PROTOCOL FOR

SOIL ATP DETERMINATION

Kuzyakov Lab

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Rev.	**Date**	**Prep.**	**Modify.**	**Check.**	**Approv.**

1. INTRODUCTION

Adenosine triphosphate (ATP) is an important energy compound in the metabolism of all living organisms. The measurement the amount of ATP in biological samples is often based on bioluminescence reaction with luciferin-luciferase, which was first recognized by McElroy (1947).

Soil ATP content is closely correlated with other indices of microbial biomass, and can serve as an independent estimate of microbial biomass content in the soil. The factor most commonly used to convert measured ATP values to total biomass carbon is 250 (Holm-Hansen Karl, 1978).

Problems occur in the extraction of ATP from soil, such as obtaining the maximum release of ATP from living cells, low ATP recovery due to ATP hydrolysis by ATPases in soil and sorption of ATP onto soil colloids.

This protocol is a compilation and modification of protocols 1) developed by Prof. Philip Brookes in the dept. of Prof. Luo Yu and 2) developed in the dept of Prof. R. Joergensen. An acid extraction method (Jenkinson and Oades, 1979 later improved by Tate and Jenkinson, 1982 and Qiu et al., 2016) based on a trichloroacetic acid/phosphate/imidazole (Redmile-Gordon et al, 2011) reagent is used to extract ATP. Mild ultrasonification (at 60% power) is applied to disrupt the microbial cells. The anion (phosphate) and the cation (imidazolium (from Imidazole)) are strongly sorbed on positive and negative soil sites, thus blocking sites, which would otherwise adsorb microbial ATP.

Even using these reagents some ATP may still be adsorbed onto the soil, so it is necessary to calculate the amount recovered. This is done by adding a known amount of ATP to extractant B (see below) and comparing the counts obtained with those from extractant A without added ATP.

2. SCOPE

All staff and students in the lab.

3. SAFETY

1) Any specific health and safety issues relating to soil sample handling should be determined and appropriate protective measures adopted.
2) Laboratory coat, safety glasses, protective sleeves and gloves must be worn at all times.

3) Some of the reagents used in ATP extraction and analysis are highly toxic, carcinogenic and mutagenic. It is essential to follow conditions laid out in the COSSH risk assessment (special assessment).
4) Disposal of all these chemicals is through a specialist contractor via the safety office.
5) Sonication can give off aerosols. The sonicator should be placed in a fume hood or a respirator worn.
6) If imported soils are being used, ensure adherence to imported soils guidelines.

4. MATERIALS, REAGENTS AND EQUIPMENT

Apparatus

Beakers

Conical flask

Volumetrics

Measuring cylinder

Stirring plate

Magnetic stirrer

50 mL centrifuge tubes (The tubes must be centrifuge tubes in order to withstand sonification)

Soil, adjusted to 40-50 % WHC, sieved to 2 mm, unless experimental conditions dictate otherwise

Spatula

Tissue paper

Balance (2 decimal places)

Sonicator

2 glass pipettes of 25 mL

20 mL scintillation vials

Centrifuge

Small beaker

Wash bottle of deionised water

Box full of ice

Plastic bag to collect the filters

MicroPette Plus 10 µL, 100 µL, 200 µL

Multilable plate reader (with Luminescence mode)

96-well microplate (black, non-transparent)

Reagents

1. 0.1 mM (di-Na salt tetra hydrate - Sigma A-5394) ATP solution (in water)
2. Extractant stock solution (for 1 litre):
   - 174.9 g trichloroacetic acid (TCA)
   - 89.6 g $Na_2HPO_4*12H_2O$ (disodium hydrogen orthophosphate dodecahydrate)
   - 40.8 g Imidazole
3. Deionised water
4. Sodium arsenate buffer
   - 31.2 g $Na_2HAsO_4*7H_2O$ (arsenic acid sodium salt hepta hydrate) (Sigma A756)
   - 10 ml 0.2 *M* disodium EDTA (Diaminoethanetetra-acetic acid disodium salt)
   - 2.46 g $MgSO_4*7H_2O$ (magnesium sulphate)
5. Sodium hydroxide – 1M
6. Luciferin-luciferase preparation

5. PROCEDURE

5.1 Reagents preparation

0.1 mM (di-Na salt tetra hydrate - Sigma A-5394) ATP solution

- Weigh out 59.9 mg of ATP. (N.B. check MW of each batch – it can vary depending on bound water).
- Adjust to 1000 mL with deionised water.
- Store at -15 °C.

Note: It is advisable to prepare 100 or 200 mL of ATP solution and pipette small portion (5-10 mL) it into 15mL plastic tubes for freezing.

Extractant stock solution (for one litre)

- Weigh out 174.9 g of TCA in the fume hood, wearing gloves, laboratory coat, goggles and sleeve protection.

- Dissolve the TCA in 500 mL deionised water in the conical flask in the fume hood, using a magnetic stirrer and a stirring plate
- Weigh out 89.6 g of sodium phosphate
- Add to the conical flask
- Bring up to approximately 750 mL with deionised water.
- Add 40.8 g of Imidazole, whilst solution is being stirred
- Transfer to a volumetric flask and make up solution to 1000 mL, with distilled water.
- Store at -15 °C.

This gives a solution 1.07 M with respect to TCA, 0.25 M to phosphate and 0.6 M to imidazole.

Normally it is advisable to prepare 5 L of extractant stock solution so that the same batch of extractant is used throughout an experiment.

Extractant A

- Pipette 5 mL of distilled water into a 1 L volumetric.
- Make up to 1 L with extractant stock solution.

Extractant B

- Pipette 5 ml of 0.1 mM ATP.
- Make up to 1 L with extractant stock solution.

100 pmol ATP 50 μL^{-1} standard solution

- Pipette 1 mL of 0.1 mM ATP solution into a 50 mL volumetric flask.
- Make to volume with extractant A.

This gives 10 pmol ATP 5 μL^{-1} standard solution 1.

Sodium arsenate buffer (for 1 litre)

- Dissolve 31.2 g sodium arsenate in 800 ml deionised water.
- Add 10 ml 0.2 *M* EDTA.
- Then add 2.46 g magnesium sulphate in 100 ml deionised water
- Adjust the pH to 7.40 with 1 *M* H_2SO_4.
- Make up to 1000 ml with deionised water.
- This will give a solution 0.1 *M* with respect arsenate, 0.01 *M* with respect to Mg^{2+} and 0.002 *M* to EDTA.

- *N.B. At the end of the assay, the contents of the scintillation vials, and any other waste sodium arsenate buffer, MUST be emptied in to a waste bottle and disposed of by the safety officer.*

1M Sodium hydroxide

Dissolve 40g Sodium hydroxide in 1L deionised water or use 1M NaOH solution from box.

Luciferin-luciferase preparation

- Dissolve 250 mg of FLE (firefly lantern extract – FLE250) in 25 mL of deionised water.
- Mix it very carefully and slow to avoid foam formation.
- Leave it in a fridge overnight to avoid autofluorescence (Lundin and Thore, 1975).
- Store it in solution max. 2 weeks.
- *NB. It is possible to prepare several portion of luciferin-luciferase solution, e.g. 10, 10 and 5 ml – if you do not need the whole 25 ml for two weeks.*

5.2 Extraction

- For each replicate (normally 3), weigh 2 soil samples each of 2.5 g dry weight equivalent (Moist weight = dry weight/Dry matter)
- Place one in a 50 mL tube labelled A, the other in a tube labelled B.
- Prepare 6 empty tubes: 3 blanks for extractant A and 3 blanks for extractant B. l

Arrange them as in Figure 1:

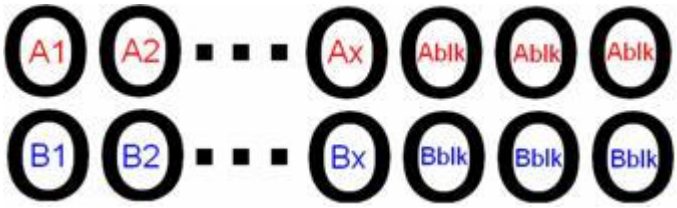


Figure 1: order of samples

- Treat each tube singly since it is important that the extractants are in contact with the soil for the same amount of time.
- Extractant A (25 mL) is added to the A tubes. Extractant B (25 mL) is added to the B tubes. Use one glass volumetric pipette for extractant A and a second pipette for extractant B.

- Immediately after adding 25 mL of extractant to the first tube, sonify for 2 min with VCX750 using a 12.5 mm probe at 60% power.
- Cool the tube in ice, for at least 5 min.
- Treat the next tube in the same way.
- Centrifuge tubes for 10 min at 5 000 rev at 8 ^{0}C or filter using Whatman 44 filter paper until 5-10 mL of filtrate is obtained. Store the extracts at -15 °C. *Note: Extracts can be stored up to one year without any changes in ATP content.*

NB: For calcareous soils check to see if pH of the filtrate is less than 2. If not, repeat the extraction, using less soil.

5.3 Standards

- Prepare standard solutions in 15mL tubes according to the scheme (table 1).
- Keep them on ice before pipetting.

Table 1. Standard solutions preparation scheme

ATP concentration, pmol 5 µL^{-1}	Standard solution 1, mL	Extractant A, mL
0	-	10
0.5	0.5	9.5
1	1	9
2	2	8
4	4	6
6	6	4
8	8	2
10	10	-

5.4 Measurement

- Pipette 150µL sodium arsenate buffer to 96-well microtiter plate.
- Pipette 13µL 1M NaOH to 96-well microtiter plate.
- Pipette 10µL extract to 96-well microtiter plate (*Note: It should be placed directly in the center of each well to obtain good results*).
- Pipette 100µL Luciferin-luciferase solution (FLE). *NB: Do it as fast as possible.*
- Measure the ATP with the Victor 3 Multilable plate reader in Luminescence mode.

6. CALCULATIONS

For calculation follow the algorithm, described in tabl.2.

Table 2. Calculation algorithm for soil ATP (after Jorgensen et al., 2015)

Step	***Calculation***	***Description***	***Unit***
1	Sample (A,B), Blank (A,B)	RLU data (Relative Light Unit) from plate reader	*(RLU)*
2	A/m, B/m, Blank/m	M=slope; Sample (RLU-data)/Slope (Standard curve)	*pmol/5µL*
3	blA=A*(B*,blB*)	Blank A minus (not blA) → background signal	*pmol/5µL*
4	(B-A)/blB	Sample B minus Sample A/ Blank B → recovery rate	*1=100%*
5	A*/recovery rate	→ correction	*pmol/5µL*
6	/5*100	Conversion to mL	*pmol/mL*

7	*(dilution+water content)/1000	Conversion to nmol related to total weight of the sample	*nmol*
8	/DM	Result in terms of 1 g dry matter (DM) of the sample	*nmol/g*

Example of calculation:

RLU

m=1617

(Slope)

A1=3235

B1=6612

blA=2000

blB=4780

Standard curve

y=mx+b (b=0)

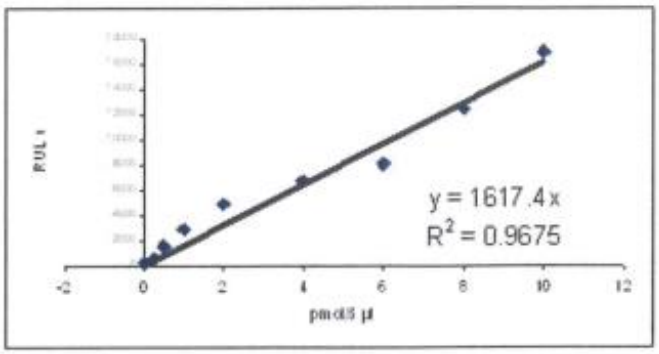
y=mx ↔ x=y/m

Soil:

DM=3g

Water content=0.5g

Total – 3.5 g



blA/m

2000/1617=1.24 pmol/5µL (background value)

blB/m

4780/1617=2.96 → -blA=1.72 pmol/5µL (measured ATP concentration in buffer B, blB*)

A/m

3235/1617=2.00 → -blA=0.76 pmol/5µL (A*)

B/m

6612/1617=4.09 → -blA=2.85 pmol/5µL (B^*)

Recovery:

(B-A)/blB to be

(2.85-0.76)/2.5=0.84 (so 84%)

→A^*/0.84=0.905 pmol/5µL

→/5*1000=181 pmol/mL

→ x (dilution+water content)/1000

181 pmol/mL*(25 mL+0.5mL)/1000= 4.61 nmol/3.00 g

→/DM

4.61 nmol/3.00 g = **1.54 nmol/g →amount of nmol ATP per gram soil (DM)**

References

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