Assay Protocol for Endogenous GPCR Activation in Human HepG2 Cells Expressing AEQ-GFP in Mitochondria (cAP-0053AEQ-GFP-Mito)

Purpose

This protocol describes a **bioluminescence-based calcium mobilization assay** to measure endogenous GPCR activation in **HepG2 cells stably expressing mitochondrial-targeted AEQ-GFP (cAP-0053AEQ-GFP-Mito)** using **aequorin reconstitution with coelenterazine**.

Materials Required

1. **Cells:** HepG2 cells expressing AEQ-GFP in mitochondria (cAP-0053AEQ-GFP-Mito)
2. **Culture Medium:** Universal Growth Medium (cAP-1B)
3. **GPCR Ligands:** Based on commonly expressed GPCRs in HepG2 (see list below)
4. **Coelenterazine h (or native coelenterazine):** For reconstitution of aequorin
5. **HBSS (Hanks' Balanced Salt Solution) + 0.1% BSA (assay buffer)**
6. **$CaCl_2$ (if performing in low-calcium conditions before Ca^{2+} reintroduction)**
7. **Luminometer (e.g., Berthold MicroLumat LB 96P or similar plate reader)**
8. **White 96-well or 384-well microplates (luminescence-compatible)**
9. **Trypsin/EDTA (for cell detachment)**
10. **Probenecid (optional, to prevent dye leakage if needed)**

Procedure

Day 1: Cell Seeding

1. **Thaw and Culture Cells:**
    - Recover cells from liquid nitrogen and culture in **Universal Growth Medium (cAP-1B)** at **37°C, 5% CO_2** until ~80% confluent.
    - Passage cells using **trypsin/EDTA** as needed.
2. **Seed Cells for Assay:**
    - Detach cells and seed in a **white 96-well plate** at **50,000–80,000 cells/well** in **100 µL growth medium**.
    - Allow cells to adhere overnight (~16–24 hr).

Day 2: Aequorin Reconstitution & GPCR Stimulation

1. **Prepare Assay Buffer:**
    - **HBSS + 0.1% BSA** (pH 7.4).
    - (Optional) Add **5 mM probenecid** to prevent dye leakage.
2. **Reconstitute Aequorin with Coelenterazine h:**
    - Dilute **coelenterazine h** in assay buffer to **5 µM** (final concentration).
    - Remove growth medium and add **100 µL/well of coelenterazine h solution**.
    - Incubate **2–4 hr at 37°C** (or **overnight at RT** for better signal stability).
3. **Prepare GPCR Ligands:**
    - Dilute ligands in assay buffer at **10X final desired concentration** (accounting for 1:10 dilution upon addition to cells).
    - Example concentrations for HepG2-expressed GPCRs:
        - **ATP (P2Y receptors):** 1–10 µM
        - **Lysophosphatidic acid (LPA1–3 receptors):** 100 nM–1 µM
        - **Endothelin-1 (ETAR/ETBR):** 10–100 nM
        - **Bradykinin (B1/B2 receptors):** 100 nM–1 µM
        - **Prostaglandin E2 (EP1–4 receptors):** 100 nM–1 µM
        - **Acetylcholine (muscarinic receptors, e.g., M3):** 1–10 µM
        - **Thrombin (PAR1/PAR4 receptors):** 0.1–1 U/mL
4. **Run the Assay (Luminometer Setup):**
    - Pre-equilibrate plate at **37°C** for 10 min.
    - Set up luminometer to inject **10 µL of 10X ligand** per well (total volume = 110 µL).
    - Measure luminescence **immediately after ligand addition** (1–2 sec intervals for 30–60 sec).

Data Analysis

- **Peak Luminescence:** Measure maximum signal after ligand addition.
- **Area Under the Curve (AUC):** Integrate signal over time for total response.
- **Normalization:** If needed, normalize to baseline or positive control (e.g., ionomycin/Ca^{2+} ionophore for maximum response).

Commonly Expressed GPCRs in HepG2 Cells

HepG2 cells express a variety of endogenous GPCRs, including:

- **Purinergic receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14)** – Respond to ATP/ADP/UTP
- **Lysophosphatidic acid receptors (LPA1–3)** – Respond to LPA
- **Endothelin receptors (ETAR, ETBR)** – Respond to endothelin-1
- **Bradykinin receptors (B1, B2)** – Respond to bradykinin
- **Prostanoid receptors (EP1–4, FP, IP, TP)** – Respond to prostaglandins (PGE2, PGF2α)
- **Muscarinic acetylcholine receptors (M3, M5)** – Respond to acetylcholine
- **Protease-activated receptors (PAR1, PAR4)** – Respond to thrombin
- **Sphingosine-1-phosphate receptors (S1PR1–3)** – Respond to S1P
- **Chemokine receptors (CXCR4, CCR2, CCR5)** – Respond to chemokines (CXCL12, CCL2)

Troubleshooting

- **Low Signal:** Increase coelenterazine incubation time or concentration.
- **High Baseline Noise:** Reduce coelenterazine exposure to light (light-sensitive).
- **No Response:** Verify GPCR expression in HepG2; test positive control (e.g., ATP or ionomycin).

Conclusion

This protocol allows functional assessment of **endogenous GPCR activation** in **HepG2 cells (cAP-0053AEQ-GFP-Mito)** via **mitochondrial Ca^{2+}-dependent aequorin luminescence**.