***Dictyostelium discoideum* as a model to study inositol pyrophosphate and inorganic polyphosphate**.

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Abstract

The yeast *Saccharomyces cerevisiae* has given us much information on the metabolism and function of inositol polyphosphates and inorganic polyphosphate. To expand our knowledge of the metabolic as well as functional connections between inositol polyphosphates and inorganic polyphosphate, we have refined and developed techniques to extract and analyse these molecules in a second eukaryotic experimental model, the amoeba *Dictyostelium discoideum*. This amoeba, possessing a well-defined developmental program, is ideal to study physiological changes in the levels of inositol polyphosphate and inorganic polyphosphate, since levels of both molecules increase at late stages of differentiation. We detail here the methods used to extract inositol polyphosphates using perchloric acid and inorganic polyphosphate using acidic phenol. We also present the post-extraction procedures to visualise and quantify these molecules by polyacrylamide gel electrophoresis and by malachite green assay.

Keywords

Inositol polyphosphate; Extraction; DAPI staining; Toluidine blue; Electrophoresis; polyP

1. Introduction

The linear polymer of phosphate groups (PO_4^{3-}) called inorganic polyphosphate (polyP) is primarily studied in bacteria, but its presence in eukaryotes is well documented [1, 2]. Conversely, while inositol can be synthesized in bacteria [3], only nucleated cells have exploited the inositol ring to generate inositol polyphosphates (IPs). These are molecules with differentially arranged phosphate groups, attached to inositol through phosphodiester bonds [4]. Furthermore, inositol can also contain diphosphate moieties, i.e. highly energetic phosphoanhydride bonds; these species are called inositol pyrophosphates (PP-IPs) [5, 6]. Recent studies primarily executed in the yeast *Saccharomyces cerevisiae* have demonstrated that the metabolism of polyP and PP-IPs is interconnected [7, 8]. This yeast has been instrumental in the elucidation of the inositol polyphosphate metabolic pathway, which from phospholipase C-generated Ins(1,4,5)P_3 produces the fully phosphorylated IP_6 (inositol hexakisphosphate or phytic acid) and the PP-IPs IP_7 and IP_8 (diphosphoinositol pentakisphosphate and bis-diphosphoinositol tetrakisphosphate) [9-13]. Similarly, yeast has been fundamental to the identification of many physiological functions attributed to IPs and PP-IPs, such as vesicular trafficking and telomere length [14-16]. The studies of *S. cerevisiae* polyP metabolism have been facilitated by the accumulation of this polymer in the yeast

vacuole and thus by relatively simple extraction and analysis procedures. The vacuolar transporter chaperone (VTC) complex, specifically the subunit Vtc4, is responsible for polyP synthesis in yeast; a recent work revealed that the IPs/PP-IPs regulate polyP synthesis by binding to the SPX domain of VTC proteins [8].

While VTC homologous proteins are present in other eukaryote clades such as the kinetoplastida (trypanosomes) [17], they are absent from the majority of eukaryotes. In another eukaryotic organism, the social amoeba *Dictyostelium discoideum*, polyP is synthesized by polyphosphate kinase (DdPPK or simply PPK), an enzyme of bacterial origin obtained by horizontal gene transfer [18]. In *D. discoideum* polyP is important for general fitness, since amoebae lacking this polymer grow slowly [19]. Interestingly, polyP dramatically accumulates during the amoeba's development and is stored in the spores. It has also been reported that polyP is somehow secreted into the culture medium. The absence of polyP accumulation during the development of ppk1 mutant cells is offset by an increase of both ATP and PP-IPs, suggesting that these three phosphate-rich molecules interactively balance each other [19].

While *D. discoideum* has become a polyP experimental model only recently, the amoeba has been instrumental for the initial discovery, during the 1980s and early 1990s, of many IPs and their metabolism [20-22]. Furthermore, it was *D. discoideum* where the PP-IPs were originally discovered [23]. These discoveries were facilitated by the amoeba's high levels of IP_6, IP_7, and IP_8. These highly phosphorylated IPs species stain very well with toluidine blue once resolved by polyacrylamide gel electrophoresis (PAGE) [24]. Therefore, PAGE analysis of *D. discoideum* cell extracts easily reveals PP-IPs species and opens new investigative opportunities [25]. Interestingly, polyP analysis is similarly performed by toluidine staining or alternatively by DAPI staining once resolved by PAGE [26]. Thus these two phosphate-rich families of molecules share similar extraction and analysis protocols, although slightly different procedures are required to optimise the analysis of polyP or PP-IPs.

Here, we present the optimal protocol to extract and analyse by PAGE both polyP and PP-IPs from *D. discoideum*. Perchloric acid extraction is preferred to purify small acidic molecules such as IPs, while phenol extraction is better suited to purify the larger polyP polymer. A high acrylamide concentration is required for PAGE to resolve IPs that stain strongly with toluidine blue, while polyP can additionally be visualised by negative DAPI staining. We will also discuss the classical malachite green assay to quantify polyP once it is degraded to phosphate.

2. Materials

2.1. Cell culture

HL5 medium (in one litre of water dissolve: peptone 14 g ; yeast extract 7 g; glucose 13.5 g; KH_2PO_4 0.5 g; Na_2HPO_4 0.5 g. Formedium #HLG0102), filter sterilized or autoclaved (store at 4°C) (*see* **Note 1**).

KK2 buffer (20 mM KH_2PO_4/K_2HPO_4 pH 6.8), filter sterilized or autoclaved (store at 4°C).

KK2 plates 1.5% agar in KK2 buffer, autoclaved. Store at 4°C.

The *D. discoideum* strains used, namely wild type axenic strain (AX2) and the strains deleted for ppk1 and IP_6-kinase (i6ka), are available through the DictyBase Stock Center (http://dictybase.org).

2.2. Extraction of inositol polyphosphates

Perchloric acid 1 M (*see* **Note 2**).

Potassium carbonate 1 M (*see* **Note 2**).

Ethylenediaminetetraacetic acid (EDTA) 0.5 M pH 8.0 (Invitrogen #15575-038).

2.3. Extraction of inorganic polyphosphate

LETS buffer (0.1 M LiCl; 0.01 M Na_2EDTA; 0.01 M Tris-Cl pH 7.4; 0.2% SDS).

Phenol, saturated with 0.1 M citrate buffer, pH 4.3. Store at 4˚C (Sigma-Aldrich #P4682).

Chloroform.

Ethanol, both pure and 70% solution in water.

Tris/Borate/EDTA buffer (TBE) (10x solution: 450 mM Tris-borate; 10 mM EDTA).

2.4. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide (40%, 19:1 acrylamide:bis-acrylamide) (National Diagnostic #EC-850).

Ammonium persulfate, 10% solution in water. Store at -20°C.

N,N,N,N'-Tetramethyl-ethylenediamine (TEMED) (Sigma-Aldrich #T9281).

polyP standards, 10 mM in water. Store at 4˚C (*see* **Note 3**).

IP_6 standard, 1 mM in water. Store at 4˚C. (Calbiochem #407125) (*see* **Note 4**).

Orange G (Sigma-Aldrich #O3756).

Loading buffer (50% glycerol, 0.01% Orange G, 1 mM EDTA).

2.5. Toluidine blue staining

Toluidine blue (Acros organics #348601000) (*see* **Note 5**).
Staining solution: 0.1 % Toluidine blue in 20 % MeOH, 2 % glycerol. Store at room temperature.
Destaining solution: 20% MeOH.
Photo Scanner (Epson Perfection 4990 PHOTO).

2.6. DAPI staining
DAPI aliquots (200 µl) 10 mg/ml in water. Store at -20˚C.
Staining solution: freshly prepared 20% MeOH, 2% glycerol, 2 µg/ml DAPI (one DAPI aliquot into 1 L of solution).
Destaining solution: 20% MeOH, 2% glycerol.
UV 300 nm transillumination recording chamber.

2.7. Malachite green assay of inorganic phosphate
Molybdate solution: 28 mM ammonium heptamolybdate (Fluka #09880) in 2.1 M sulfuric acid.
Malachite green solution: 0.76 mM malachite green (Sigma-Aldrich #213020) in 0.35% polyvinyl alcohol. Store at 4˚C. (*see* **Note 6**).
Polyphosphatase buffer (5 mM Tris-HCl pH 7.0, 1.5 mM $MgCl_2$).
Recombinantly produced His-ScDdp1 and His-ScPpx1. Store at -20^{o}C. (*see* **Note 7**).

3. Methods
3.1. *D. discoideum* growth and starvation procedures.
1. Grow *D. discoideum* cells axenically in HL5 media at 22˚C (120 rpm) to a cell density not exceeding 4x10^6 cells/ml, ideally between 1-3x10^6 cells/ml (*see* **Note 8**).
2. To induce development, collect 10^7 cells at a cell density of 1-5x10^6 cells/ml.
3. Wash twice in KK2 buffer and plate onto 55 mm diameter KK2 plates.
4. After 8 h or 24 h, collect the cells manually by adding 500 µl of KK2 buffer to the plates.
5. Scrape the cells out of the plates using a plastic spatula.
6. Transfer the cell suspension to a 1.5 ml microtube.
7. Wash the plate once more as before and pool the cells.
8. Wash the cells once in KK2 buffer.

3.2. Inositol polyphosphates extraction.

1. Grow the cells to a density of 1-4x10^6 cells/ml.
2. Centrifuge 0.5-2x10^7 cells (1000 g, 2 min, 22 ˚C).
3. Wash the cells once with KK2 buffer and centrifuge as before.
4. Resuspend the cell pellets in 2 volumes or at least 50 µl of 1 M perchloric acid containing 5 mM EDTA. Prepare freshly (*see* **Note 2**).
5. Vortex at 4 ˚C for 10 min.
6. Centrifuge at 16000 g for 5 min at 4˚C.
7. Transfer the supernatant to a new microtube.
8. Add 1 volume of 1 M potassium carbonate containing 5 mM EDTA. Prepare freshly (*see* **Note 2**) (*see* **Note 9**).
9. Leave the tubes on ice for at least 2 h with occasional shaking and the lid open.
10. Centrifuge at 16000 g for 5 min at 4˚C.
11. The recovered supernatant contains the inositol polyphosphates (*see* **Note 10**).

3.3. Extraction of polyP

1. Grow the cells to a density of 1-4x10^6 cells/ml.
2. Centrifuge 2-4x10^7 cells (1000 g, 2 min, 22 ˚C).
3. Wash the cells once with KK2 buffer and centrifuge as before.
2. Resuspend the cells pellet in 1.5-2 volumes of LETS buffer, or at least 200 µl.
3. Add one volume of phenol (pH 4.3) to the cell suspension.
4. Vortex the samples at 4 ˚C for 5 min.
5. Centrifuge the samples at 16000 g for 5 min at 4 ˚C.
6. Transfer the aqueous top layer to a new 1.5 ml microtube.
7. Add 2 volumes of chloroform and mix vigorously.
8. Vortex the samples at 4 ˚C for 5 min.
9. Centrifuge at 5000 g for 5 min at 4 ˚C.
10. Transfer the aqueous phase to a new 1.5 ml microtube.
11. Add 2.5 volumes of ethanol.
12. Keep the samples at -80 ˚C for 2 h or overnight.
13. Centrifuge the samples at 16000 g for 5 min at 4 ˚C.
14. Wash the pellets once with 70 % ethanol.
15. Resuspend the pellets in water or 1x TBE.

16. Measure RNA concentration by recording the absorbance at 260 nm (*see* **Note 11**).

3.4. Inositol phosphates analysis by PAGE and toluidine blue staining

1. Prepare 38 ml of gel solution by mixing polyacrylamide:bis-acrylamide stock (40%), TBE and water to obtain a final polyacrylamide concentration of 33-35% (*see* **Note 12**).
2. Add 200 µl of 10 % APS and 20 µl of TEMED just prior to pouring the gel.
3. Pour the gel slowly, avoiding the formation of air bubbles, and fit the comb.
4. Wait for the gel to set (around 20-30 min) at room temperature (*see* **Note 13**).
5. Allow the gel to set completely in the cold room to prevent it from drying.
6. Pre-run the gel at 3 mA for 30-60 min.
7. Add sample buffer to the samples to be analysed for a final glycerol concentration of 10%.
8. Load the samples onto the gel.
9. Run the gel in the cold room at 500-600 Volt and 5-6 mA for around 16-18 h (*see* **Note 14**).
10. After the dye reaches two thirds of the gel, turn off the power supply (*see* **Note 14**).
11. Remove the gel from the plates and transfer it to a plastic box.
12. Add the toluidine blue staining solution to the box.
13. Incubate the gel at room temperature for 30 min with gentle shaking.
14. Recover the staining solution (*see* **Note 15**).
15. Rinse the gel with 20% methanol.
16. Destain the gel in 20% methanol until the desired staining is obtained changing the 20% methanol every 30 minutes.
17. Scan the gel.

3.5. PAGE analysis of polyP and DAPI staining

1. The same procedure as in Section 3.3 must be followed to prepare the gels, except that lower polyacrylamide concentrations (between 20% and 30% acrylamide) should be used (*see* **Note 16**).
2. Run the gel in the cold room at 400-500 Volt and 4-5 mA for around 16-18 h (*see* **Note 14**).
3. Pour one about litre of 20% methanol, 2% glycerol solution into a plastic box.
4. Add one 200 µl aliquot of 10 mg/ml DAPI and mix to complete the staining solution.
5. Remove the gel from the apparatus and incubate it in the staining solution for 40 min with gentle shaking and illumination.

6. Pour off the staining solution and rinse the gel twice with 250 ml of destaining solution.
7. Destain for 30 min with gentle shaking.
8. Use the UV transilluminator to image the gel (*see* **Note 15**).

3.6. Quantification of polyP using the Malachite Green detection assay
1. PolyP is extracted as described in Section 3.3.
2. Prepare serial dilutions of polyP samples in polyphosphatase buffer to a final volume of 200 µl.
3. Split the sample into two wells of a 96-well plate and add 0.01 µg/µl of recombinant ScPpx1 and ScDdp1 to one of the aliquot.
4. In the meantime, prepare serial dilutions of KH_2PO_4 from 0.05 to 5 nmoles in polyphosphatase buffer.
5. Add 100 µl of each dilution into the 96-well plate.
6. Incubate at 37 ˚C for 1 h.
7. Prepare the malachite green reagent just before use by mixing 860 µl of molybdate solution and 640 µl of malachite green solution.
8. Add 150 µl of malachite green reagent to all the samples.
9. Incubate the plate at room temperature for 5 min.
10. Read the absorbance at 595-600 nm.
11. Express the amount of free phosphate released by His-ScPpx1/His-ScDdp1 relative to the initial amount of RNA in the sample (nmoles Pi/µg of RNA).

4. Notes
1. All solutions are prepared using milliQ or equivalent ultrapure water (18 MΩ-cm); all solutions and or reagents are stored at room temperature (unless otherwise indicated).
2. EDTA (0.5 mM) is freshly added to the required amount (a few ml) of perchloric acid and potassium carbonate 1 M stock solutions. These prepared aliquots can be kept on ice for few hours.
3. Commercial polyP is often unavailable or discontinued. During the writing of the current chapter, Sigma-Aldrich offers polyP (sodium phosphate glass) Type 45 (45 phosphate groups as average polymeric length) (polyP45; S4379). In the past Sigma-Aldrich has offered polyP of variable polymeric length from 5 to 75.
4. Several commercially available IP_6 contain 5-10 % of other inositol polyphosphates such as IP_5 and IP_7, which can be easily detected by toluidine staining. Therefore, it is advisable to

check the purity of an IP_6 standard by PAGE before using it in important experiments. This is particularly relevant if the aim is to use IP_6 for enzymatic assays or binding experiments.

5. Commercial source of toluidine blue differ in the amount of dye that they possess. Therefore it is important to assess the staining ability of each batch of toluidine blue.

6. Polyvinyl alcohol is difficult to dissolve. Heating and overnight stirring are often necessary. Filter the solution once fully dissolved.

7. Constructs to generate the recombinant proteins His-ScDdp1 and His-ScPpx1 are available from the Saiardi laboratory on request.

8. It is important not to overgrow *D. discoideum* liquid culture, since high cell density can induce differentiation. A detailed description of how to handle amoeba cells can be found on DictyBase (http://dictybase.org).

9. The pH of the samples should be between 6 and 8. The pH can be measured using pH test strips and adjusted accordingly using either perchloric acid or potassium carbonate containing EDTA.

10. It is difficult to precisely normalize inositol polyphosphates by the amount of extracted cells/material. We can only rely on the initial number of cells that were plated to induce starvation. While cells do not divide in KK2, they aggregate to form slugs and fruiting bodies at the end of development, changing the amoeba physiology, cell shape, and cell number. Furthermore, it is complicated to collect the same percentage of biological material especially at the late stage of development, since *D. discoideum* cell architecture changes dramatically during development.

11. The described extraction co-purifies polyP and RNA, therefore measuring the absorbance of nucleic acid at 260 nm allows normalisation to the amount of extracted material.

12. The size of the glass plates and spacer in which the gel is prepared will determine the amount of gel solution to be prepared. To nicely resolve inositol polyphosphates we prepare 24 cm long, 1 mm thick polyacrylamide gels. For these gel dimensions we usually prepare 38 ml of gel solution.

13. Polyacrylamide polymerization is an exothermic process, and highly concentrated gels warm considerably during polymerization.

14. The correct voltage and amperage should be adjusted accordingly to the gel length and concentration used. The gel should be stopped when the orange G dye arrives at 2/3 of the length of the gel. IP_6, IP_7 and IP_8 run faster than Orange G and may run out if the dye is allowed to run further than 2/3 of gel length.

15. The staining solution can be used until the dye is exhausted. We usually prepare a fresh solution after 15-20 stainings.
16. Because of the polymeric nature of polyP, high polyacrylamide concentrations (e.g. 30 %) can be used to properly resolve small polymeric polyP species, while 20 % or lower polyacrylamide gel concentration is ideal to resolve large polymeric polyP species. Using different acrylamide concentrations dramatically affects gel migration thus voltage and amperage should be adjusted accordingly.
17. PolyP induced DAPI photo-bleaching [26] can take a couple of minutes to complete, therefore it is opportune to record several imagines. After DAPI staining the gel can be subsequently stained with toluidine blue as described above.

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Legends to the figures.
Fig. 1 Representative IPs profiles of *D. discoideum* AX2 and the IP_6-kinase knockout (i6ka). Inositol polyphosphates extracted from 2.10^7 cells by perchloric acid were loaded onto a 35.5% polyacrylamide gel. Inositol hexakisphosphate (IP6) and polyphosphate of mean chain length of 13 phosphate residues (polyP13) were loaded as standards. The gel was stained with toluidine blue, scanned, and processed using Adobe Photoshop.
Fig. 2 polyP profiles of D. discoideum AX2 and the polyphosphate kinase knockout (ppk1). polyP was extracted using the acidic phenol protocol and loaded onto a 25% polyacrylamide gel. polyP13 was used as a standard and Orange G was used to follow the migration. The gels were satined with DAPI. A. polyP was extracted from cells in the vegetative state and 70 µg of RNA were loaded on the gel. B. Cells were collected at different times during development on KK2 plates, polyP was extracted, and 15 µg of RNA were loaded on a gel.
Fig. 3 Quantification of polyP using the malachite green assay. A. Representative standard curve obtained with 0.05 to 5 nmoles of free phosphate. B. polyP was extracted as in A. and quantified as the amount of Pi released after the action of the recombinant polyphosphatases Ppx1 and Ddp1.

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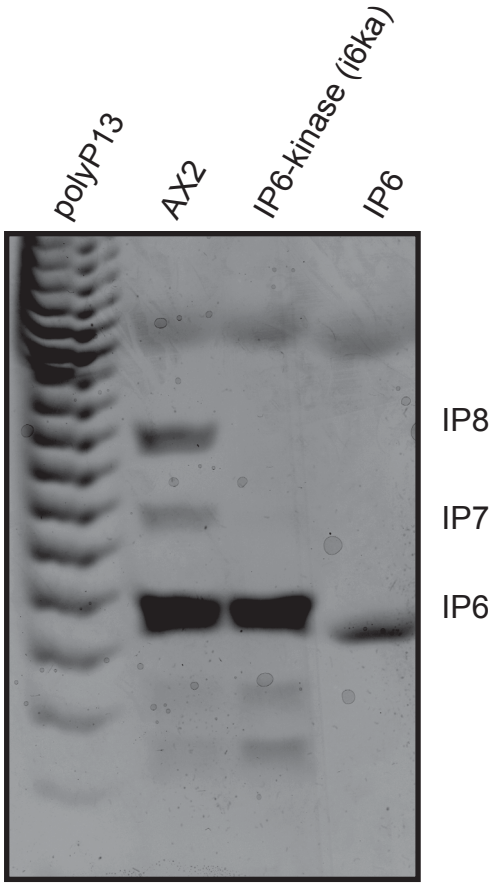
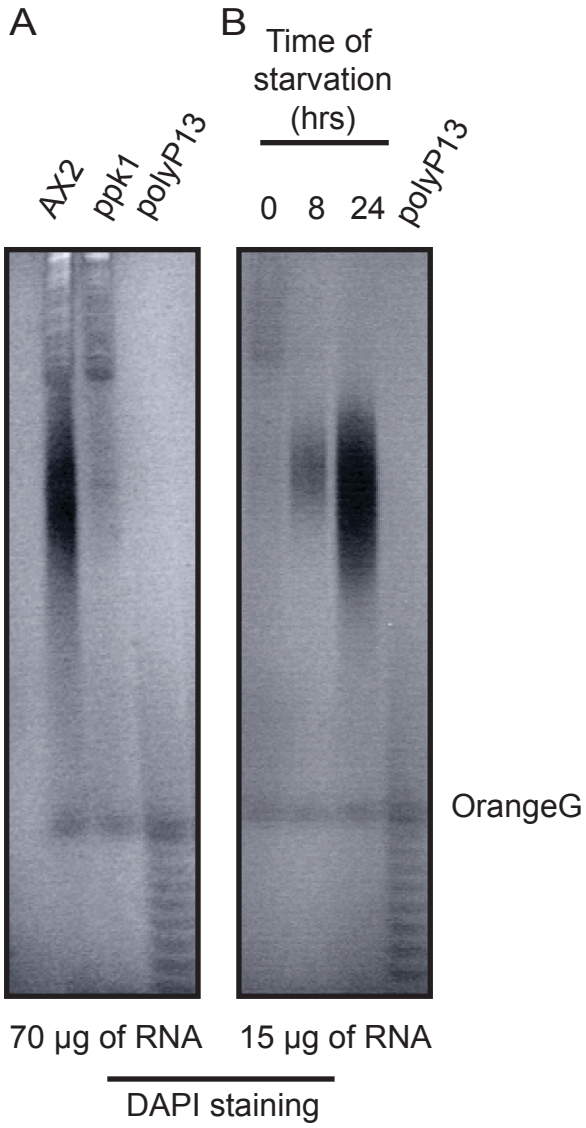
Figure 1.



Toluidine blue staining

Figure 2.


A
B
Time of starvation (hrs)
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polyP13
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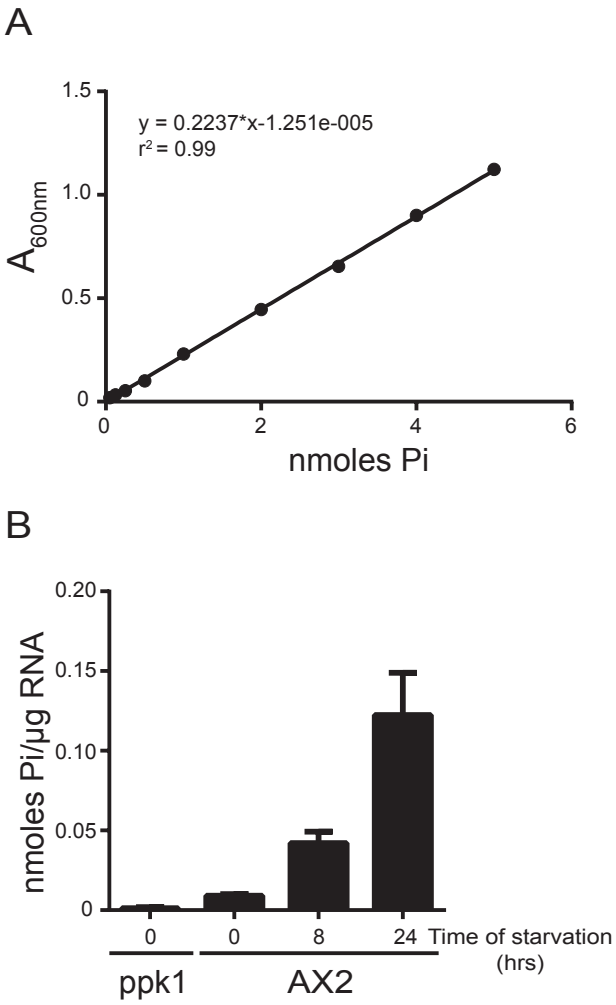
8
24
polyP13
OrangeG
70 µg of RNA
15 µg of RNA
DAPI staining

Figure 3.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r2 = 0.99
A600nm
nmoles Pi
B
nmoles Pi/µg RNA
Time of starvation (hrs)
ppk1
AX2