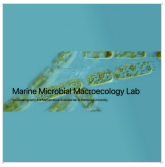

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Inorganic polyphosphate in microalgae: A DAPI-based quantification in microtiter plate

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

The DAPI-based fluorometric quantification of polyphosphate in microalgae has been widely used in field samples since the method was published by Martin P. et. al., where fluorescence of DAPI-stained samples is analyzed in quartz cuvettes by spectrofluorometer. In order to minimize the photobleaching of DAPI and reduce the consumption of reagent, time and labor, we have now scaled this method to 96-well black microtiter plate. Regarding to the matrix effects in microplate, the calculation has been modified accordingly.
Our method permits processing large numbers of samples by using only 250 uL of extracted sample and 30 uL of DAPI (100 uM). A lid with black film can protect all DAPI-stained samples from photobleaching.

Martin, Patrick & Van Mooy, Benjamin. Fluorometric Quantification of Polyphosphate in Environmental Plankton Samples: Extraction Protocols, Matrix Effects, and Nucleic Acid Interference. Applied and Environmental Microbiology. http://doi.org/10.1128/AEM.02592-12

PROTOCOL CITATION

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https://protocols.io/view/inorganic-polyphosphate-in-microalgae-a-dapi-based-banbidan

KEYWORDS

DAPI, polyphosphate, microtiter plate, microplate, microalgae, fluorescence

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GUIDELINES

1. Polyphosphate samples are collected on precombusted GFF filters and rinsed by oxalate reagent.
2. Keep samples frozen at -20 ºC before processed.
3. Total particulate phosphorus (TPP) measurement is recommended prior to the extraction of polyphosphate. The level of TPP helps to estimate the volume of extraction solution.
4. Different species or different sample locations (for field samples) require different numbers of extraction. A preliminary extraction efficiency test helps to obtain optimized number of extraction for extracting the most amount of polyphosphate with the least number of extraction.

MATERIALS

NAME	CATALOG #	VENDOR
Tris Buffer 1M pH 7.0	BP1756-500	Fisher Scientific
Sodium phosphate glass type 45	S4379-500MG	Sigma Aldrich
Proteinase-K	BP1700-500	Fisher Scientific
RNase A: 500 U/mL; RNase T1: 20000 U/mL	AM2288	Fisher Scientific
TURBO DNase 2 U/uL	AM2239	Fisher Scientific
DAPI: 4'6-Diamidino-2-phenylindole dihydrochloride	D1306	Fisher Scientific

MATERIALS TEXT

FreeZone® 2.5 L Benchtop Freeze Dryers
Labconco® 700202000

Sterile Disposable Filter Units with PES Membrane
Thermo Scientific™ Nalgene™ Rapid-Flow™ 5964520

Microbalance
Cubis series
Sartorius MSE6.6S-000-DM

VWR® Advanced Hot Plates
VWR 97042-658

VWR ANALOG VORTEX MIXER
VWR 10153-838
With tube insert

Varioskan LUX Multimode Microplate Reader

Thermo Fisher VL0L00D0

VWR® Vials, Borosilicate Glass, with Phenolic Screw Cap

22.18 mL

VWR 66012-044

24-400 cap: VWR 89076-764

Falcon® Centrifuge Tubes

Polypropylene, Sterile, 15 mL

Corning® 352096

Falcon® Centrifuge Tubes

Polypropylene, Sterile, 50 mL

Corning® 352070

Screw-Cap Centrifuge Tube

5 mL

VWR 10002-738

Microcentrifuge Tubes

1.7 mL/0.6 mL

Axygen Scientific MCT-175-C/MCT-060-L-C

Maxymum Recovery® Snaplock Microcentrifuge Tube
2.0 mL, Polypropylene, Clear, Nonsterile,
Axygen® MCT-200-L-C

BT Barrier Pipet Tips
Pre-Sterile
Neptune® BT1250, BT100, BT10

General-purpose benchtop centrifuge
IEC CENTRA CL2
Thermo 00427 0F

CENTRIFUGE 5430 R
Eppendorf MP2231000510

SHAKING INCUBATOR
71L
Corning® LSE™ 6753

Finnpipette Stepper Pipette
Thermo Scientific™ 4540000

Finntip™ Stepper Pipette Tips
500 uL
Thermo Scientific™ 9404170

96-Well Black Microplates
Polystyrene
Greiner Bio-One 655076

Black Vinyl Films for Fluorescence and Photoprotection
VWR 89087-692

And other general instruments:

- Autoclave tape
- Ultrasonic cleaner
- Pipettor
- Pipet tips (1~5 mL)
- Shaker
- 1 L volumetric flask

EQUIPMENT

NAME	CATALOG #	VENDOR
General-purpose benchtop centrifuge	00427 0F	
SHAKING INCUBATOR	6753	
96-Well Black Microplates	655076	
VWR® Advanced Hot Plates	97042-658	VWR international Ltd
VWR® Vials, Borosilicate Glass, with Phenolic Screw Cap	66012-044	
Microcentrifuge Tubes	MCT-175-C/MCT-060-L-C	VWR international Ltd
Maxymum Recovery® Snaplock Microcentrifuge Tube	MCT-200-L-C	VWR international Ltd
Finnpipette Stepper Pipette	4540000	Fisher Scientific
Varioskan LUX Multimode Microplate Reader	VL0L00D0	
Falcon® Centrifuge Tubes	352070	VWR international Ltd
FreeZone® 2.5 L Benchtop Freeze Dryers	700202000	VWR international Ltd
Falcon® Centrifuge Tubes	352096	VWR international Ltd
Microbalance	MSE6.6S-000-DM	Fisher Scientific
BT Barrier Pipet Tips	BT1250, BT100, BT10	VWR international Ltd
CENTRIFUGE 5430 R	MP2231000510	
Finntip™ Stepper Pipette Tips	9404170	Fisher Scientific
Black Vinyl Films for Fluorescence and Photoprotection	89087-692	
VWR ANALOG VORTEX MIXER	10153-838	VWR international Ltd
Screw-Cap Centrifuge Tube	10002-738	VWR international Ltd
Sterile Disposable Filter Units with PES Membrane	5964520	Fisher Scientific

Preparation of reagents

1 Tris buffer [M]**20 mM** pH**7.0**

1.1 In a 1 L volumetric flask, top **20 mL** [M]**1 M** pH**7.0** Tris buffer to 1 L with MilliQ

1.2 Filter through Rapid-flow and store at **Room temperature**

2 PolyP primary standard stock

2.1 Weigh one glass pellet of polyP (45) and write down the weight.

2.2 Transfer the pellet into a 100 mL graduated cylinder.

2.3 Dilute to 100 mL with Tris [M]**20 mM** pH**7.0**

2.4 Aliquot primary stock into 10~50 uL per microtube with Stepper and store at **-20 °C**

3 PolyP secondary standard stock
If the pellet is far more than 10 mg, dilute primary to secondary to bring down the concentration before preparing working standard

4 Proteinase K [M]**20 mg/ml**

4.1 Add **25 mL** MilliQ directly into the original bottle of Proteinase K, vortex to mix

4.2 Aliquot 600 uL to microtubes (around 45 microtubes) and keep frozen at **-20 °C**

5 DAPI primary stock [M]**14.3 mM**

Add **2 mL** MilliQ directly into the original package and keep frozen at **-20 °C**

Preliminary extraction efficiency test

6 Prepare boiling bath.

7 Transfer sample into glass vial

8 If the sample has less than 3 ug total particulate phosphate, use **2 mL** Tris Buffer **[M]20 mM** **pH7.0** for each extraction.
Otherwise, use **4 mL** Tris Buffer **[M]20 mM** **pH7.0** for each extraction.

9 Add **2 mL** or **4 mL** Tris buffer **[M]20 mM** **pH7.0** , sonicate. 15s

10 Keep in boiling bath. 5m

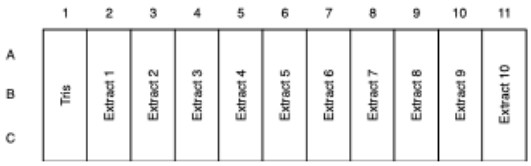
11 Sonicate 15s

12 Transfer extract to a 5 mL centrifuge tube, label the tube with number of extraction.

13 **go to step #9 Repeat until total extract number reaches 10.**

14 For all extract:
13300 rpm, Room temperature 00:05:00

15 Load microtitre plate with **250 µl** extract (triplicate).
Tris buffer **[M]20 mM** **pH7.0** is used as blank.



16 Prepare DAPI working solution **[M]100 uM**
Dilute **2.1 µl** of **[M]14.3 mM** DAPI stock with **300 µl** MilliQ in a foil wrapped 600 uL microtube and vortex.

17 In the dimmed room with only red light bulb on, add **30 µl** **[M]100 uM** DAPI to each sample in the plate.

18 Adhere black film on the top of a microplate lid and cover the plate with this lid.

19 Shake at room temperature 7m

20 Read fluorescence: excitation at 410 nm and emission at 550 nm

21 Plot fluorescence intensity versus number of extraction.
The number of extract (N) is the stationary point where the fluorescence of stained extract stops decreasing or the derivative of the fluorescence after that point is close to zero.

Extraction of polyphosphate from samples

22 Prepare boiling bath.

23 Transfer samples into glass vials

24 Add same amount of Tris buffer [M]**20 mM** pH**7.0** as preliminary test and then sonicate 15s

25 Place vials in boiling bath 5m

26 Sonicate 15s

27 Remove extract to a centrifuge tube, and then **go to step #24 until total extract reaches N+1** .

28 Combine extract 1~N into the same tube, transfer extract N+1 into another tube.

Enzyme treated extract

29 Centrifuge the mixture of 1~N extract **133000 rpm, Room temperature 00:05:00**

30 Transfer **4 mL** supernatant to a 5 mL centrifuge tube, add **40 µl** RNase and **40 µl** DNase

31 Incubate at **37 °C** , shake continuously 10m

32 Add **80 µl** Proteinase

33 Incubate at **37 °C** , shake continuously. 30m

Enzyme treated standard amended extract

34 Prepare PolyP working standard **7.6 uM**
Based on the actual concentration of PolyP (45) primary or secondary standard stock, dilute a certain volume of stock with **1 mL** Tris buffer **20 mM** **7.0**

35 Transfer **1680 µl** of enzyme treated extract into a 5 mL centrifuge tube

36 Add **320 µl** **7.6 uM** polyP working standard to **1680 µl** of enzyme treated extract, vortex.

Enzyme treated N+1 extract

37 Centrifuge extract "N+1" **13300 rpm, Room temperature 00:05:00**

38 Transfer **1 mL** supernatant into a 2 mL tube, add **10 µl** RNase and **10 µl** DNase

39 Incubate at **37 °C** , shake continuously 10m

40 Add **20 µl** Proteinase

41 Incubate at **37 °C** , shake continuously 30m

Load microtiter plate

42 Load **250 mL** samples to the microplate. Organize samples as shown in the following scheme:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
B	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
C	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
D	B1	B2	B3	B4	B5	B6				B1	B2	B3
E	B1	B2	B3	B4	B5	B6				B4	B5	B6
F	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
G	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
H	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6

S: Enzyme treated extract
A: Enzyme treated standard amended extract
B: Enzyme treated N+1 extract

43 Prepare DAPI working solution [M]**100 uM**

Dilute **2.1 µl** of [M]**14.3 mM** DAPI stock with **300 µl** MilliQ in a foil wrapped 600 uL microtube and vortex.

44 In a dimmed room with only red bulb on, add **30 µl** DAPI working solution [M]**100 uM** to each sample in the microplate **except for those labelled with UN**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
B	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
C	S1	S2	S3	S4	S5	S6	A1	A2	A3	A4	A5	A6
D	B1	B2	B3	B4	B5	B6				B1 (UN)	B2 (UN)	B3 (UN)
E	B1	B2	B3	B4	B5	B6				B4 (UN)	B5 (UN)	B6 (UN)
F	S1 (UN)	S2 (UN)	S3 (UN)	S4 (UN)	S5 (UN)	S6 (UN)	A1 (UN)	A2 (UN)	A3 (UN)	A4 (UN)	A5 (UN)	A6 (UN)
G	S1 (UN)	S2 (UN)	S3 (UN)	S4 (UN)	S5 (UN)	S6 (UN)	A1 (UN)	A2 (UN)	A3 (UN)	A4 (UN)	A5 (UN)	A6 (UN)
H	S1 (UN)	S2 (UN)	S3 (UN)	S4 (UN)	S5 (UN)	S6 (UN)	A1 (UN)	A2 (UN)	A3 (UN)	A4 (UN)	A5 (UN)	A6 (UN)

S: Enzyme treated extract
A: Enzyme treated standard amended extract
B: Enzyme treated N+1 extract
UN: no DAPI

45 Adhere black film on the top of a microplate lid and cover the plate with this lid.

46 Shake at room temperature. 7m

47 Read fluorescence: excitation at 410 nm and emission at 550 nm

Calculation

48 Definition of symbol

Symbol	Definition
M	Concentration of standard in one microplate well (nmol)
DF_{amend}	Dilution factor in amended extract $V_{extract}/(V_{extract} + V_{std})$
N	extraction times
V_{Tris}	Volume of Tris per extraction (mL)
V_s	Volume of sample loaded in microplate, i.e. 0.25 mL
MW	Molecular weight of polyP, i.e. 101.9617
DF_{enzyme}	Dilution factor in enzyme treatment

49

Signal	Color of wells
$SignalA(DAPI)$	
$SignalA(DAPI + std)$	
$SignalB(DAPI)$	
$SignalA(unstained)$	
$SignalA(+std,\ unstained)$	
$SignalB(unstained)$	

50

$$Signal(polyP) = [SignalA(DAPI) - SignalB(DAPI)] - [SignalA(unstained) - SignalB(unstained)]$$

$$Signal(polyP + std) = [SignalA(DAPI + std) - SignalB(DAPI)] - [SignalA(+std,\ unstained) - SignalB(unstained)]$$

$$polyP\ /\text{well} = M \cdot DF_{amend} \cdot \frac{Signal(polyP)}{signal(polyP+std) - DF_{amend} \cdot Signal(polyP)}$$

$$polyP/\text{filter} = 101.96 \cdot (0.001) \cdot V_{Tris} \cdot N \cdot DF_{enzyme} \cdot (polyP\ /\text{well})/V_s$$