GPCR Activity Assay Protocol (Aequorin-GFP System)

Objective:
Measure GPCR activation through calcium-mediated bioluminescence using co-expressed Aequorin-GFP calcium-sensor proteins in HEK293 or CHO-K1 cells expressing GPCR-Gαqi9 constructs.

Materials & Reagents

Cell Culture

- HEK293 or CHO-K1 cells co-expressing:
  - GPCR of interest
  - Gαqi9 chimera (to direct GPCR coupling to calcium signaling)
  - Aequorin-GFP fusion protein
- Appropriate cell culture medium (e.g., DMEM/F12, 10% FBS, antibiotics)

Assay Reagents

- Coelenterazine-h (Aequorin substrate; stock solution typically 1 mM in ethanol, protected from light)
- HBSS buffer (Hank's Balanced Salt Solution, with calcium and magnesium)
- HEPES (20 mM, pH 7.4)
- Bovine serum albumin (BSA, fatty acid-free; 0.1%)
- Ligands (test compounds, positive agonists, and negative controls)

Equipment

- Luminometer capable of injection and simultaneous detection (e.g., Berthold Mithras, PerkinElmer EnVision, FlexStation, or similar systems)
- White opaque 96- or 384-well plates suitable for luminescence detection

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Detailed Protocol

1. Cell Preparation and Plate Seeding (Day before assay)

- Grow HEK293 or CHO-K1 cells co-expressing GPCR, Gαqi9, and Aequorin-GFP until approximately 70–80% confluent.

- Harvest cells, count, and seed into white, opaque-bottom assay plates at optimized densities:
    - **96-well plates:** 30,000–50,000 cells/well
    - **384-well plates:** 10,000–20,000 cells/well
- Incubate plates overnight (37°C, 5% CO_2).

2. Loading Cells with Coelenterazine-h (Day of assay)

- Prepare Coelenterazine-h working solution:
    - Dilute the 1 mM stock to **5 µM final** in assay buffer (HBSS + 20 mM HEPES + 0.1% BSA, pH 7.4).
    - Protect solution from light.
- Gently remove culture medium from assay plates and replace with Coelenterazine-h solution (100 µL/well in 96-well plates, ~30 µL/well in 384-well plates).
- Incubate cells protected from light:
    - **1–2 hours at room temperature (optimal for stable signal generation).** *(Optionally: 37°C incubation can shorten loading time but signal might decay more rapidly.)*

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3. Luminescent Measurement (Aequorin-GFP assay)

Preparation of Ligands

- Prepare ligands at 2× or 3× final concentration in assay buffer for injection.
- Include:
    - Positive agonist (known activator)
    - Vehicle control (assay buffer or vehicle alone)
    - Test ligands at serial dilutions for dose-response curves

Measurement using Luminometer

- Load assay plate into luminometer.
- Record baseline luminescence for 5–10 sec.
- Automatically inject ligand (typically 25–50 µL/well in 96-well, or ~10 µL/well in 384-well).
- Immediately measure luminescence for **30–60 seconds** after injection, capturing peak luminescence values.

Recommended instrument settings:

- Injection speed: Medium-fast
- Measurement interval: 0.5–1 sec
- Integration time per reading: 0.5–1 sec

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4. Data Analysis

- Determine the peak luminescence (RLU, relative luminescence units) after ligand injection.
- Calculate fold induction:

  Fold induction=RLU (ligand)RLU (vehicle control)\text{Fold induction} = \frac{\text{RLU (ligand)}}{\text{RLU (vehicle control)}}Fold induction=RLU (vehicle control)RLU (ligand)

- Plot dose-response curves (Fold induction vs. ligand concentration) and calculate EC_{50} using non-linear regression (e.g., GraphPad Prism).

Critical Tips and Considerations

- **Stable vs. Transient Expression:**
  Stable cell lines generally yield more consistent results than transient transfections, especially important for high-throughput screening.
- **Coelenterazine Handling:**
  Coelenterazine-h is light-sensitive and should always be handled and incubated in dim light or darkness.
- **Cell Health:**
  Avoid over-confluence or stressed cells; optimal density and health strongly influence assay reproducibility.
- **Controls:**
  Always include known agonists and negative (vehicle-only) controls.
- **Optimization:**
  Cell density, coelenterazine concentration, incubation duration, and ligand injection volumes should be optimized for best signal-to-noise ratio.