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Dear Editor,

Please find attached the revised version of our manuscript, entitled "A fast, simple assay for inorganic polyphosphate in bacteria" for consideration for publication in the Journal of Visualized Experiments, along with a rebuttal document specifically addressing the reviewers' and editor's comments.

Thank you for your consideration,

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TITLE:

Assaying for Inorganic Polyphosphate in Bacteria

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KEYWORDS:
Polyphosphate, stress response, exopolyphosphatase, Gram-negative bacteria, Gram-positive bacteria, mycobacteria

SUMMARY:
We describe a simple method for rapid quantification of inorganic polyphosphate in diverse bacteria, including Gram-negative, Gram-positive, and mycobacterial species.

ABSTRACT:
Inorganic polyphosphate (polyP) is a biological polymer found in cells from all domains of life, and is required for virulence and stress response in many bacteria. There are a variety of methods for quantifying polyP in biological materials, many of which are either labor-intensive or insensitive, limiting their usefulness. We present here a streamlined method for polyP quantification in bacteria, using a silica membrane column extraction optimized for rapid processing of multiple samples, digestion of polyP with the polyP-specific exopolyphosphatase ScPPX, and detection of the resulting free phosphate with a sensitive ascorbic acid-based colorimetric assay. This procedure is straightforward, inexpensive, and allows reliable polyP quantification in diverse bacterial species. We present representative polyP quantification from the Gram-negative bacterium (*Escherichia coli*), the Gram-positive lactic acid bacterium (*Lactobacillus reuteri*), and the mycobacterial species (*Mycobacterium smegmatis*). We also include a simple protocol for nickel affinity purification of mg quantities of ScPPX, which is not currently commercially available.

INTRODUCTION:

Inorganic polyphosphate (polyP) is a linear biopolymer of phosphoanhydride-linked phosphate units that is found in all domains of life[1-3]. In diverse bacteria, polyP is essential for stress response, motility, biofilm formation, cell cycle control, antibiotic resistance, and virulence[4-11].

45 Studies of polyP metabolism in bacteria therefore have the potential to yield fundamental
46 insights into the ability of bacteria to cause disease and thrive in diverse environments. In many
47 cases, however, the methods available for quantifying polyP in bacterial cells are a limiting
48 factor in these studies.
49
50 There are several methods currently used to measure polyP levels in biological materials. These
51 methods typically involve two distinct steps: extracting polyP and quantifying the polyP present
52 in those extracts. The current gold standard method, developed for the yeast *Saccharomyces*
53 *cerevisiae* by Bru and colleagues[12], extracts polyP along with DNA and RNA using phenol and
54 chloroform, followed by ethanol precipitation, treatment with deoxyribonuclease (DNase) and
55 ribonuclease (RNase), and digestion of the resulting purified polyP with the *S. cerevisiae* polyP-
56 degrading enzyme exopolyphosphatase (ScPPX)[13] to yield free phosphate, which is then
57 quantified using a malachite green-based colorimetric assay. This procedure is highly
58 quantitative but labor-intensive, limiting the number of samples that can be processed in a
59 single experiment, and is not optimized for bacterial samples. Others have reported extracting
60 polyP from a variety of cells and tissues using silica beads ("glassmilk") or silica membrane
61 columns[6,14-18]. These methods do not efficiently extract short chain polyP (less than 60
62 phosphate units)[12,14,15], although this is of less concern for bacteria, which are generally thought
63 to synthesize primarily long-chain polyP[3]. Older methods of polyP extraction using strong
64 acids[19,20] are no longer widely used, since polyP is unstable under acidic conditions[12].
65
66 There are also a variety of reported methods for quantifying polyP. Among the most common is
67 4',6-diamidino-2-phenylindole (DAPI), a fluorescent dye more typically used to stain DNA. DAPI-
68 polyP complexes have different fluorescence excitation and emission maxima than DAPI-DNA
69 complexes[21,22], but there is considerable interference from other cellular components, including
70 RNA, nucleotides, and inositol phosphates[12,15,16,23], reducing the specificity and sensitivity of
71 polyP measurements made using this method. Alternatively, polyP and adenosine diphosphate
72 (ADP) can be converted into adenosine triphosphate (ATP) using purified *Escherichia coli* polyP
73 kinase (PPK) and the resulting ATP quantified using luciferase[14,17,18]. This allows the detection of
74 very small amounts of polyP, but requires two enzymatic reaction steps and both luciferin and
75 very pure ADP, which are expensive reagents. ScPPX specifically digests polyP into free
76 phosphate[6,12,13,24], which can be detected using simpler methods, but ScPPX is inhibited by DNA
77 and RNA[12], necessitating DNase and RNase treatment of polyP-containing extracts. Neither PPK
78 nor ScPPX are commercially available, and PPK purification is relatively complex[25,26].
79
80 PolyP in cell lysates or extracts can also be visualized on polyacrylamide gels by DAPI negative
81 staining[27-30], a method that does allow assessment of chain length, but is low-throughput and
82 poorly quantitative.
83
84 We now report a fast, inexpensive, medium-throughput polyP assay that allows rapid
85 quantification of polyP levels in diverse bacterial species. This method begins by lysing bacterial
86 cells at 95 °C in 4 M guanidine isothiocyanate (GITC)[14] to inactivate cellular phosphatases,
87 followed by a silica membrane column extraction optimized for rapid processing of multiple
88 samples. The resulting polyP-containing extract is then digested with a large excess of ScPPX,

89 eliminating the need for DNase and RNase treatment. We include a protocol for
90 straightforward nickel affinity purification of mg quantities of ScPPX. Finally, polyP-derived free
91 phosphate is quantified with a simple, sensitive, ascorbic acid-based colorimetric assay[24] and
92 normalized to total cellular protein. This method streamlines the measurement of polyP in
93 bacterial cells, and we demonstrate its use with representative species of Gram-negative
94 bacteria, Gram-positive bacteria, and mycobacteria.
95
96 **PROTOCOL:**
97
98 **1. Purifying Yeast Exopolyphosphatase (ScPPX)**
99
100 1.1. Transform the *E. coli* protein overexpression strain BL21(DE3)[31] with plasmid pScPPX2[6] by
101 electroporation[32] or chemical transformation[33].
102
103 1.2. Inoculate 1 L of lysogeny broth (LB) containing 100 µg mL^{-1} ampicillin in a 2 L unbaffled flask
104 with a single colony of BL21(DE3) containing pScPPX2 and incubate overnight at 37 °C without
105 shaking, to an absorbance at 600 nm (A_{600}) of approximately 0.3, as measured in a
106 spectrophotometer.
107
108 1.3. Start the culture shaking (180 rpm) and incubate for 30 min at 37 °C, during which time the
109 cells will grow to an A_{600} of 0.4 - 0.5.
110
111 1.4. Add isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and an
112 additional 100 µg mL^{-1} ampicillin, then incubate for 4 h at 37 °C with shaking at 180 rpm.
113
114 NOTE: This overexpression protocol generates a large amount of soluble ScPPX. However,
115 ScPPX overexpression is very forgiving, and a variety of other common protein overexpression
116 protocols have been used to successfully purify ScPPX.
117
118 1.5. Transfer the cells to a 1 L centrifuge bottle and harvest them by centrifuging for 20 min at
119 5000 x g at 4 °C. Remove the supernatant and transfer the cell pellet to a 50 mL conical tube.
120
121 NOTE: Cell pellets can be stored indefinitely at -80 °C.
122
123 1.6. Thaw the pellet on ice, then resuspend it in 9 mL of 50 mM HEPES, 0.5 M NaCl, and 5 mM
124 imidazole (pH 8) for a total volume of about 10 mL.
125
126 1.7. Add (final concentrations) 1 mg mL^{-1} lysozyme, 2 mM $MgCl_2$, and 50 units mL^{-1} of an RNA-
127 and DNA-degrading endonuclease (see **Table of Materials**) and incubate for 30 min on ice.
128
129 NOTE: ScPPX binds nucleic acids (data not shown), so nuclease treatment is essential during
130 purification.
131
132 1.8. Using a sonicator with a microtip (see **Table of Materials**), lyse the cells by 2 cycles of

133 sonication on ice at 50% power, pulsing 5 s on and 5 s off, with a 2 min rest between cycles.
134
135 NOTE: Use appropriate hearing protection during sonication. Similarly to the case with
136 overexpression, a variety of cell lysis methods are acceptable for ScPPX purification.
137
138 1.9. Remove insoluble debris by centrifuging the lysate for 20 min at 20,000 x g at 4 °C. Filter
139 the resulting supernatant (approximately 10 mL) through a 0.8 µm pore size cellulose acetate
140 syringe filter.
141
142 1.10. Load the lysate onto a nickel-charged 5 mL chelating column (see **Table of Materials**)
143 using either a peristaltic pump or a large syringe.
144
145 1.11. Rinse the column with 50 mL of 50 mM HEPES, 0.5 M NaCl, 5 mM imidazole (pH 8).
146
147 1.12. Rinse the column with 50 mL of 50 mM HEPES, 0.5 M NaCl, 20 mM imidazole (pH 8).
148
149 1.13. Elute ScPPX with 50 mM HEPES, 0.5 M NaCl, and 0.5 M imidazole (pH 8), collecting 15 1-
150 mL fractions. Test these elution fractions for protein content with the Bradford protein assay[34]
151 (see **Table of Materials**) and run an SDS-PAGE gel[35] to confirm which fractions contain purified
152 ScPPX (molecular weight = 45 kDa).
153
154 1.14. Pool the fractions containing pure ScPPX and adjust the concentration of the pooled
155 protein to about 2 mg mL^{-1} with 50 mM HEPES and 0.5 M NaCl. Higher protein concentrations
156 may precipitate in the next step.
157
158 1.15. Exchange the purified ScPPX into storage buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 10%
159 [v/v] glycerol) by dialysis. Place pooled ScPPX fractions in a sealed length of 12,000 - 14,000 Da
160 molecular weight cutoff dialysis membrane tubing (see **Table of Materials**) and suspend in 1 L
161 of storage buffer with continuous stirring at 4 °C for at least 4 h. Repeat this step with fresh
162 storage buffer for a total of 3 buffer changes.
163
164 1.16. Transfer the purified, dialyzed protein to a centrifuge tube or tubes and centrifuge for 20
165 min at 20,000 x g at 4 °C to remove any insoluble aggregates. Carefully transfer the supernatant
166 to a clean 15 mL conical tube.
167
168 1.17. Adjust the concentration of the purified ScPPX to 1 mg mL^{-1} with storage buffer, add 0.1%
169 (w/v) protease-free bovine serum albumin (BSA; final concentration), and store for up to 6
170 months at 4 °C.
171
172 NOTE: ScPPX loses more than 90% of its activity when it is frozen[13].
173
174 **2. Harvesting Samples for Polyphosphate Extraction**
175
176 2.1. Grow bacteria under the conditions of interest for determining polyP content. For this

177 protocol, grow *Lactobacillus reuteri*[36] overnight at 37 °C without shaking in malic enzyme
178 induction (MEI) medium[37] without cysteine (MEI-C).
179
180 2.2. Harvest enough cells by centrifugation in a 1.5 mL microcentrifuge tube to total 50 – 100 µg
181 of cellular protein (see step 3 below). For *E. coli*, this is 1 mL of a log phase culture at an A_{600} of
182 0.2 - 0.4. For *L. reuteri*, this is 1 mL of an overnight culture. Adjust as necessary for other species
183 of interest.
184
185 2.3. Completely remove the supernatant from the cell pellets.
186
187 2.4. Resuspend the cell pellets in 250 µL of GITC lysis buffer (4 M guanidine isothiocyanate, 50
188 mM Tris-HCl, pH 7) and lyse by incubation at 95 °C for 10 min. Store lysates at -80 °C.
189
190 NOTE: Be consistent with lysis time, since extended incubation at high temperature may result
191 in degradation of polyP by hydrolysis[38]. Lysates can be stored indefinitely at -80 °C.
192
193 CAUTION: Guanidine isothiocyanate is a chaotropic salt and should be handled with gloves and
194 disposed of as hazardous waste.
195
196 **3. Measuring the Protein Content of Cell Lysates**
197
198 3.1. Prepare BSA standards containing 0, 0.1, 0.2, and 0.4 mg mL^{-1} of BSA in GITC lysis buffer.
199
200 NOTE: It is important to make the BSA standards in GITC, since GITC influences the color
201 development of the Bradford assay. BSA standards can be stored indefinitely at -20 °C.
202
203 3.2. Aliquot 5 µL of thawed, well-mixed cell lysates and of BSA standards to separate wells in a
204 clear 96-well plate.
205
206 3.3. Add 195 µL of Bradford reagent[34] to each well and measure absorbance at 595 nm (A_{595}) in
207 a plate reader (see **Table of Materials**).
208
209 3.4. Calculate the amount of protein in each well by comparison to the BSA standard curve,
210 using the formula $y = mx + b$, where y is A_{595}, x is µg of BSA, m is the slope of the standard
211 curve, and b is the y-intercept of the standard curve. Multiply the resulting value by 0.05 to
212 determine the total mg of protein in each sample.
213
214 **4. Extracting Polyphosphate**
215
216 4.1. Add 250 µL of 95% ethanol to each GITC-lysed sample and vortex to mix.
217
218 4.2. Apply that mixture to a silica membrane spin column and centrifuge for 30 s at 16,100 x g.
219
220 4.3. Discard the flow-through, then add 750 µL of 5 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM

221 EDTA, 50% ethanol and centrifuge for 30 s at 16,100 x g.
222
223 4.4. Discard the flow-through and centrifuge for 2 min at 16,100 x g.
224
225 4.5. Place the column in a clean 1.5 mL microfuge tube and add 150 µL of 50 mM Tris-HCl (pH
226 8).
227
228 4.6. Incubate at room temperature for 5 min, then elute polyP by centrifuging for 2 min at
229 8,000 x g.
230
231 NOTE: If desired, the eluates can be stored at -20 °C for at least 1 week.
232
233 **5. Digesting Polyphosphate**
234
235 5.1. Prepare standards containing 0, 5, 50, or 200 µM potassium phosphate in 50 mM Tris-HCl
236 (pH 8).
237
238 NOTE: Potassium phosphate standards can be stored indefinitely at room temperature.
239
240 5.2. Aliquot 100 µL of each phosphate standard and of extracted polyP samples into separate
241 wells of a clear 96-well plate.
242
243 5.3. Prepare a master mix containing (per sample): 30 µL of 5x ScPPX reaction buffer (100 mM
244 Tris-HCl, 25 mM $MgCl_2$, 250 mM ammonium acetate, pH 7.5)[13], 19 µL of H_2O, and 1 µL of
245 purified ScPPX (1 mg mL^{-1}).
246
247 5.4. Add 50 µL of the master mix to each well of the 96-well plate. Incubate for 15 min at 37 °C.
248
249 NOTE: If desired, the digested polyP samples can be stored at -20 °C indefinitely.
250
251 **6. Detecting Free Phosphate**[24]
252
253 6.1. Prepare detection solution base by dissolving 0.185 g of antimony potassium tartrate in
254 200 mL of water, adding 150 mL of 4 N H_2SO_4, then adding 1.49 g of ammonium
255 heptamolybdate. Stir to dissolve and then bring to a final volume of 456 mL. Filter the solution
256 to remove particulates and store protected from light at 4 °C for up to 1 month.
257
258 6.2. Prepare a stock solution of 1 M ascorbic acid. Store protected from light at 4 °C for up to 1
259 month.
260
261 6.3. Prepare a fresh working stock of detection solution each day by mixing 9.12 mL of
262 detection solution base with 0.88 mL of 1 M ascorbic acid. Allow the detection solution to come
263 to room temperature before use.
264

265 6.4. Add 50 µL of detection solution to each sample and standard in the 96-well plate and
266 incubate at room temperature for about 2 min to allow color development.
267
268 6.5. Measure absorbance at 882 nm with a plate reader and calculate the phosphate
269 concentration of each sample by comparison to the potassium phosphate standard curve.
270
271 CAUTION: The detection solution contains toxic salts and strong acids. Wear gloves and treat
272 excess solution as toxic waste.
273
274 **7. Calculating Cellular Polyphosphate Content**
275
276 7.1. Convert the phosphate concentrations determined in step 6.5 to nanomoles of polyP-
277 derived phosphate in each entire cell lysate according to the following formula:
278
279 $$\text{nmol polyP} = 1.5 \times (\mu\text{M phosphate} / 10)$$
280
281 NOTE: The standard curve-based method in step 6 determines the concentration of phosphate
282 (in µM) in each 100 µL polyP sample aliquoted in step 5.2. To convert this concentration to a
283 number of nmol, 100 µL is divided by 10^6 to give a volume in L, multiplied by the concentration
284 (µmol L^{-1}), then multiplied by 1000 (the number of nmol in a µmol). This reduces to dividing the
285 concentration by 10. The total extract volume prepared in step 4 is 150 µL, so it is necessary to
286 multiply the resulting value by 1.5 to calculate the nmol of phosphate present in the entire
287 extract.
288
289 7.2. Normalize cellular polyP content to total cellular protein by dividing nmol polyP by the mg
290 total protein in each sample determined in step 3.4. PolyP levels are expressed in terms of the
291 concentration of individual polyP-derived free phosphate.
292
293 NOTE: In some cases, the amount of polyP-derived phosphate present in a sample may fall
294 outside the linear range of the phosphate standard curve (step 6.5). If the levels of polyP are
295 very high, the excess polyP-containing eluate from step 4.6 can be diluted 1:10 or 1:100, as
296 necessary, and then measured again as described in steps 5 through 7.
297
298 **REPRESENTATIVE RESULTS:**
299 The key steps of the protocol are diagrammed in simplified form in **Figure 1**.
300
301 To demonstrate the use of this protocol with Gram-negative bacteria, wild-type *E. coli*
302 MG1655[39] was grown to mid-log phase in LB rich medium at 37 °C with shaking (200 rpm), then
303 rinsed and incubated for an additional 2 h in morpholinopropanesulfonate-buffered (MOPS)
304 minimal medium[40] containing 4 g L^{-1} glucose and 0.1 mM K_2HPO_4, a condition known to induce
305 production of polyP[14,29]. As shown in **Figure 2A**, we detected no polyP in wild-type *E. coli* grown
306 in LB and 192 ± 14 (mean ± standard deviation [SD]) nmol polyP mg^{-1} total protein in MOPS,
307 consistent with previous reports[14,29]. As expected, a Δ*ppk* mutant[6], which lacks polyP kinase[41],
308 produced no polyP in either medium, a Δ*ppx* mutant[6], which lacks exopolyphosphatase[42],

produced approximately the same amount of polyP as the wild-type, and a Δ*phoB* mutant[29], which is defective in phosphate transport, produced significantly less polyP than the wild-type[14,29,43].

To demonstrate the use of this protocol with Gram-positive bacteria, wild-type *L. reuteri* ATCC PTA 6475[36] and an isogenic *ppk1* null mutant lacking polyP kinase, constructed using oligo-directed recombineering[44], were grown overnight in MEI-C at 37 °C without shaking. As shown in **Figure 2B**, the wild-type accumulated 51 ± 6 nmol polyP mg^{-1} total protein under these conditions, while the *ppk1* mutant contained less than half this amount. *L. reuteri* contains a second polyP kinase, encoded by the *ppk2* gene[45], which presumably accounts for the polyP present in the *ppk1* null mutant.

To demonstrate the use of this protocol with mycobacteria, *Mycobacterium smegmatis* strain SMR5[46] was grown to mid-log phase in Hartmans-de Bont (HdB) medium[47], then rinsed and diluted five-fold in HdB or HdB with 2% ethanol. These cultures were then incubated overnight at 37 °C with shaking (180 rpm). As shown in **Figure 2C**, in the absence of ethanol, *M. smegmatis* accumulated 141 ± 52 nmol polyP mg^{-1} total protein, while ethanol treatment resulted in a three-fold increase to 437 ± 102 nmol polyP mg^{-1} total protein. This result was expected, since ethanol has previously been reported to elevate polyP levels in *M. smegmatis*[48].

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of polyphosphate extraction and measurement procedure. The essential steps of the polyP quantification protocol are illustrated. Bacterial cell pellets are lysed at 95 °C in GITC (guanidine isothiocyanate). Small aliquots of the lysates are assayed for protein content using the Bradford assay. PolyP is extracted using silica membrane columns and then digested with ScPPX. The resulting free phosphate is quantified using the ascorbic acid assay. Total polyP-derived phosphate is normalized to total protein for each sample. A_{595} = absorbance at 595 nm; A_{882} = absorbance at 882 nm.

Figure 2: Representative results with diverse bacteria. (**A**) *E. coli* MG1655 wild-type and isogenic Δ*ppk*, Δ*ppx*, and Δ*phoB* mutants were grown at 37 °C with shaking (200 rpm) to A_{600} = 0.2 - 0.4 in rich LB medium (black circles) and then shifted to minimal medium (MOPS containing 4 g L^{-1} glucose and 0.1 mM K_2HPO_4) for 2 h (white circles; n = 3, ± SD). (**B**) *L. reuteri* ATCC PTA 6475 (circles) and an isogenic *ppk1* null mutant (triangles) were grown overnight at 37 °C in MEI-C medium without shaking (n = 3, ± SD). (**C**) *M. smegmatis* SMR5 was grown at 37 °C with shaking (180 rpm) to A_{600} = 1 in HdB medium with (closed squares) or without (open squares) 2% ethanol (n = 3, ± SD). PolyP levels are expressed in terms of the concentration of individual polyP-derived free phosphate.

DISCUSSION:

The protocol described here simplifies and accelerates quantification of polyP levels in diverse bacteria, with a typical set of 24 samples taking about 1.5 h to fully process. This permits rapid screening of samples and analysis of mutant libraries, and simplifies kinetic experiments

353 measuring the accumulation of polyP over time. We have demonstrated that the protocol
354 works effectively on representatives of three different phyla: proteobacteria, firmicutes, and
355 actinobacteria, which are notorious for their resilient, difficult nature to lyse cell walls[49].
356
357 Detection of polyP by digestion with ScPPX is more specific and more sensitive than
358 fluorescence detection with DAPI, and is cheaper, more technically straightforward than
359 conversion of polyP to ATP using PPK. Although ScPPX is inhibited by DNA and RNA[12], we have
360 overcome this by using a very large excess of enzyme (1 μg per sample) to achieve complete
361 digestion even in bacteria containing more than 6000 nmol polyP mg^{-1} protein[29]. Other
362 published methods use 10 to 15 ng of ScPPX per reaction[12,24]. Our protocol for ScPPX
363 purification typically yields more than 5 mg of pure ScPPX per liter of overexpression culture,
364 which is enough for more than 5000 assays. We have found that several different protocols for
365 nickel-affinity purification of His-tagged proteins work well to purify ScPPX overexpressed from
366 pScPPX2 (data not shown), and there are a wide variety of such protocols available to suit labs
367 with varying technical capacities.
368
369 Previous protocols using ScPPX digestion for polyP detection have often relied on malachite
370 green-based colorimetric assays to quantify free phosphate[6,12]. These assays are sensitive, but
371 typically require carefully timed sequential addition of two or three separate reagents to make
372 accurate measurements[24,50,51]. The ascorbic acid-based detection system used here[24] has a
373 wider linear range of detection than malachite green with no loss of sensitivity, only involves
374 addition of a single reagent, and does not require precise timing.
375
376 There are several steps that are critical to the success of this protocol. First, bacterial samples
377 should be lysed in GITC immediately after harvesting, to ensure that polyP levels do not change
378 after the time of sampling and to rapidly inactivate cellular phosphatases. Second, do not
379 incubate GITC lysates at 95 °C for more than 10 min, and store them immediately afterwards at
380 -80 °C to minimize possible polyP hydrolysis. Third, be careful when pipetting samples into 96-
381 well plates for either protein or phosphate measurements not to splash or drip anything from
382 one sample into another. The colorimetric assays, particularly for phosphate, are very sensitive,
383 and small amounts of contamination can strongly skew the results. Finally, some reagents used
384 in this protocol (*i.e.* ScPPX, detection solution) have limited shelf lives. Prepare fresh ScPPX
385 every 6 months and fresh detection solution every month.
386
387 One limitation of our method is that silica columns do not bind polyP less than 60 phosphate
388 units long very well[12,14,15]. Although bacteria typically synthesize mostly long-chain polyP[3], we
389 recommend preliminary experiments using polyacrylamide gel electrophoresis[27,30] to assess
390 chain length in a given bacterial species before using our procedure. For species where short-
391 chain polyP makes up a substantial fraction of the polyP pool, our protocol is not appropriate
392 and we recommend extracting polyP by a different method (*e.g.*, phenol-chloroform
393 extraction[12]), and would also recommend supplementing ScPPX digestion with commercially
394 available *S. cereviseae* pyrophosphatase (PPA1)[24], since ScPPX does not digest pyrophosphate[13]
395 and this has a proportionally higher impact on measurements of shorter-chain polyP. As an
396 alternative to ScPPX, acid hydrolysis[38] could be used to hydrolyze polyP to free phosphate, but

397 this would require additional DNase, RNase, and purification steps to ensure that only polyP-
398 derived phosphate was being measured. In some cases, it may be useful to use an alternative
399 extraction method to test whether the efficiency of polyP extraction with GITC varies between
400 strains or conditions, particularly with bacteria known to have thick cell walls, such as
401 mycobacteria. If levels of polyP below the limit of detection of this assay are important for
402 studies of a particular species, it may be necessary to use a different detection scheme, such as
403 using PPK to convert polyP to ATP, which can be detected extremely sensitively using
404 commercially available luciferase-based assays[14,17,18].
405
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410
411 **DISCLOSURES:**
412 The authors have nothing to disclose.
413
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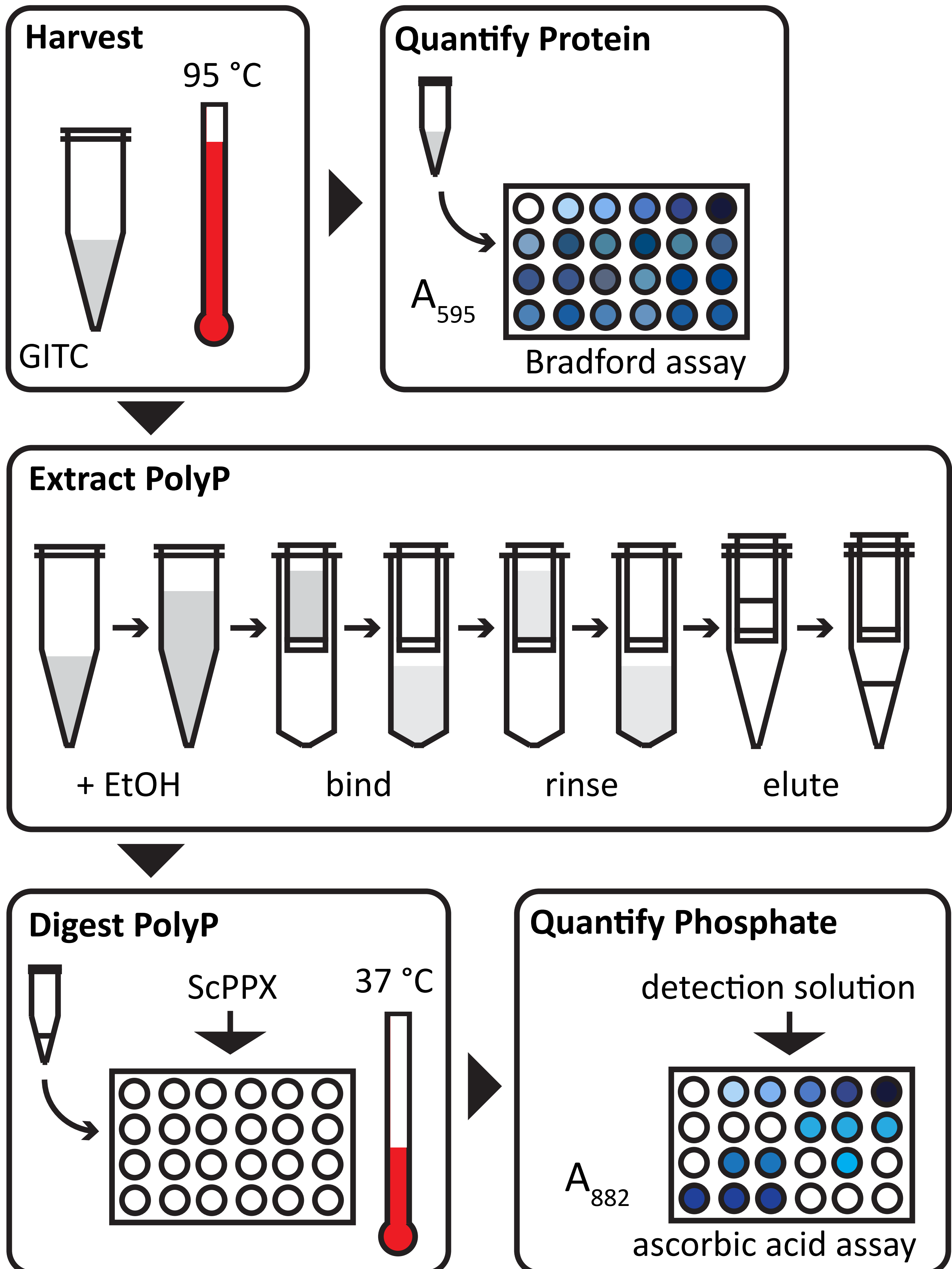

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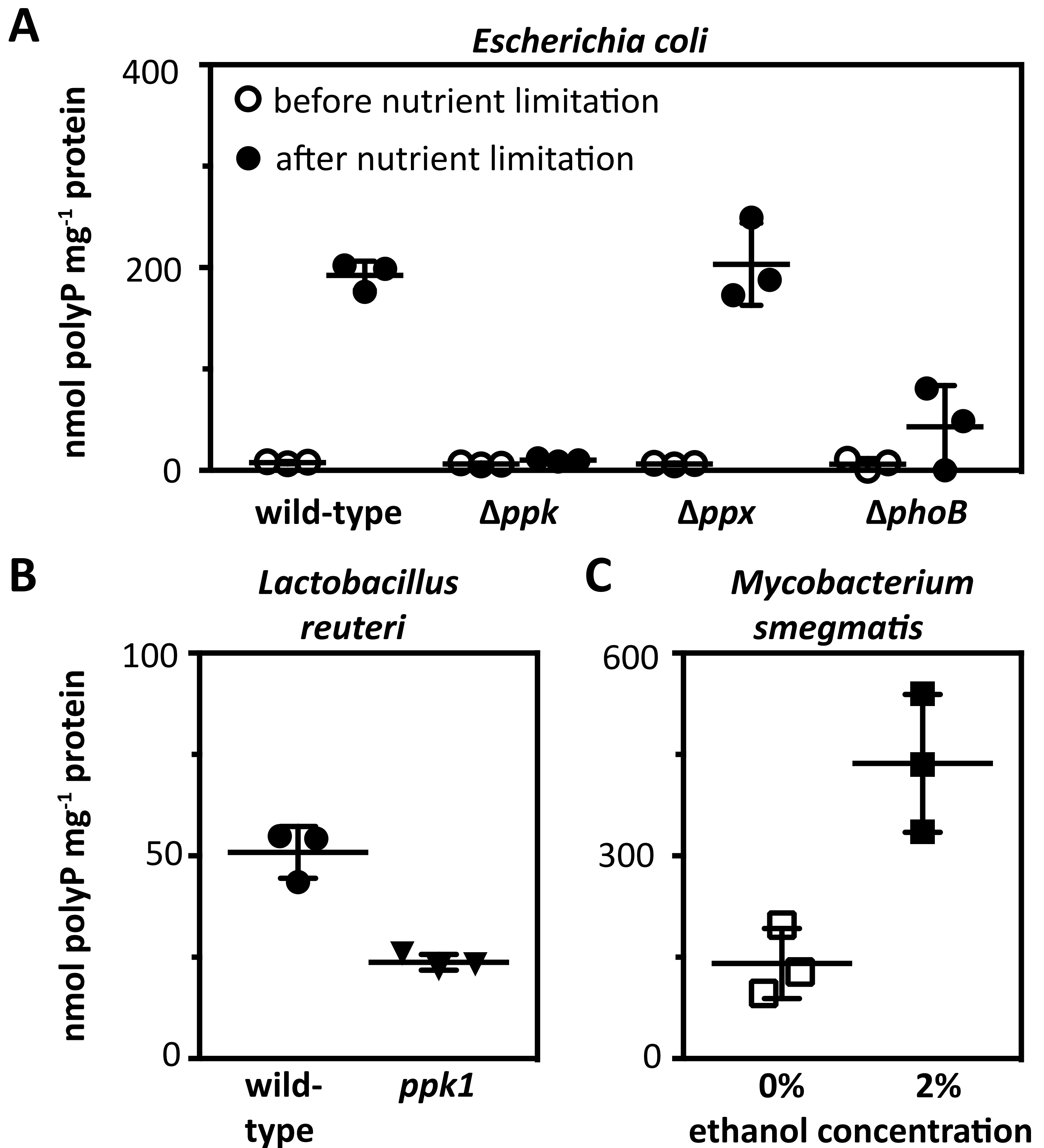

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
E. coli BL21(DE3)	Millipore Sigma	69450	
plasmid pScPPX2	Addgene	112877	available to academic and other non-profit institutions
LB broth	Fisher Scientific	BP1427-2	*E. coli* growth medium
ampicillin	Fisher Scientific	BP176025	
isopropyl β-D-1-thiogalactopyranoside (IPTG)	Gold Biotechnology	I2481C	
HEPES buffer	Gold Biotechnology	H-400-1	
potassium hydroxide (KOH)	Fisher Scientific	P250500	for adjusting the pH of HEPES-buffered solutions
sodium chloride (NaCl)	Fisher Scientific	S27110	
imidazole	Fisher Scientific	O3196500	
lysozyme	Fisher Scientific	AAJ6070106	
magnesium chloride ($MgCl_2$)	Fisher Scientific	BP214-500	
Pierce Universal Nuclease	Fisher Scientific	PI88700	Benzonase (Sigma-Aldrich cat. # E1014) is an acceptable substitute
Model 120 Sonic Dismembrator	Fisher Scientific	FB-120	other cell lysis methods (e.g. French Press) can also be effective
5 mL HiTrap chelating HP column	GE Life Sciences	17040901	any nickel-affinity chromatography column or resin could be substituted
nickel(II) sulfate hexahydrate	Fisher Scientific	AC415611000	for charging HiTrap column
0.8 µm pore size cellulose acetate syringe filters	Fisher Scientific	09-302-168	
Bradford reagent	Bio-Rad	5000205	
Tris buffer	Fisher Scientific	BP1525	

Spectrum Spectra/Por 4 RC Dialysis Membrane Tubing 12,000 to 14,000 Dalton MWCO	Fisher Scientific	08-667B	other dialysis membranes with MWCO < 30,000 Da should also work
hydrochloric acid (HCl)	Fisher Scientific	A144-212	for adjusting the pH of Tris-buffered solutions
potassium chloride (KCl)	Fisher Scientific	P217500	
glycerol	Fisher Scientific	BP2294	
10x MOPS medium mixture	Teknova	M2101	*E. coli* growth medium
glucose	Fisher Scientific	D161	
monobasic potassium phosphate (KH_2PO_4)	Fisher Scientific	BP362-500	
dibasic potassium phosphate (K_2HPO_4)	Fisher Scientific	BP363-500	
dehydrated yeast extract	Fisher Scientific	DF0886-17-0	
tryptone	Fisher Scientific	BP1421-500	
magnesium sulfate heptahydrate	Fisher Scientific	M63-50	
manganese sulfate monohydrate	Fisher Scientific	M113-500	
guanidine isothiocyanate	Fisher Scientific	BP221-250	
bovine serum albumin (protease-free)	Fisher Scientific	BP9703100	
clear flat bottom 96-well plates	Sigma-Aldrich	M0812-100EA	any clear 96-well plate will work
Tecan M1000 Infinite plate reader	Tecan, Inc.	not applicable	any plate reader capable of measuring absorbance at 595 and 882 nm will work
ethanol	Fisher Scientific	04-355-451	
silica membrane spin columns	Epoch Life Science	1910-050/250	
ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific	BP120500	
1.5 mL microfuge tubes	Fisher Scientific	NC9580154	

ammonium acetate	Fisher Scientific	A637-500
antimony potassium tartrate	Fisher Scientific	AAA1088922
4 N sulfuric acid (H_2SO4)	Fisher Scientific	SA818-500
ammonium heptamolybdate	Fisher Scientific	AAA1376630
ascorbic acid	Fisher Scientific	AC401471000

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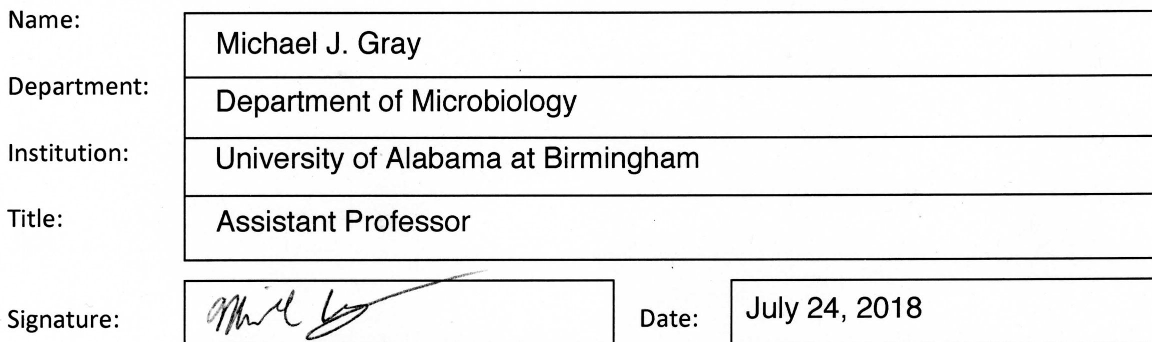

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We thank the editor and reviewers for their helpful and thoughtful comments. We have addressed the stated concerns, and this document contains a point-by-point response to those comments.

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.

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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.

<u>Reviewers' comments:</u>
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.