***in vivo* ATP:ADP Ratio Assay**

**Adapted from BioChain *in vitro* assay (Catalog # Z5030042)**

**ATP Standard Curve Preparation:**

* Sigma (Cat A6559)
  + This is a 0.1M (100mM) stock solution stored at -20oC
* Remove ATP standard stock and thaw on ice
* Generate the following dilutions

|  |  |  |  |
| --- | --- | --- | --- |
| **[ATP Molar]** | **uL of ddH20** | **uL of ATP Standard** | **uL of Previous Dilution to add** |
| **10-3** | 900uL | 100uL | ---------------- |
| **10-4** | 900uL | ---------------- | 100ul of 10-3 |
| **10-5** | 900uL | ---------------- | 100ul of 10-4 |
| **10-6** | 900uL | ---------------- | 100ul of 10-5 |
| **10-7** | 900uL | ---------------- | 100ul of 10-6 |
| **10-8** | 900uL | ---------------- | 100ul of 10-7 |
| **10-9** | 900uL | ---------------- | 100ul of 10-8 |
| **10-10** | 900uL | ---------------- | 100ul of 10-9 |

**Reagent Preparation:**

* Remove ATP assay buffer, ATP substrate, ATP co-substrate, ATP enzyme and ADP enzyme from -20oC and allow to thaw on ice
* Once thawed, prepare a master mix for ATP reaction
  + Per well of the 96 well plate you will need:
    - 95uL of ATP assay buffer
    - 1uL of ATP substrate
    - 1uL of ATP co-substrate
    - 1uL of ATP enzyme
      * When calculating needs, remember each sample should be run in triplicate and these reagents will also need to be added to the ATP standards!
* Add 90uL of the ATP master mix to the appropriate wells of a COSTAR, white walled, transparent, flat bottom 96 well plate (On plate reader, labeled as COS96)
  + This is so as soon as the samples are processed, they can be added and the assay can begin immediately…
  + In addition, add the appropriate amount of ddH2O to the wells in which sample dilutions are needed…
    - Troubleshooting revealed that for one cortical hemisphere from a P35 mouse, an undiluted and a 1:2 diluted sample will place you in the linear portion of the ATP standard curve
      * Since 100uL of the sample are needed for the reaction, this means that wells designated for the 1:2 diluted samples will have 50uL of ddH2O already added (again so the actual brain sample additions mark the immediate start of the reaction…)

**Other Preparations:**

* All tools for dissection should be cooled in LN2
* All sample tubes, etc should be labeled appropriately and stored on ice

**Tissue Acquisition:**

* Anesthetize animal using appropriate amount of Fatal Plus via ip injection
  + Adult Mouse ~200uL
    - Record amount in anesthetic log
* Check reflexes
* Cervically dislocate and remove brain as quickly as possible
* Cut brain into hemispheres
  + One hemisphere is immediately placed into a 2mL screw cap eppendorf tube and dropped into LN2 (Flash frozen)
    - This sample can be banked and used in future experiments
  + The other hemisphere is further dissected leaving behind only forebrain and mostly cortex
    - This sample is also placed into a 2mL screw cap tube and flash frozen

**Sample Preparation (Done in Cold Room):**

* Place forebrain / cortical sample into a mortar containing LN2 and use the pestal to grind the tissue until a fine powder remains
* Once the LN2 is finished vaporizing, use a spatula to collect the powdered sample and place in an epi tube
* Reconstitute the sample in 600uL of ddH2O
  + This is enough for triplicate undiluted samples (100uL each), 1:2 diluted samples in triplicate (50uL) each AND leftovers so that a protein assay can be carried out so ATP levels can be reported as \_\_\_\_\_ / mg...
* Sonicate the samples at 5 watts for 10 seconds

**ATP Assay:**

* Immediately add the appropriate amount of brain lysate to the 96 well plate (in triplicate)
  + No dilution = add 100uL
  + 1:2 dilution = add 50uL of sample to the wells already containing 50uL of ddH2O
* Once brain sample have been added, add 100uL (in triplicate) of the ATP standards
* Mix the reagents in the plate by tapping
* Incubate the plate for 10 minutes
  + Note: For optimal enzyme action, carry this 10’ incubation out at room temperature in a dark place
* Following incubation, read luminescence in the plate reader with a top down read and the following parameters:
  + Gain = 150
  + Integration time = 1000ms

**ADP Assay:**

* While ATP is being assessed in plate reader, prepare an ADP Master mix
  + For each well of a 96 well plate you will need:
    - 12uL ddH2O
    - 1uL of ADP enzyme
* Once ATP data is saved, add 10uL per well of the ADP master mix
* Tap the plate to mix reagents
* Incubate for 10’ at room temperature
* Read luminescence on plate reader with the exact same parameters as used for the ATP analysis