

Assay Protocol for Endogenous GPCR Activation in Human Aortic Smooth Muscle Cells (AoSMCs) Expressing AEQ-GFP in Mitochondria (cAP-0026AEQ-GFP-Mito)

Purpose

This protocol describes a **functional bioluminescence assay** to measure endogenous GPCR activation in **human adult aortic smooth muscle cells (AoSMCs) stably expressing mitochondrial-targeted AEQ-GFP (cAP-0026AEQ-GFP-Mito)** using **aequorin-based calcium mobilization**.

Materials Required

1. **Cells:** Human AoSMCs expressing AEQ-GFP in mitochondria (cAP-0026AEQ-GFP-Mito)
 2. **Culture Medium:** SMCs Growth Medium (cAP-24)
 3. **GPCR Ligands:** Based on commonly expressed GPCRs in AoSMCs (see list below)
 4. **Coelenterazine h (or native coelenterazine):** For reconstitution of aequorin (5 μ M final concentration)
 5. **Assay Buffer:** HBSS + 0.1% BSA (pH 7.4)
 6. **CaCl₂ (for calcium reintroduction if needed)**
 7. **Luminometer (e.g., Berthold MicroLumat LB 96P or equivalent)**
 8. **White 96-well or 384-well microplates (luminescence-compatible)**
 9. **Trypsin/EDTA (for cell detachment)**
 10. **Probenecid (optional, 5 mM, to prevent dye leakage)**
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Procedure

Day 1: Cell Seeding

1. **Thaw and Culture Cells:**
 - Recover cells from liquid nitrogen and culture in **SMCs Growth Medium (cAP-24)** 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 AoSMC-expressed GPCRs:**
 - **Endothelin-1 (ETAR/ETBR):** 10–100 nM
 - **Angiotensin II (AT1R):** 100 nM–1 µM
 - **Norepinephrine (α1-AR, β2-AR):** 1–10 µM
 - **Thrombin (PAR1/PAR4):** 0.1–1 U/mL
 - **Serotonin (5-HT2A/2B receptors):** 1–10 µM
 - **ATP (P2Y receptors):** 1–10 µM
 - **Prostaglandin F2α (FP receptor):** 100 nM–1 µM
 - **Sphingosine-1-phosphate (S1PR1–3):** 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).
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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).
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Commonly Expressed GPCRs in Human AoSMCs

AoSMCs express a variety of endogenous GPCRs, including:

- **Endothelin receptors (ETAR, ETBR)** – Vasoconstriction (ET-1)
 - **Angiotensin II receptor (AT1R)** – Vasoconstriction (Ang II)
 - **Adrenergic receptors (α 1-AR, β 2-AR)** – Norepinephrine-mediated contraction/relaxation
 - **Protease-activated receptors (PAR1, PAR4)** – Thrombin-induced proliferation
 - **Serotonin receptors (5-HT2A, 5-HT2B)** – 5-HT-mediated vasoconstriction
 - **Purinergic receptors (P2Y1, P2Y2, P2Y6, P2Y12)** – ATP/ADP-mediated signaling
 - **Prostanoid receptors (FP, TP, EP1–4)** – $\text{PGF}_2\alpha$ /TXA2/PGE2 signaling
 - **Sphingosine-1-phosphate receptors (S1PR1–3)** – S1P-mediated migration/proliferation
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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 AoSMCs; test positive control (e.g., ATP or ionomycin).
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Conclusion

This protocol allows functional assessment of **endogenous GPCR activation** in **AoSMCs (cAP-0026AEQ-GFP-Mito)** via **mitochondrial Ca^{2+} -dependent aequorin luminescence**.