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## Purification and Quality Control of Recombinant Septin Complexes for Cell-Free Reconstitution

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1 **TITLE:**  
2 Purification and Quality Control of Recombinant Septin Complexes for Cell-Free Reconstitution  
3

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25 **SUMMARY:**

26 *In vitro* reconstitution of cytoskeletal proteins is a vital tool to understand the basic functional  
27 properties of these proteins. The present paper describes a protocol to purify and assess the  
28 quality of recombinant septin complexes, which play a central role in cell division and migration.  
29

30 **ABSTRACT:**

31 Septins are a family of conserved eukaryotic GTP-binding proteins that can form cytoskeletal  
32 filaments and higher-order structures from hetero-oligomeric complexes. They interact with  
33 other cytoskeletal components and the cell membrane to participate in important cellular  
34 functions such as migration and cell division. Due to the complexity of septins' many interactions,  
35 the large number of septin genes (13 in humans), and the ability of septins to form hetero-  
36 oligomeric complexes with different subunit compositions, cell-free reconstitution is a vital  
37 strategy to understand the basics of septin biology. The present paper first describes a method  
38 to purify recombinant septins in their hetero-oligomeric form using a two-step affinity  
39 chromatography approach. Then, the process of quality control used to check for the purity and  
40 integrity of the septin complexes is detailed. This process combines native and denaturing gel  
41 electrophoresis, negative stain electron microscopy, and interferometric scattering microscopy.  
42 Finally, a description of the process to check for the polymerization ability of septin complexes  
43 using negative stain electron microscopy and fluorescent microscopy is given. This demonstrates

44 that it is possible to produce high-quality human septin hexamers and octamers containing  
45 different isoforms of septin\_9, as well as *Drosophila* septin hexamers.

46

#### 47 **INTRODUCTION:**

48 The cytoskeleton has been classically described as a three-component system consisting of actin  
49 filaments, microtubules, and intermediate filaments<sup>1</sup>, but recently, septins have been  
50 acknowledged as a fourth component of the cytoskeleton<sup>1</sup>. Septins are a family of GTP-binding  
51 proteins that are conserved in eukaryotes<sup>2</sup>. Septins are involved in many cellular functions such  
52 as cell division<sup>3</sup>, cell-cell adhesion<sup>4</sup>, cell motility<sup>5</sup>, morphogenesis<sup>6</sup>, cellular infection<sup>7</sup>, and the  
53 establishment and maintenance of cell polarity<sup>8</sup>. Despite their important functions, how septins  
54 are involved in such processes is poorly understood.

55

56 The septin family of proteins is subdivided into several subgroups (four or seven, depending on  
57 the classification) based on protein sequence similarity<sup>2</sup>. Members of different subfamilies can  
58 form palindromic hetero-oligomeric complexes, which are the building blocks of filaments and  
59 which, in turn, assemble into higher-order structures such as bundles, rings, and meshworks<sup>1,9–  
60 12</sup>. Further molecular complexity arises from the presence of different splice variants, an example  
61 being human SEPT9, where there is evidence for specific functions of different splice variants<sup>13–  
62 15</sup>. Additionally, the length of the hetero-oligomers depends on species and cell type. For  
63 instance, *Caenorhabditis elegans* septins form tetramers<sup>16</sup>, *Drosophila melanogaster* septins  
64 form hexamers<sup>17</sup> (**Figure 1A**), *Saccharomyces cerevisiae* septins form octamers<sup>18</sup>, and human  
65 septins form both hexamers and octamers<sup>19</sup> (**Figure 1A**). The ability of septin isoforms and splice  
66 variants from the same subfamily to substitute each other in the complex and the (co-)existence  
67 of differently sized hetero-oligomers have made it difficult to delineate the cellular functions of  
68 different hetero-oligomeric complexes<sup>12</sup>.

69

70 Another interesting ability of septins is their ability to interact with many binding partners in the  
71 cell. Septins bind the plasma membrane and membranous organelles during interphase and cell  
72 division<sup>20–22</sup>. In dividing cells, septins cooperate with anillin<sup>23–25</sup> and actin and myosin during  
73 cytokinesis<sup>26,27</sup>. At the late stages of cytokinesis, septins seem to regulate the endosomal sorting  
74 complexes required for the transport (ESCRT) system for midbody abscission<sup>28</sup>. Additionally,  
75 there is also evidence of septin located on the actin cortex and actin stress fibers of cells in  
76 interphase cells<sup>29–31</sup>. In specific cell types, septins also bind and regulate the microtubule  
77 cytoskeleton<sup>32,33</sup>.

78

79 All of these features make septins a very interesting protein system to study, but also a  
80 challenging one. The combination of the large number of septin subunits (13 genes in humans  
81 without counting splice variants<sup>2</sup>) with the potential of septin subunits from the same subfamily  
82 to substitute each other and form differently sized hetero-oligomers makes it difficult to draw a  
83 conclusion on the cellular function of a specific septin by genetic manipulation. Furthermore, the  
84 multiple interactions of septins make interpreting the effects of common research tools such as  
85 drugs<sup>34</sup> directed at cytoskeletal or membrane components a hard task.

86

87 A way to overcome this situation is to complement research in cells with *in vitro* (cell-free)  
88 reconstitution of septins. *In vitro* reconstitution allows for the isolation of a single type of septin  
89 hetero-oligomers with a specific subunit composition and length<sup>18,35–37</sup>. This complex can then  
90 be studied in a controlled environment, either alone to discover the basic structural and  
91 physicochemical properties of septins<sup>38–40</sup>, or in combination with desired partners such as model  
92 biomembranes<sup>11,41,42</sup>, actin filaments<sup>10,27</sup>, or microtubules<sup>32,36</sup> to decipher the nature of their  
93 interactions.

94  
95 Therefore, a reliable method to purify different septin complexes efficiently is vital for septin  
96 research. However, even using the same protocol, different purifications can give proteins with  
97 different activity/functionality or even integrity. For commercially available proteins such as  
98 enzymes, the functionality and enzymatic activity are carefully validated<sup>43</sup>. Implementing careful  
99 quality control for cytoskeletal or structural proteins such as septins can be challenging, but it is  
100 essential to make experiments across labs comparable.

101  
102 This paper describes a robust method to purify high-quality recombinant septins in their hetero-  
103 oligomeric form based on the simultaneous expression of two vectors containing mono- or bi-  
104 cistronic constructs (**Table 1**) in *Escherichia coli* cells. The method consists of a two-step affinity  
105 chromatography approach to capture septin hetero-oligomers containing both a his<sub>6</sub>-tagged  
106 septin and a Strep-II-tagged septin (**Figure 1B,C**). This protocol, first described in Iv et al.<sup>10</sup>, has  
107 been used to purify *Drosophila* septin hexamers<sup>11,27,35</sup>, human septin hexamers<sup>10</sup>, and several  
108 human septin octamers containing different native (isoform 1, 3, and 5)<sup>10,32</sup> or mutated SEPT9  
109 isoforms<sup>32</sup>. Furthermore, a description of a set of techniques to assess the quality of the purified  
110 septins is given. First, the integrity and correct stoichiometry of the septin subunits is checked  
111 using denaturing electrophoresis and transmission electron microscopy (TEM). Then, the  
112 presence of hetero-oligomers of the correct molecular mass and the presence of monomers or  
113 smaller oligomers indicative of complex instability are examined by native electrophoresis and  
114 mass photometry via interferometric scattering microscopy (iSCAT). Finally, the last step consists  
115 of the assessment of the polymerizing activity of the septins using fluorescence microscopy and  
116 TEM.

117  
118 [Place **Figure 1** here]

119  
120 **PROTOCOL:**

121  
122 **1. Purification of septin hetero-oligomers**

123  
124 1.1. Co-transformation of bacterial cells with the expression vectors

125  
126 1.1.1. Select a combination of one pnEA and one pnCS plasmid<sup>44</sup> that will be used for expression.  
127 Choose the combination depending on the desired subunit composition of the septin hetero-  
128 oligomer<sup>10,35</sup> and whether or not fluorescent tagging is required.

129

130 NOTE: C-terminally tagged monomeric superfolder GFP (msfGFP)-tagged SEPT2 (for human  
131 septins) or msfGFP- or monomeric enhanced GFP (mEGFP)-DSep2 (for *Drosophila* septins) is used  
132 here (**Table 1**).

133

134 1.1.2. Pipette 1  $\mu\text{L}$  of each plasmid ( $\sim 1 \text{ ng}/\mu\text{L}$ ) into 100  $\mu\text{L}$  of competent BL21 *Escherichia coli* cells  
135 and incubate on ice for 20 min.

136

137 1.1.3. Place the cells in a water bath at 42  $^{\circ}\text{C}$  for 40 s and then immediately incubate them for 3  
138 min on ice.

139

140 1.1.4. Add 0.9 mL of lysogeny broth (LB) medium to the cell suspension and let the cells grow for  
141 1 h at 37  $^{\circ}\text{C}$ . Plate 100  $\mu\text{L}$  of cells on warm LB-agar plates containing 100  $\mu\text{g}/\text{mL}$  ampicillin and  
142 100  $\mu\text{g}/\text{mL}$  spectinomycin and incubate overnight at 37  $^{\circ}\text{C}$ .

143

144 1.2. Grow bacterial pre-culture

145

146 1.2.1. Fill a 250 mL Erlenmeyer flask with 100 mL of Terrific broth (TB) or LB medium containing  
147 100  $\mu\text{g}/\text{mL}$  ampicillin and 100  $\mu\text{g}/\text{mL}$  spectinomycin.

148

149 1.2.2. Pick out a single colony from the LB-agar plate with a sterile inoculation loop and transfer  
150 it to fresh media from step 1.2.1.

151

152 1.2.3. Incubate at 37  $^{\circ}\text{C}$  in a rotary shaker incubator, either overnight or for at least 6 h.

153

154 NOTE: From this culture, a glycerol stock can be prepared by mixing the bacterial suspension 1:1  
155 with glycerol and stored at  $-80^{\circ}\text{C}$ . This stock can be used in step 1.2.2. instead of a freshly  
156 transformed colony.

157

### 158 1.3. Bacterial culture and protein expression induction

159

160 1.3.1. Transfer 100 mL of grown bacteria into 5 L of TB or LB containing 50  $\mu\text{g}/\text{mL}$  ampicillin and  
161 50  $\mu\text{g}/\text{mL}$  spectinomycin.

162

163 1.3.2. Grow this culture at 37  $^{\circ}\text{C}$  in a shaker incubator until it reaches an optical density (OD)  
164 measured at a wavelength of 600 nm in the range of 2–3 for unlabeled septins or 0.6–0.8 for  
165 msfGFP/mEGFP-labelled septins and induce protein expression by adding a final concentration  
166 of 0.5 mM IPTG. The lower OD for the labeled septins is to avoid reaching the death phase in their  
167 longer expression time, as detailed in the next step.

168

169 1.3.3. Incubate the cells expressing unlabeled septin hetero-oligomers for 3 h at 37  $^{\circ}\text{C}$  or the cells  
170 expressing msfGFP-labelled hetero-oligomers overnight at 17  $^{\circ}\text{C}$ .

171

172 NOTE: The short protein expression time for unlabeled complexes, facilitated by the use of the  
173 richer TB medium, is chosen to prevent protein degradation. The longer expression time

174 combined with lower temperature for labeled complexes is chosen to allow for correct folding of  
175 the msfGFP tag.

176

#### 177 1.4. Bacterial lysis and lysate clarification

178

179 NOTE: From this point onwards in the purification procedure, keep the protein-containing  
180 solution on ice or at 4 °C at all times to prevent proteolytic protein degradation or loss of activity.

181

182 1.4.1. Collect the cultured cells by centrifuging at 4,000 x *g* for 20 min at 4 °C. Discard the  
183 supernatant.

184

185 1.4.1.1. Optionally, snap-freeze the pellet in this step and store at –80 °C for around 6 months. If  
186 this option is chosen, make sure to thaw the pellet on ice before continuing.

187

188 1.4.2. Dissolve the pellet in 100 mL of lysis buffer (**Table 2**) and lyse the cells. Choose one of the  
189 two options below

190

191 1.4.2.1. Sonicate in 7 cycles of 30 s ON and 59 s OFF with a tip sonicator using 30% amplitude  
192 (note that the settings are sonicator-dependent).

193

194 1.4.2.2. Break down the cells in the French press by passing them at least 3x.

195

196 1.4.3. Clarify the cell lysate by centrifuging at 20,000 x *g* for 30 min at 4 °C and keep the  
197 supernatant. It is recommended to start with step 1.5.1. during this centrifugal step.

198

199 1.4.4. Optionally, take a sample for denaturing electrophoresis, as described in section 2.

200

#### 201 1.5. Affinity chromatography for His-tagged proteins

202

203 NOTE: This step yields complexes containing human SEPT2 or *Drosophila* Sep1 using a nickel  
204 column (**Figure 1B**).

205

206 1.5.1. Equilibrate a pre-packed nickel sepharose high-performance chromatography column with  
207 septin buffer (**Table 2**).

208

209 1.5.2. Load the clarified supernatant onto the column at 1 mL/min and wash the bound protein  
210 with at least three column volumes of septin buffer.

211

212 1.5.3. Elute the septin complexes with 50% HisTrap elution buffer (**Table 2**) at 1 mL/min while  
213 collecting 0.5 mL fractions to yield an imidazole concentration of 250 mM.

214

215 1.5.4. Pick the fractions containing septin complexes, as indicated by the optical absorbance of  
216 the eluate at 280 nm monitored online with a fast protein liquid chromatography (FPLC) system  
217 or after the purification with a microvolume spectrophotometer.

218

219 NOTE: Imidazole absorbs light at 280 nm. This probably explains why the protein peak does not  
220 go back to zero absorbance after the septin elution (**Figure 2A**).

221

## 222 1.6. Affinity chromatography for Strep-II-tagged proteins

223

224 NOTE: This step yields complexes containing either human SEPT7 (hexamers), human SEPT9  
225 (octamers), or *Drosophila* peanut using a Strep-Tactin column (**Figure 1B**). The chromatography  
226 column is based on a modified biotin-streptavidin system. The protein is tagged with modified  
227 biotin (Strep-II-tag), and the column contains an engineered streptavidin (Strep-Tactin). Despite  
228 being modified from the biotin-streptavidin system, there is no interference between the Strep-  
229 Tactin-Strep-II-tag system and the biotin-streptavidin system. The described system is used to  
230 avoid interference with reconstitution assays using biotin and streptavidin.

231

232 1.6.1. Equilibrate a pre-packed StrepTactin sepharose high-performance chromatography  
233 column with septin buffer (**Table 2**). Load the septin-containing fractions recovered from the  
234 nickel column at 1 mL/min and wash the bound protein with at least three column volumes of  
235 septin buffer.

236

237 1.6.2. Elute the septin complexes with 100% StrepTrap elution buffer (**Table 2**) at 1 mL/min while  
238 collecting 0.5 mL fractions to yield a concentration of 2.5mM desthiobiotin.

239

240 NOTE: The desthiobiotin in the StrepTrap elution buffer must be dissolved fresh.

241

242 1.6.3. Pick the fractions containing septin complexes, as indicated by the optical absorbance of  
243 the eluate at 280 nm monitored online with an FPLC system or after the purification with a  
244 microvolume spectrophotometer.

245

246 NOTE: The denaturing electrophoresis is usually done at this point with samples of the column  
247 washings and septin fractions. The order of columns can be inverted with indistinguishable  
248 results, i.e., the clarified lysate after step 1.4. can be subjected to Strep-Tactin affinity  
249 chromatography followed by nickel affinity chromatography.

250

## 251 1.7. Dialysis and storage

252

253 1.7.1. To remove the desthiobiotin from the final storage solution, dialyze the septin complexes  
254 in a ~1:300 sample-to-buffer volume ratio against septin buffer (**Table 2**) supplemented with 1  
255 mM DTT overnight, or for at least 4 h, at 4 °C using a 30 kDa MWCO dialysis membrane.

256

257 1.7.2. Optionally, concentrate the septins using a 30 kDa MWCO centrifugal concentration  
258 column up to the desired concentration. Aim for a concentration of 5–7 µM, as measured via the  
259 solution optical absorbance at 280 nm and using a theoretical extinction coefficient calculated  
260 via ProtParam (**Table 3**).

261

262 1.7.3. Aliquot the protein complexes into the desired aliquot size, snap-freeze the aliquot, and  
263 store it at  $-80^{\circ}\text{C}$ .

264  
265 NOTE: It is recommended not to store the protein for more than 6 months. In addition, it is  
266 recommended to perform regular quality control experiments, especially if the protein is stored  
267 for longer than the recommended time.

## 268 **2. Quality control of purity and integrity of the septin hetero-oligomer**

270  
271 NOTE: The hetero-oligomer quality control consists of a set of biochemical and imaging  
272 techniques that allow for the detection of the mass and integrity of the septin complexes present  
273 in the solution.

274  
275 2.1. Denaturing electrophoresis to check for the formation of the septin hetero-oligomer with  
276 the correct components

277  
278 2.1.1. Mix  $10\ \mu\text{L}$  of the selected fractions with  $10\ \mu\text{L}$  of 2x SDS sample buffer, load them onto a  
279 precast 4%–15% TGX gel, and fill the system with Tris/glycine/SDS running buffer.

280  
281 2.1.2. Run the electrophoresis for 35 min at 200 V and stain the gel (**Table of Materials**) to  
282 visualize the results. The molecular weights of the individual septin proteins and septin hetero-  
283 oligomeric complexes can be found in **Table 3**.

284  
285 2.1.3. Measure the relative intensity of each band inside each lane containing purified septins in  
286 a contrast-inverted image. Do this by calculating the mean intensity of equally sized rectangles  
287 around each band and of an equally sized rectangle on a region without any band in the same  
288 lane. Then, normalize the values by dividing the intensity of each band by the intensity of the  
289 region without bands.

290  
291 NOTE: If the intensity is saturated (for example, values of 255 for an 8-bit image on a contrast-  
292 inverted image), skip the lane.

293  
294 2.2. Ensemble-averaged native size distribution *via* native electrophoresis

295  
296 2.2.1. Prepare 800 mL of anode buffer and 200 mL of light blue cathode buffer the day before  
297 and store them in the fridge. To prepare the anode buffer, dilute 40 mL of 20x running buffer  
298 with 760 mL of type-I deionized water (I-water). To prepare the light blue cathode buffer, dilute  
299 10 mL of 20x running buffer and 1 mL of 20x cathode additive with 189 mL of I-water. The running  
300 buffer and cathode additive come with a kit (**Table of Materials**).

301  
302 2.2.2. Prepare  $10\ \mu\text{L}$  of the sample by mixing  $\sim 500\ \text{ng}$  of septin with the needed amount of sample  
303 buffer ( $2.5\ \mu\text{L}$  in this case, due to the use of a 4x sample buffer; see **Table of Materials**) and  
304 enough I-water to reach a volume of  $10\ \mu\text{L}$ .

305

306 2.2.3. Load the samples onto the gel and fill the system with the ice-cold anode and cathode  
307 buffers.

308  
309 2.2.4. Run the electrophoresis for around 115 min at 150 V with a power supply that does not  
310 stop at low currents and stain the gel (**Table of Materials**) to visualize the results. The molecular  
311 weights of the single proteins and complexes calculated based on the sequence can be found in  
312 **Table 3**.

313  
314 2.3. Single-molecule mass distribution using mass photometry via interferometric scattering  
315 microscopy

316  
317 2.3.1. Wash #1.5 glass slides by sonicating them in an ultrasonic cleaner for 5 min in I-water, 5  
318 min in isopropanol, and finally, 5 min in I-water.

319  
320 2.3.2. Dry two glass slides with a gentle stream of nitrogen gas and place a 7  $\mu$ L drop of 0.01%  
321 Poly-L-Lysine (PLL) solution on the center of one of the slides. Then, place the center of the other  
322 slide on top of the PLL drop, orienting the two slides orthogonally for easy separation. Incubate  
323 for 30 s.

324  
325 2.3.2.1. Wash by immersing in a beaker with I-water 1x and by directly applying a stream of I-  
326 water 2x. Then, dry the two slides with a flow of nitrogen gas. These slides can be stored  
327 afterward for around 6 weeks at room temperature in dry conditions.

328  
329 NOTE: Label the side of the slide that is treated with the PLL-PEG to correctly run the experiment.

330  
331 2.3.3. Just before the experiment, cut a piece of 2 x 2, 3 x 2, or 3 x 3 gaskets (that yield 4, 6, or 9  
332 imaging chambers/slide, respectively) and stick it on the PLL-PEG-treated part of a glass slide  
333 while avoiding the glass slide and the gaskets contacting any dirty surface. Place the slide on a  
334 light-duty wiper tissue and press on the gaskets with a pipette tip to stick them with the  
335 protecting plastic still on the gaskets.

336  
337 2.3.4. Warm septin buffer (**Table 2**) to room temperature and thaw the proteins in hand (keep  
338 them on ice afterward).

339  
340 NOTE: iSCAT shows the signal of some detergents and small molecules that resemble protein  
341 signals<sup>45</sup>. DTT is one of those small molecules, and that is why it is not used for this experiment.  
342 There is only a trace of DTT coming from the stored septin.

343  
344 2.3.5. Place the slide with gaskets on the commercial mass photometry system containing 19  $\mu$ L  
345 of septin buffer and focus the microscope using the autofocus option. Follow the manufacturer's  
346 instructions to check if the found focus is correct.

347 2.3.5.1. Optimize the frame rate and the exposure time by pressing on **Optimize illumination** and  
348 refocus with the autofocus option with the new settings. The standard 100x objective that is part  
349 of the setup is used here.

350  
351 2.3.6. Create or load a project folder to store the data using **File > New Project** or **File > Load**  
352 **Project**.

353  
354 2.3.7. Pipette 1  $\mu\text{L}$  of sample onto the 19  $\mu\text{L}$  of septin buffer drop (step 2.3.5) used to focus and  
355 mix while minimizing the movement of the slide by not touching anything while doing so. Then,  
356 record a 6,000-frame video by clicking on **Record**.

357  
358 2.3.7.1. For correct analysis, record the following samples: septin buffer, protein mass standard  
359 for the calibration of the signal-to-mass ratio (if a recent calibration is available and the  
360 environmental conditions have not changed, this sample can be skipped), and 250 nM of septin  
361 complexes diluted in septin buffer without DTT (this gives a final concentration of  $\sim 12.5$  nM).

362  
363 2.3.8. Analyze the videos using the manufacturer's software to obtain the protein mass  
364 distribution. Check for good quality data as follows.

365  
366 2.3.8.1. If the peaks of different septin hetero-oligomers sizes are overlapping too much or too  
367 many events are detected ( $>3,500$  events for a 6,000-frames video with the regular field of view  
368 of 128 pixels x 34 pixels spanning  $10.8 \mu\text{m}$  x  $2.9 \mu\text{m}$ ), decrease the final septin concentration and  
369 measure again.

370  
371 2.3.8.2. If there are not enough counts of single molecules measured (at least 2,500–3,500 for a  
372 6,000-frame video with the regular field of view), increase the septin concentration and measure  
373 again.

374  
375 2.4. Direct imaging of septin complexes via negative stain transmission electron microscopy

376  
377 2.4.1. Dilute samples to a concentration of about 50 nM in septin buffer and prepare the staining  
378 solution (2% uranyl formate or uranyl acetate in I-water).

379  
380 NOTE: Uranyl formate must be prepared fresh.

381  
382 2.4.2. Pipette 4  $\mu\text{L}$  of diluted septins onto a glow discharged electron microscopy grid and  
383 incubate for 30 s.

384  
385 2.4.3. Remove most of the protein solution using a filter paper and wash the grid 2x with septin  
386 buffer and 1x with I-water to remove loosely adsorbed septins.

387  
388 2.4.4. Stain with 2% uranyl acetate or uranyl formate solution in I-water for 1 min, absorb the  
389 staining solution with a filter paper, and air-dry the grid for a few minutes.

390  
391 2.4.5. Screen the grid using a properly aligned transmission electron microscope to search for  
392 regions of enhanced stain and collect about 100 images within these selected areas.

393

394 2.4.6. Collect images at a magnification of at least 50,000x to obtain a pixel size of about 2 Å/pixel  
395 and with a defocus varying from  $-1\ \mu\text{m}$  to  $-2\ \mu\text{m}$ . Use an acceleration voltage of 200 kV.  
396 Preferably, use an automated procedure to collect the data, which will depend on the acquisition  
397 software available.

398

399 2.4.7. Perform 2D image processing using dedicated software

400

401 2.4.7.1. Box out at least 2,000 particles using a dedicated software<sup>46</sup>.

402

403 2.4.7.2. Perform two-dimensional alignment and classification iteratively until classes are  
404 obtained without further improvement. The first alignment and classification step should be  
405 reference-free to avoid any bias in the classification.

406

407 2.4.7.3. Use the averages obtained from the first reference-free classification as new references  
408 to carry out an extra round of classification. Repeat this process iteratively until no further  
409 improvement is achieved. Ensure that each class is based on 50 to 100 picked particles, and  
410 individual subunits are clearly visible. Different software tools can be used (Spider, Eman, or  
411 Relion)<sup>46-48</sup>.

412

### 413 **3. Septin functional quality control via polymerization analysis**

414

415 NOTE: The functionality quality control consists of a set of imaging techniques that allow for the  
416 detection of polymerized septin complexes. Below, unlabelled septins are referred to as “dark”  
417 septins, and the buffer used for polymerizing unlabelled septins is referred to as “dark” septin  
418 polymerization buffer (SPB).

419

420 3.1. Septin bundle imaging via fluorescence microscopy

421

422 3.1.1. Prepare the 5x fluoSPB (**Table 2**) and a septin mix consisting of 90% dark septin and 10%  
423 msfGFP-septin at six times higher concentration than the desired final concentration in septin  
424 buffer + 1 mM DTT. A typical concentration for this assay is 300 nM, and, therefore, the  
425 concentration is 1,800 nM for this mix.

426

427 3.1.2. Polymerize the septin by mixing, in this specific order, I-water (enough to top up to the  
428 final desired volume), 20% 5xfluoSPB (a final dilution of 1:5), 0.05  $\mu\text{M}$  PCD, and 16.67% septin  
429 mix (a final dilution of 1:6). For 10  $\mu\text{L}$ , mix 6.23  $\mu\text{L}$  of I-water, 2  $\mu\text{L}$  of 5xfluoSPB, 0.1  $\mu\text{L}$  of PCD  
430 (with a stock of 5  $\mu\text{M}$ ), and 1.67  $\mu\text{L}$  of septin mix. Incubate this mix for at least 30 min at room  
431 temperature.

432

433 3.1.3. Add the samples to an imaging chamber washed with fluoSPB (**Table 2**) and image the  
434 septin bundles. PLL-PEG-passivated flow channels, as described in previous research<sup>10,32</sup>, work  
435 well for this experiment.

436

### 437 **3.2. Septin bundle imaging via negative stain transmission electron microscopy**

438  
439 3.2.1. Prepare the 5x darkSPB (**Table 2**) and a septin mix consisting of 100% dark septin at six  
440 times higher concentration than the desired final concentration in septin buffer + 1 mM DTT. A  
441 typical concentration for this assay is 300 nM, and, therefore, the concentration is 1,800 nM for  
442 this mix.

443  
444 3.2.2. Polymerize the septin by mixing, in this specific order, I-water (enough to top up to the  
445 final desired volume), 20% 5xdarkSPB, and 16.67% septin mix. For 5  $\mu$ L, mix 3.16  $\mu$ L of I-water, 1  
446  $\mu$ L of 5xfluSPB, and 0.83  $\mu$ L of septin mix. Incubate this mix for at least 30 min at room  
447 temperature.

448  
449 3.2.3. Add 3–5  $\mu$ L of sample to a glow-discharged electron microscopy grid and incubate for 1  
450 min. Then, wash the grid 2x with darkSPB (**Table 2**) by absorbing the liquid with a filter paper and  
451 adding a drop of darkSPB buffer, wash 1x with I-water, incubate for  $\sim$ 30 s with 2% uranyl acetate,  
452 blot the stain, and air-dry the sample for a few minutes.

453  
454 3.2.4. Image the septin bundles at 120 kV and magnifications between 5,000x and 60,000x with  
455 a defocus of between 1–2  $\mu$ m.

456  
457 **REPRESENTATIVE RESULTS:**

458 As mentioned in the protocol, 5 L of *E. coli* cells co-transformed with the two septin-expressing  
459 plasmids were grown, and the expression of septins was induced by adding IPTG. After 3 h, the  
460 cells were collected by centrifugation, resuspended in lysis buffer, and lysed by sonication. The  
461 lysate was then clarified by centrifugation, and the clarified solution was applied to a HisTrap  
462 column (**Figure 2A**). After the first purification, the septin-containing fractions were pooled and  
463 applied onto a StrepTrap column (**Figure 2B**). This typically yields around 3–5 mL of  $\sim$ 1  $\mu$ M septin  
464 complex. Before pooling the septin-containing fractions, denaturing gel electrophoresis can be  
465 used to check for the integrity of the septin subunits and the equimolar stoichiometric ratio  
466 between the different septin subunits forming the complex. (**Figure 3A**). If the gel shows similarly  
467 intense bands corresponding to the molecular weights (**Table 3**) of the septin subunits, the  
468 protocol can be continued. If not, it is recommended to start the protocol again. In the example  
469 shown for human septin octamer with SEPT9\_i1, **Figure 3A** clearly shows bands corresponding  
470 to SEPT9\_i1, SEPT6, SEPT7, and SEPT2 (in the order from top to bottom) with similar intensities;  
471 the 99% confidence interval of the normalized intensity was  $1.128 \pm 0.048$  for SEPT2,  $1.092 \pm$   
472  $0.034$  for SEPT6,  $1.108 \pm 0.040$  for SEPT7, and  $1.067 \pm 0.029$  for SEPT9. If SEPT2 is tagged with  
473 msfGFP, it will shift up very close below SEPT9\_i1. Depending on the electrophoresis system used  
474 and the presence of the C-terminal TEV-Strep tag for SEPT7 (which makes it migrate more slowly  
475 than untagged SEPT7), the SEPT7 and SEPT6 bands sometimes merge due to their comparable  
476 molecular weights. The next step is to pool the fractions and dialyze them against septin buffer  
477 with DTT. After the dialysis, if the concentration is too low ( $<2 \mu$ M) or a higher concentration is  
478 needed for the experiments, a concentration step can be included, as described in the protocol.  
479 Concentrations below 1  $\mu$ M usually indicate the bad functional quality of the septins. A final  
480 septin complex concentration between 3.5  $\mu$ M and 7  $\mu$ M works well for most *in vitro* assays.

481 These concentrations are usually obtained when the volume after concentration reaches 0.5–1  
482 mL.

483  
484 [Place **Figure 2** here]

485  
486 To continue with the quality control, native electrophoresis, as described in the protocol, was  
487 performed (**Figure 3B**). In the gels, a major band corresponding to the intact hetero-oligomers  
488 and, usually, a minor band corresponding to trimers or tetramers can be observed. Human  
489 hexamers are found a little bit above the 242 kDa marker band, while octamers are found above  
490 the 480 kDa band, above their calculated molecular mass. The location of these bands was  
491 checked by western blot analysis of eukaryotic cell extracts<sup>32</sup>. Tagging with msfGFP couples each  
492 SEPT2 with an msfGFP protein. This causes an increase in the molecular weight of septin  
493 complexes of 53.4 kDa (26.7 kDa/msGFP molecule). Nevertheless, on the native electrophoresis  
494 gel, the apparent molecular weight of the msfGFP-tagged complexes is indistinguishable from  
495 that of the untagged complexes.

496  
497 A complementary technique to test whether the septin complexes are intact is mass photometry  
498 by iSCAT microscopy. iSCAT monitors the light scattering of molecules landing on a glass slide  
499 amplified by interference with reference light, typically the reflection of the laser on the bottom  
500 of the glass slide. Then, a background subtraction approach is used to give contrast to the  
501 particles. Due to this correction, the signal shows positive and negative values depending on  
502 whether the particles land on the glass or move away from it<sup>49</sup>. The detected signal is directly  
503 proportional to the molecular weight of proteins<sup>50</sup>. Therefore, a signal-to-mass calibration with  
504 a mass standard can determine the mass of the sample proteins. **Figure 3C** shows an example of  
505 human septin octamers containing SEPT9\_i1. Most of the detected single particles (~50%) are of  
506 a molecular weight expected for complete octamers containing SEPT9\_i1 (423 kDa) (**Figure 3C**).  
507 There are also particles with masses between 150–300 kDa, but no clear peak is observed,  
508 indicating the possible presence of other septin species in low abundance. Similarly, most of the  
509 detected single particles for mEGFP-tagged *Drosophila* hexamers are of a molecular weight  
510 expected for intact hexamers (361 kDa) (**Figure 3D**). An additional clear peak at 241 kDa indicates  
511 the presence of stable tetramers containing two peanut proteins, one DSep1 and one mEGFP-  
512 DSep2. Finally, both the human and the fly septin complexes show a peak around 80 kDa that  
513 could be a mix of monomers and dimers, possibly amplified by a trace of DTT or any other small  
514 molecule that aggregates, showing a peak in the positive side of the plot<sup>45</sup>.

515  
516 [Place **Figure 3** here]

517  
518 Given that both native gels and iSCAT provide ensemble-averaged information only, class  
519 averaging of transmission electron microscopy images of single septin oligomers was used to  
520 check the integrity and purity of the complexes by direct visualization. In TEM images of septin  
521 complexes in septin buffer, rods of 24 nm (hexamers) or 32 nm (octamers) in length can be  
522 observed. An example of a human septin octamer containing SEPT9\_i1 can be seen in **Figure 3E**.  
523 When class averaging them, each of the subunits can be observed and counted, as seen for the  
524 msfGFP-tagged human octamer with SEPT9\_i1 in **Figure 3F**. In case the oligomer is fluorescently

525 labeled, extra densities that correspond to the SEPT2-msfGFP can be observed at the end of the  
526 rods (**Figure 3F**).

527

528 The combination of the above techniques proves that octamers (or hexamers) with the correct  
529 stoichiometric ratio and high purity can be purified using the described protocol. Finally, the last  
530 quality control check is for the functionality of the septin complexes in terms of their  
531 polymerization ability. In the presence of low salt concentration (<150 mM KCl with the described  
532 buffer<sup>9</sup>), if septins are not in the presence of other proteins or negatively charged lipid  
533 membranes, they self-assemble into bundles<sup>9</sup>. Septins are prevented from polymerization by  
534 keeping them in the storage buffer, which has a high (300 mM) KCl concentration. The septin  
535 hetero-oligomers are then diluted at a 1:6 volume ratio in a buffer of the same composition but  
536 without KCl to achieve a final KCl concentration of 50 mM. To do fluorescence imaging, this buffer  
537 is complemented with an oxygen scavenging system to protect from photobleaching and with a  
538 blinking suppressor. In TIRF microscopy, small clusters of proteins can be observed within the  
539 shallow TIRF field (~100 nm; **Figure 4A,B**). On a confocal microscope, large clusters of filamentous  
540 structures can be seen floating higher up in solution (**Figure 4C**). Finally, with TEM, small bundles  
541 of septin (**Figure 4D**), corresponding to the clusters observed by TIRF, and large bundles (**Figure**  
542 **4E**), corresponding to the structures observed by confocal microscopy, can be observed. The  
543 insets of **Figure 4D,E** reveal that both types of structures consist of long, thin filaments that run  
544 in parallel, forming bundles with tapered ends. Together, the fluorescence and TEM images prove  
545 that the purified septin complexes can polymerize into filaments, which in turn self-assemble into  
546 bundles.

547

548 [Place **Figure 4** here]

549

#### 550 **FIGURE AND TABLE LEGENDS:**

551 **Figure 1: Purification strategy.** (A) Schematics of the septin hetero-oligomers that exist in human  
552 (left) and *Drosophila* (right) cells. Numbers denote septin subunits from the indicated groups,  
553 and P denotes peanut. Human SEPT9 can be any of its isoforms. The septin subunits have an  
554 asymmetric shape and are longitudinally associated with two distinct interfaces, the NC:NC and  
555 the G:G interface, as denoted by NC and G, respectively, on top of the human hexamer. (B,C)  
556 Schematic illustration of the two-step chromatography strategy, shown for (B) human septin  
557 hexamers and (C) octamers. H indicates the his-tags, while S indicates the Strep-II-tags.

558

559 **Figure 2: Example chromatograms corresponding to the purification of dark human septin**  
560 **octamers\_9i1.** (A) HisTrap column chromatogram. After the septin elution peak, the absorbance  
561 does not go back to zero, likely due to the presence of imidazole in the buffer. The pooled fraction  
562 went from the start of the elution peak until the absorbance stabilized at around 250 mL. (B)  
563 StrepTrap column chromatogram. The pooled fraction went from the start of the elution peak  
564 until the absorbance went back to around 0 at 50 mL.

565

566 **Figure 3: Examples of results of the oligomer quality control.** (A) Example of denaturing gel  
567 showing different fractions of the elution peak from the purification of dark human septin  
568 octamers\_9i1. (B) Example of native electrophoresis of different septin complexes. (C,D)

569 Different examples of histogram results of mass photometry at 12.5 nM of septin complexes: (C)  
570 dark human septin octamers\_9i1 and (D) DSep1-msfGFP *Drosophila* septin hexamers. Lines are  
571 Gaussian fits. (E) TEM image of 25 nM dark human septin octamers\_9i1 in septin buffer. Scale  
572 bar = 200 nm. (F) Class average image of SEPT2-msfGFP human septin octamers\_9i1. The msfGFP  
573 tags are visible as fuzzy densities on the two ends. Scale bar = 10 nm. Panels (E) and (F) are the  
574 copyright of The Company of Biologists and have been adapted from Iv et al.<sup>10</sup> with permission.  
575

576 **Figure 4: Examples of results of the polymerization ability quality control.** (A) TIRF image of 300  
577 nM human septin hexamers (10% msfGFP-labelled hexamers) in fluoSPB. (B) TIRF image of 300  
578 nM human septin octamers containing SEPT9\_i1 (10% msfGFP-labelled octamers9\_i1) in fluoSPB.  
579 (C) Confocal maximum-intensity projections of Z-stacks across ~30  $\mu\text{m}$  with 0.5  $\mu\text{m}$  of spacing of  
580 300 nM human septin octamers\_9i3 in fluoSPB. (A–C) Scale bar = 10  $\mu\text{m}$  and inverted grayscale.  
581 (D,E) Example TEM images of (D) small and (E) large bundles of human septin octamers\_9i1 in  
582 darkSPB. Insets show regions where clear filaments running parallel within the bundle can be  
583 observed. Scale bars = 500 nm. Panels (C–E) are copyright of The Company of Biologists and have  
584 been adapted from Iv et al.<sup>10</sup> with permission.  
585

586 **Table 1: List of plasmids.** Plasmids to purify septin oligomers following this protocol. All plasmids  
587 have been deposited in Addgene (first column).  
588

589 **Table 2: List of buffers.** Buffer compositions used for the purification and quality control of septin  
590 oligomers.  
591

592 **Table 3: Molecular weights and extinction coefficients.** List of molecular weights (MW) and  
593 optical extinction coefficients ( $\epsilon$ ) at a wavelength of 280 nm calculated with ProtParam based on  
594 the sequences of the complex, assuming linear fusion of the septin subunits, the different septin  
595 complexes, and the unique septin subunits (only MW) that can be purified with the plasmids  
596 listed in **Table 1**.  
597

## 598 **DISCUSSION:**

599 The method described here allows for the robust purification and quality control of pre-formed  
600 septin hetero-oligomers. Some of the key issues to consider for the correct application of the  
601 method are as follows. During the elution steps in the chromatographic separations, it is  
602 important to use the recommended (or lower) flow rate to minimize the dilution of the septin  
603 complexes. Additionally, to maximize the recovery of protein during the final concentration step,  
604 the concentrator column is oriented in such a way that the solution is not pushed against the  
605 filter (when there is only a filter on one side). If the solution goes directly to the filter, the protein  
606 sticks much more to it, substantially decreasing the final yield. It is also important to consider  
607 that the concentration step is not always necessary. Picking fractions only from a narrow range  
608 around the peak in the chromatogram usually gives a high enough stock concentration (>3,000  
609 nM) for many reconstitution applications (which usually operate between 10–300 nM). Finally,  
610 for the quality control of the functionality of the septin complexes by fluorescence microscopy,  
611 it is important to correctly passivate the surface of the microscopy slides, since septin complexes

612 avidly stick to glass. Passivation of the glass slides can be done either via PLL-PEG  
613 functionalization or by the formation of neutral (100% DOPC) supported lipid bilayers<sup>11,32</sup>.

614

615 Compared to the original purification protocol first described in Iv et al.<sup>10</sup>, there is a change in  
616 the buffer compositions (**Table 2**). The concentration of MgCl<sub>2</sub> has been reduced from 5 mM to  
617 2 mM, and the concentration and pH of Tris-HCl have been reduced from 50 mM to 20 mM and  
618 from 8.0 to 7.4, respectively. These changes were made to make the buffer conditions compatible  
619 with studies of the interactions of human septins with lipid bilayers, actin filaments, and  
620 microtubules<sup>10,11,32</sup>. This is because the authors formed supported lipid bilayers and polymerized  
621 actin in the F-buffer, whose composition is identical to that of darkSPB, apart from the presence  
622 of ATP in the F-buffer. The buffer change did not produce any changes in the quality or lifetime  
623 of the purified septins compared to the original buffers.

624

625 This method of purification still has several limitations. First, different purification attempts can  
626 vary in yield (0.5–1 mL of 2–5 μM septin complexes) and functional quality, as checked by the  
627 bundle formation ability of the purified septin complexes. That is why it is very important to  
628 consistently perform the quality checks described in this paper. Controlling very well the times  
629 of expression and the optical density of the bacterial culture can help mitigate the difference in  
630 yield. Second, this purification pipeline cannot distinguish between trimers and hexamers or  
631 between tetramers and octamers (**Figure 1B**). However, the quality control experiments can be  
632 used to prove that the majority of septin complexes are in their long oligomer form. In case an  
633 even narrow oligomer size distribution is required, size exclusion chromatography can be  
634 inserted in between step 1.6. and step 1.7. of the purification protocol. This optional step,  
635 however, dramatically decreases the yield, and it is not recommended unless it is strictly needed.  
636 A last, more fundamental, limitation comes from the use of *E. coli* as an expression system for  
637 recombinant septin complexes. Naturally, this system does not allow for post-translational  
638 modifications (PTMs), which have been reported in animal cells, such as phosphorylation,  
639 acetylation, and sumoylation<sup>6,51–53</sup>. These posttranslational modifications could be added by  
640 implementing a similar purification strategy in insect or human cells. Furthermore, this paper has  
641 only discussed the reconstitution of septins by themselves, but studies in cells indicate that  
642 regulatory proteins such as proteins from the Borg family<sup>54,55</sup> and anillin<sup>24,25,56</sup> can have  
643 substantial yet poorly understood effects on the assembly and functions of septins and are,  
644 therefore, important to eventually incorporate in *in vitro* studies. Protocols for the purification  
645 of Borg proteins and anillin have been reported<sup>54,57</sup>.

646

647 The septin purification protocol reported here offers a standardized way to purify septins in their  
648 oligomer form with the correct subunit stoichiometry, offering an important advance over many  
649 earlier *in vitro* studies relying on single septin subunits. Even though some septins in specific  
650 contexts can act as a single subunit<sup>2</sup>, the current body of literature strongly suggests that, in  
651 animal cells, septins mostly function in complexes<sup>9,58</sup>. Therefore, the use of pre-formed hetero-  
652 oligomers, such as the ones described in this paper and others<sup>10,11,18,32,35–37</sup>, is of great importance  
653 to study the structural and biophysical properties of septins via *in vitro* reconstitution to dissect  
654 their functions in the cell. Furthermore, septins are self-assembling proteins with many  
655 interaction partners, including the membrane and the cytoskeleton, which makes them of great

656 interest for bottom-up synthetic biology<sup>59–61</sup> and studies of protein-induced changes in  
657 membrane biophysical properties such as curvature<sup>42,62,63</sup>.

658

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668

#### 669 **DISCLOSURES:**

670 The authors declare no competing or financial interests.

671

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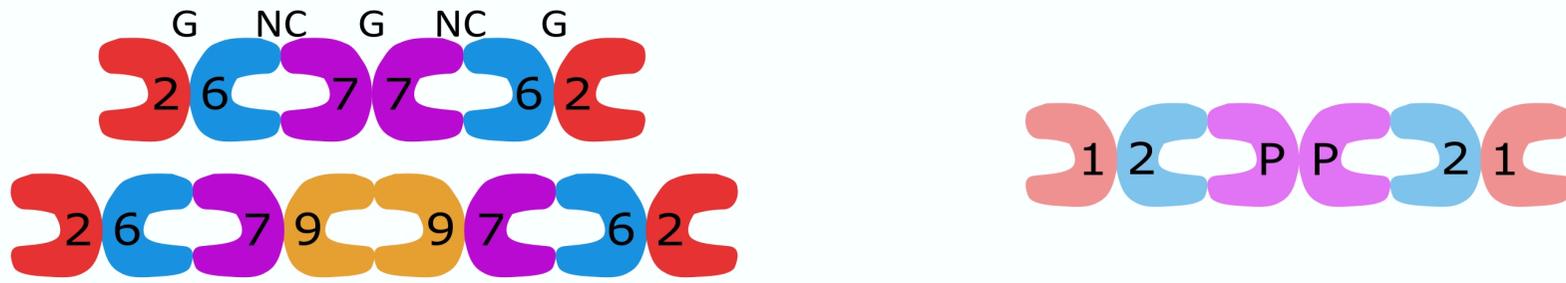
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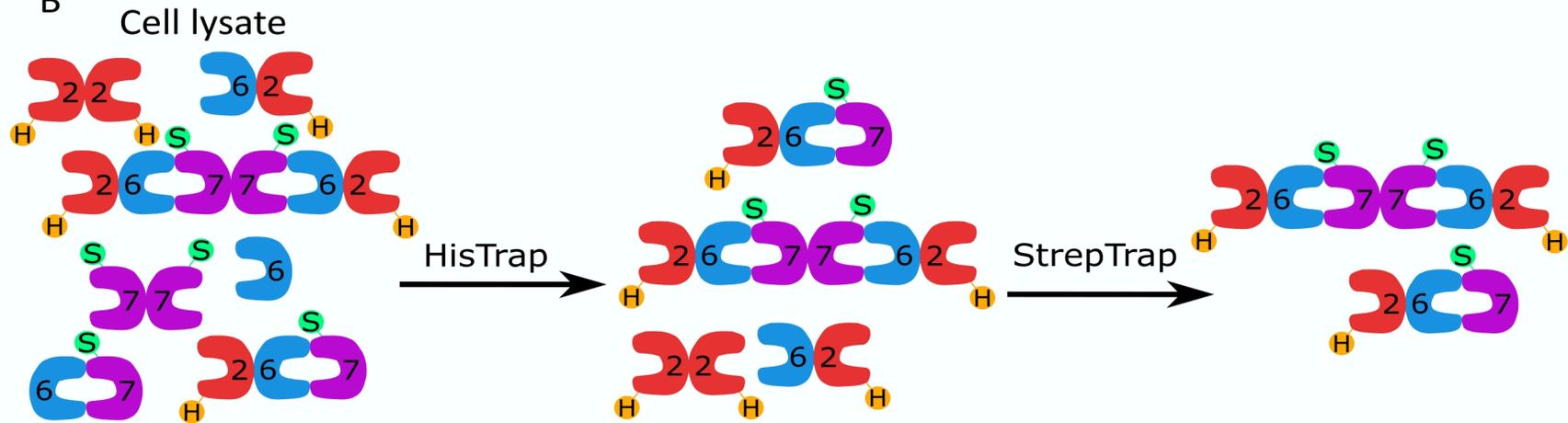
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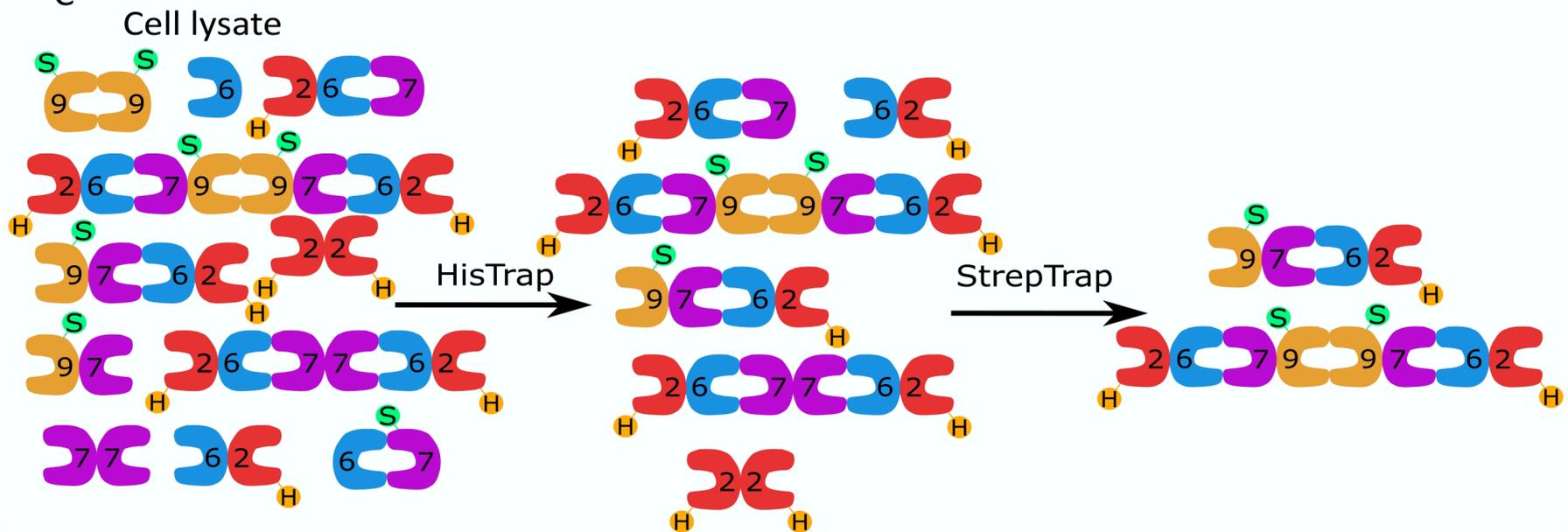
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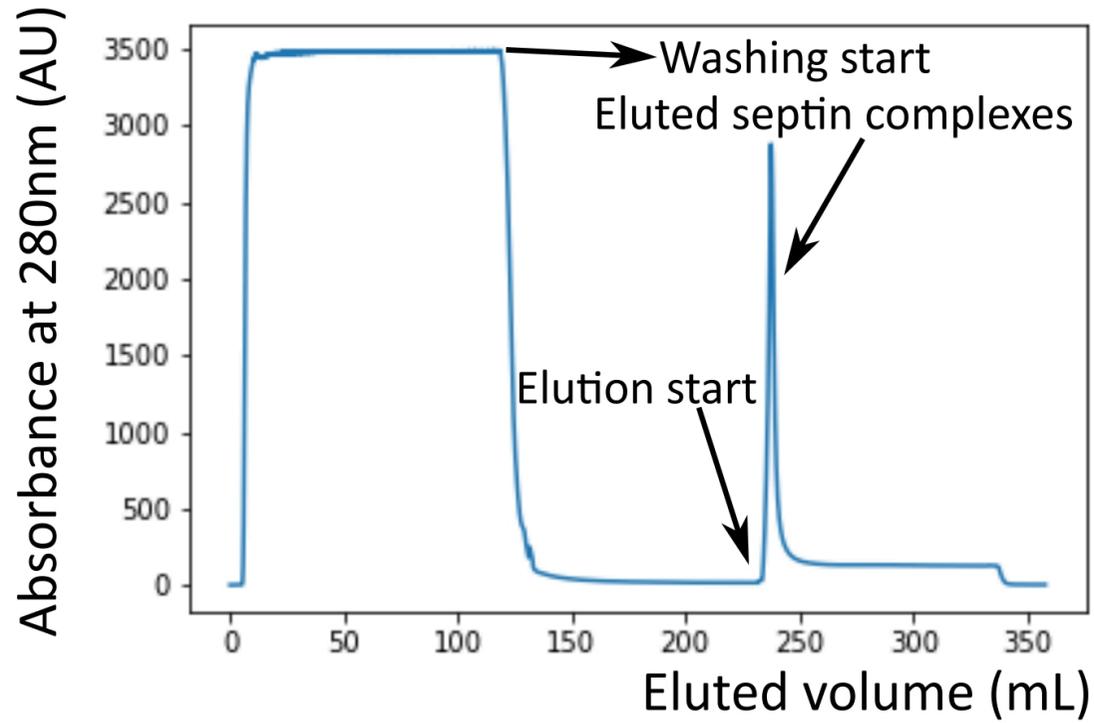


B



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**A****HisTrap Chromatogram****B****StrepTrap Chromatogram**