**We thank the editor and reviewers for their helpful and thoughtful comments. We have addressed the stated concerns, and this document contains a point-by-point response to those comments.**

**Thank you!**

Editorial comments:  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Done.**

2. Please spell out each abbreviation the first time it is used.

**Done.**

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

**Done.**

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Pierce, HiTrap, etc.

**Fixed.**

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

**We have gone through the manuscript and added details to many steps of the protocol. The specific examples mentioned are addressed below:**

1.4: What is the shaking speed?

**The shaking speed (180 rpm) has been included.**

1.5: Is the culture transformed to a centrifugation tube before centrifugation?

**Yes, and this is now noted in the protocol.**

1.13: Please describe how to test elution fractions for protein content and run SDS-PAGE gel.

**Citations have been included for the Bradford assay and for SDS-PAGE of proteins.**

2.1: Please specify the bacteria that will be grown in the video and specify the conditions used.

**Done.**

2.2: What is A600? How is it measured.

**A600 is absorbance at 600 nm, and is measured in a spectrophotometer. This is now included in the protocol.**

3.4: Please provide an equation for calculating the amount of protein.

**This equation has been included.**

6. Figure 1: Please define A595 and A882 in the figure legend.

**Done.**

7. Discussion: Please also discuss critical steps within the protocol.

**The discussion of diluting samples that are outside the standard curve has been moved out of the Discussion and into a Note for step 7, and a paragraph describing the critical steps of the protocol has been included in the Discussion.**

8. References: Please do not abbreviate journal titles.

**Fixed.**

Reviewers' comments:  
Reviewer #1:  
  
Manuscript Summary:  
This article describes quantification of polyphosphate granules in bacteria. PolyP are essential for survival during dormancy and required for virulence in many pathogenic bacteria. This method also involves purification of S. cerevisiae exopolyphosphatase (ScPPX), polyP-degrading enzyme required for this procedure. Other methods that include complex purification procedures or more direct methods (e.g., microscopy) are available, but the presented method may be useful when quick and accurate quantification is required.  
  
Major Concerns:  
None  
  
Minor Concerns:  
\* Note that text on the right for the reagent/equipment list is cut off.

**The table’s Print Area has been adjusted, and cells set to “wrap text”, which makes it print on the width of a single page.**

\* For ScPPX expression, OD when IPGT is added should be noted in case incubation time needs to be adjusted.

**IPTG is added at an OD of 0.4 – 0.5, and this is now noted in the protocol.**

\* Flask/medium volume for growth and shaking speed may not provide enough oxygen. Have other conditions been tested? What kind of flask should be used (e.g., baffled).

**We use unbaffled flasks. The overnight static growth conditions are not aerated, and allow *E. coli* to grow fermentatively to a reasonable cell density. Once the overnight culture is started shaking, the available oxygen allows the cells to respire and re-enter log phase for protein expression. We find that this protocol gives very large protein yields. Other methods of protein overexpression (*e.g.* growing cells to mid-log phase with shaking, then inducing with IPTG) have also worked well. The ScPPX overexpression and purification are very forgiving. We have included text in the manuscript to this effect.**

\* Proper PPE (e.g., ear protection) should be noted for sonication experiment.

**Done.**

\* What material is the syringe filter membrane? This information is important, in case this product is not available in few years.

**The filters are cellulose acetate, and this has been noted in the protocol.**

\* How efficient are nuclease and lysozyme reactions on ice? Shouldn't be higher temperature?

**We regularly incubate cells with lysozyme and nuclease on ice to prevent thermal damage to the proteins we are trying to purify. The lysozyme certainly is active under these conditions (visible cell lysis occurs), and addition of the nuclease as described eliminates nucleotide absorbance associated with purified ScPPX, from which we infer that the nuclease is also active under these conditions.**

\* What is used for dialysis? Volume, time, type and membrane pore size, is buffer mixed?

**The details of the dialysis procedure are now included in the protocol.**

\* How long polyP eluates can be store at -20oC?

**We have stored these extracts for up to a week with no ill effects, and have noted this in the protocol. They may be stable longer, but we have not tested it.**

\* Explain formula at 7.1

**Done.**

\* Was this method compared against other methods?

**We developed this method due to our dissatisfaction with the methods described in Gray *et al.* 2014, Cremers *et al.* 2016, and Dahl *et al.* 2017, and have informally compared it to a variety of other methods for polyP quantification (extractions, gels, *in vivo* DAPI staining), but we feel that a systematic comparison between the results obtained with our method and the wide variety of previously published methods is beyond the scope of this paper.**

\* How specific ScPPX is? Will it hydrolyze other phosphates?

**ScPPX is highly specific for polyP (see Wurst & Kornberg), and to our knowledge does not hydrolyze any other phosphates. For the purposes of this assay, it is known to be completely inactive against DNA and RNA, the other main components in the polyP-containing extracts.**

\* One potential concern is if extraction efficiency varies between conditions/strains. This is particularly problematic with mycobacteria, as they have a thick cell wall.  
  
**This is certainly a possible concern, and has been noted in the discussion, along with advice for a potential approach to control for such effects.**  
  
Reviewer #2:  
  
Manuscript Summary:  
This manuscript describes a detailed protocol for the extraction and quantification of polyP. The authors did a good job in putting together a method for the purification of S. cereviseae exoplyphosphatase, polyP extraction and digestion and phosphate determination. As long as one is in possession of a plasmid expressing ScPPX the protocol can be easily followed using simple reagents and equipment.  
  
Major Concerns:  
- I could not find plasmid pScPPX2 in the addgene website. Since this plasmid carries the exopolyphosphatase that is at the heart of the method, it is important to provide a reliable source for it.

**Addgene has finished processing the plasmid, and it is now available.**

- An alternative for the enzymatic hydrolysis of polyP is boiling in the presence of a strong acid. This would simplify the method and make it more affordable.

**Unfortunately, boiling in strong acid also hydrolyzes DNA and RNA present in the polyP-containing extracts. One possible modification might be to DNAse and RNAse treat the extracts before acid hydrolysis, but this would necessitate a second purification step to eliminate the resulting free nucleotides. This is certainly possible (an isopropanol precipitation step could be included, for example), but we disagree that it would simplify the method.**

- How do you know that the incubation at 95C in the presence of GITC during the extraction step does not affect polyP chain length?

**This is a potential concern, although polyP is most susceptible to hydrolysis at acidic pH rather than the neutral pH of the GITC lysis buffer. We have noted in the protocol the importance of being consistent with incubation time during the lysis step and the potential danger of extended incubation at high temperature.**  
  
Minor Concerns:  
- Protocol item 1.2- mention what would be the OD or aspect of the culture following overnight growth.

**The OD of the culture at this stage is approximately 0.3, and this is now noted in the protocol.**

- Protocol item 1.6- omit 'scppx overexpression'.

**Done.**

- Protocol item 1.7- state whether the concentrations are stock or final.

**Done.**

- Protocol item 1.8- specify brand and model of the sonicator, or give general instructions for the sonication.

**The brand and model of the sonicator are listed in the Table of Materials.**

- Protocol item 1.9- are you really going to pass 1 L through a syringe filter?

**At this stage, the volume of the lysate is approximately 10 mL (see step 1.6). This has been noted more clearly in the protocol.**

- Protocol item 1.15- is dialysis overnight?

**Yes. Details of the dialysis procedure are now included in the protocol.**

- Protocol item 3.4- Last sentence is redundant. An alternative: "multiply by 0.05 to determine total ug protein"

**Done.**

- Discussion, line 300- write "...works efficiently on representatives of three different phyla: proteobacteria, firmicute and actinobacteria..." instead of "...works effectively on both Gram-negative and Gram-positive species, as well as on mycobacteria..."

**Done.**

- Discussion, last para.- mention that as an alternative polyP could be hydrolyzed by boiling the sample in acid

**Done, with caveats about additional purification steps necessary.**

Reviewer #3:  
  
Manuscript Summary:  
This is a straightforward method that will be of interest to anyone working on polyphosphate. It seems to be a robust, relatively easy-to-perform method, and it is very well documented.  
  
Major Concerns:  
No major concerns. I think the protocol will be easy to follow and it is good that the authors also include a detailed protocol for producing the recombinant yeast enzyme that digests polyphosphate.  
  
Minor Concerns:  
1. Throughout the paper, the authors refer to the spin columns as "silica gel spin columns". I do not think these columns actually contain silica gel. Instead they have what the manufacturers refer to as a "silica membrane", and from inspection of these columns, what is in them doesn't look at all like silica gel to me. (Looks more like some sort of fibers matted together to make the "membrane", actually.) In any case, none of the manufacturers of these columns claim they have silica gel in them.

**The reviewer is quite right, and “gel” has been replaced with “membrane”.**  
  
2. The authors should confirm that they are using BL21 cells, not BL21(DE3) cells, for expressing the recombinant yeast enzyme.

**The reviewer is correct again; this should have been BL21(DE3). This has been changed, and a reference included.**  
  
3. I couldn't find the plasmid for expressing pScPPX2 listed in Addgene. Have the authors not submitted it yet?

**The plasmid is now available at Addgene. Their processing takes quite a while, but it actually became available the same day I received the reviews.**  
  
4. The name of the company providing the spin columns is Epoch Life Science, not Epoch Life Sciences.

**Fixed.**  
  
5. The table listing the materials doesn't print out well: Some of the lines are too long to fit on a single page, and the remainders of those lines are on a separate page.

**The table’s Print Area has been adjusted, and cells set to “wrap text”, which makes it print on the width of a single page.**