experiments.
4. Prepare 10x appropriate concentration of forskolin in Stimulation buffer.
   Note: This is the internal control for the experiment – forskolin is an activator of adenylate cyclase, enhancing the formation of cAMP. Recommended final concentration of forskolin in a CHO FlpIN cell line is 100 µM.
5. Following 30 min incubation in stimulation buffer, add 10 µl of 10x prepared ligands to

cells, for a total volume of 100 µl, 1x final concentration.

6. Incubate cells with ligand in a humidified environment at 37 °C, 5% CO_2 for 30 min.
   Note: Optimization for assay time will be necessary. Recommended initial stimulation is 30 min.
7. After 30 min, rapidly remove ligand containing media from cells.
   Note: Depending on the cell type, this may involve flicking or gentle aspiration.
8. Add 50 µl ice cold 100% ethanol to cells.
9. Allow ethanol to evaporate at room temperature (RT) or in a 37 °C oven.
   Note: Ensure the ethanol is completely evaporated before proceeding to the next step.
10. Add 75 µl Lysis buffer.
    Note: Optimization for lysis volume will be necessary, and depends on the cell type, expression level of the receptor and efficiency of coupling to the cAMP pathway. Recommended starting lysis volume in a CHO FlpIN cell line is 75 µl.
11. Incubate lysates at RT for 5-10 min on an orbital shaker.

C. Detection

1. In reduced lighting conditions, prepare detection reagents (Acceptor and Donor buffers).
2. Transfer 10 µl of cell lysate to a 384-well OptiPlate.
3. Prepare cAMP standard curve in Lysis buffer, enough for 10 µl/well to be performed in duplicate (minimum).
4. Transfer 10 µl cAMP standard curve to a 384-well OptiPlate.
5. Briefly centrifuge to draw contents to the bottom of the wells.
6. In reduced lighting conditions, add 5 µl Acceptor buffer to every well (samples and standard curve).
7. In reduced lighting conditions, add 15 µl Donor buffer to every well (samples and standard curve) (following preincubation for 30 min).
8. Seal the plate with TopSeal and wrap in foil.
   Note: Small volumes are subject to evaporation, TopSeal is essential.
9. Briefly centrifuge to draw contents to the bottom of the wells.
10. Incubate overnight at RT (8-12 h) in reduced lighting conditions.
11. Briefly centrifuge to draw contents to the bottom of the wells.
12. Analyse luminescence on a Fusion-α or Envision plate reader using standard α-screen settings.

D. Data analysis

1. Extrapolate data from the cAMP standard curve. Ideally, data should lie on the linear section of the cAMP standard curve.

a. If data falls off the bottom end of the curve (*i.e.*, high concentration of cAMP), dilute lysates further with Lysis buffer and repeat detection component of protocol.
b. If data falls off the top end of the curve (*i.e.*, low concentration of cAMP), assay should be performed again and cells lysed with a lower volume of Lysis buffer.

2. Normalize data to forskolin control.

Recipes

1. Stimulation buffer (pH 7.4, incubate at 37 °C prior to use)
   Phenol free DMEM
   0.1% w/v BSA*
   1 mM 3-isobutyl-1-methylxanthine (IBMX)**
   * BSA is not essential, but is recommended for 'sticky' ligands, *i.e.,* peptides.
   ** IBMX is a potent phosphodiesterase inhibitor. IBMX powder should be made up as a 500 mM stock in 100% DMSO. It may precipitate out of solution if DMEM is too cold, so gently heat and stir DMEM when adding IBMX.
2. Lysis buffer (pH 7.4)
   Milli-Q H_2O
   0.3% Tween 20
   5 mM HEPES
   0.1% w/v BSA
3. Acceptor buffer (prepare in Stimulation buffer)
   1% Acceptor beads (10 U/µl) (mix gently by pipetting before use)
4. Donor buffer (prepare in Stimulation buffer)***
   0.3% Donor Beads (10 U/µl) (mix gently by pipetting before use)
   0.025% biotinylated cAMP (133 U/µl)
   *** Donor buffer MUST be preincubated at RT for 30 min prior to use.
5. cAMP standard curve
   Prepare cAMP standard dilution series in Lysis buffer at 3x concentration to take into account dilution in detection plate (10 µl cAMP standard, 5 µl Acceptor buffer and 15 µl Donor buffer, final volume 30 µl). Recommended concentration range for cAMP standard (final) is 10 µM - 1 pM in half log units.
   Note: Lysates may be stored at -20 °C and cAMP accumulation can be detected at a later time, but no longer than 2 weeks following stimulation.

References

1. Koole, C., Wootten, D., Simms, J., Valant, C., Sridhar, R., Woodman, O. L., Miller, L. J., Summers, R. J., Christopoulos, A. and Sexton, P. M. (2010). Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Mol Pharmacol* 78(3): 456-465.

