

Assay Protocol for Endogenous GPCR Activation in Human Liver Stellate Cells (HLiStCs) Expressing AEQ-GFP in Mitochondria (cAP-0035AEQ-GFP-Mito)

Purpose

This protocol describes a **functional bioluminescence assay** to measure endogenous GPCR activation in **human liver stellate cells (HLiStCs) stably expressing mitochondrial-targeted AEQ-GFP (cAP-0035AEQ-GFP-Mito)** using **aequorin-based calcium mobilization**.

Materials Required

1. **Cells:** HLiStCs expressing AEQ-GFP in mitochondria (cAP-0035AEQ-GFP-Mito)
 2. **Culture Medium:** Stellate-Growth Medium (cAP-31)
 3. **GPCR Ligands:** Based on commonly expressed GPCRs in HLiStCs (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. **CaCl₂ (if performing in low-calcium conditions before Ca²⁺ 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)**
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Procedure

Day 1: Cell Seeding

1. **Thaw and Culture Cells:**
 - Recover cells from liquid nitrogen and culture in **Stellate-Growth Medium (cAP-31)** at **37°C, 5% CO₂** 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).
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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 HLiStC-expressed GPCRs:
 - **Endothelin-1 (ETAR/ETBR):** 10–100 nM
 - **Angiotensin II (AT1R):** 100 nM–1 μM
 - **Lysophosphatidic acid (LPA1–3 receptors):** 100 nM–1 μM
 - **Thrombin (PAR1/PAR4 receptors):** 0.1–1 U/mL
 - **ATP (P2Y receptors):** 1–10 μM
 - **Sphingosine-1-phosphate (S1PR1–3):** 100 nM–1 μM
 - **Prostaglandin E2 (EP1–4 receptors):** 100 nM–1 μM

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/Ca²⁺ ionophore for maximum response).

Commonly Expressed GPCRs in Human Liver Stellate Cells (HLiStCs)

HLiStCs express a variety of endogenous GPCRs, including:

- **Endothelin receptors (ETAR, ETBR)** – Respond to endothelin-1
 - **Angiotensin II receptor (AT1R)** – Respond to angiotensin II
 - **Lysophosphatidic acid receptors (LPA1–3)** – Respond to LPA
 - **Purinergic receptors (P2Y1, P2Y2, P2Y6, P2Y12, P2Y13, P2Y14)** – Respond to ATP/ADP/UTP
 - **Protease-activated receptors (PAR1, PAR4)** – Respond to thrombin
 - **Sphingosine-1-phosphate receptors (S1PR1–3)** – Respond to S1P
 - **Prostanoid receptors (EP1–4, FP, IP, TP)** – Respond to prostaglandins (PGE2, PGF2 α)
 - **Chemokine receptors (CXCR3, CXCR4, CCR5, CCR2)** – Respond to chemokines (CXCL10, CXCL12, CCL5, CCL2)
 - **Cannabinoid receptors (CB1, CB2)** – Respond to endocannabinoids (anandamide, 2-AG)
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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 HLiStCs; test positive control (e.g., ATP or ionomycin).
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Conclusion

This protocol allows functional assessment of **endogenous GPCR activation** in **HLiStCs (cAP-0035AEQ-GFP-Mito)** via **mitochondrial Ca²⁺-dependent aequorin luminescence**.