ATP release assay from cultured cells

***For determining absolute concentrations of ATP released from cultured adherent cells *in vitro*.**

Equipment/Reagents:

- Adherent cells (primary or cell line)
- Appropriate cell culture media
- Sterile culture dishes (24-well)
- Fibronectin/poly-L-lysine/gelatin
- BSA
- 1x PBS
- ARL 67156/ecto-ATPase inhibitor (Tocris)
- ATP bioluminescence assay kit HSII (Roche)

Protocol:

***Throughout the protocol it is essential to minimize agitation of the plates to avoid mechanical stimulation of the cells and subsequent ATP release into the media.**

Day 1:

1. Coat sterile culture dishes with fibronectin/poly-L-lysine/gelatin (depending on your cell type) for 30 minutes under a sterile cell culture hood.
   ***I find that this helps with keeping the cells adherent to the plate throughout the assay protocol.**
2. Wash the coated plates with 1x PBS ***three*** times and place under UV light for 10 minutes.
3. Plate adherent cells at an appropriate seeding density to achieve confluency in 24 hours. If you are transfecting cells seed the appropriate number of cells to achieve confluency by the end of your transfection protocol.
   ***For cultured human endothelial cells I find $7.5x10^4$ per well of a 24-well plate is sufficient.**

Day 2:

4. Wash cells 3x with sterile 1x HBSS using a P-1000 pipetteman to slowly change solutions.

   ***Using vacuum suction to change media pulls come of the cells up from the plate, which you don't want to happen with these assays.**
5. Add sterile basal culture media (or a physiological salt solution) containing 1% BSA in place of FBS/FCS to each well. *FBS will degrade any ATP released from the cells*.

   ***For experiments with cells in 24-well plates I use a final volume of 300µL/well. Determine the volume of ARL 67156, antagonists and/or agonists that you will stimulate your cells with and add the appropriate volume of culture media + 1% BSA to each well of your plate so as to achieve a final volume of 300µL/well.**
6. Incubate cells at 37°C in a humidified cell culture incubator for 30 minutes to allow degradation of any ATP that may have been released into the media during the wash steps.
7. Add 300µM ARL 67156 to each well of your plate to inhibit ecto-nucleotidase activity from your cells.

   ***Extracellular ATP can be rapidly degraded by these enzymes depending on the expression in your cell type.**
8. Incubate cells at 37°C in a humidified cell culture incubator for 30 minutes.
9. Treat your cells with your desired blocker, drug, etc. for an appropriate time at 37°C.*** I typically incubate my blockers at the same time as the ARL.**
10. Stimulate ATP release from your cells by adding the appropriate concentration of your agonist to the desired samples and incubate for the desired stimulation time at 37°C.
11. Collect a sample from each well by removing the extracellular media with a pipetteman using ice cold pipette tips and transfer to sterile microfuge tubes on ice.

    ***For experiments using 24-well plates, I collect 150µL of media from each well.**

12. Centrifuge all samples at 5000x RPM at 4°C to pellet any cells that may have pulled off the plate during sample collection and transfer the supernatant to sterile microfuge tubes on ice.
13. Prepare serial dilutions of ATP standard using the ATP standard supplied in the ATP bioluminescence assay kit HSII (Roche).

    ***I typically make my ATP standards by preparing 2-fold serial dilutions of the ATP standard with my largest standard at 250nM ATP.**
14. Prepare the luciferin:luciferase reagent supplied in the ATP bioluminescence assay kit HSII (Roche) following the manufacturer's instructions.
15. Pipette 50µL of each ATP standard (duplicates) and 50µL of each sample into wells of an opaque 96-well plate.
16. Using a luminometer equipped to read 96-well plates, program the hardware to inject 50µL of the luciferin:luciferase reagent into each well of your plate and to mix and read the luminescence for each sample.

    ***We use a FluoStar Omega luminometer that is equipped with an injection port for direct injection of reagents to each well in the plate. It's important to have a method for rapid acquisition following the addition of the luciferin:luciferase reagent because the HSII kit from Roche has very rapid kinetics with the signal falling off in a matter of minutes.**
17. Create a standard curve for determination of ATP concentrations in your samples using the luminescence values obtained for each of your ATP standards.
18. Calculate the concentration of ATP in each of your samples using the standard curve created in **17**.