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► **To cite this version:**

Gerard Castro-Linares, Jeffrey den Haan, Francois Iv, Carla Silva Martins, Aurélie Bertin, et al.. Purification and Quality Control of Recombinant Septin Complexes for Cell-Free Reconstitution. Journal of visualized experiments: JoVE, 2022, 184, 10.3791/63871 . hal-03703833v2

HAL Id: hal-03703833

<https://cnrs.hal.science/hal-03703833v2>

Submitted on 5 Jul 2022

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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 a 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 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 filaments¹. But recently, septins have been
50 acknowledged as a fourth component of the cytoskeleton¹. Septins are a family of GTP-binding
51 proteins that are conserved in eukaryotes². Septins are involved in many cellular functions such
52 as cell division³, cell-cell adhesion⁴, cell motility⁵, morphogenesis⁶, cellular infection⁷, and the
53 establishment and maintenance of cell polarity⁸. 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 similarity². Members of different subfamilies can
58 form palindromic hetero-oligomeric complexes that are the building blocks of filaments, which
59 in turn assemble into higher-order structures such as bundles, rings, and meshworks^{1,9-12}. Further
60 molecular complexity arises from the presence of different splice variants, an example being
61 human SEPT9, where there is evidence for specific functions of different splice variants¹³⁻¹⁵.
62 Additionally, the length of the hetero-oligomers depends on species and cell type. For instance,
63 *Caenorhabditis elegans* septins form tetramers¹⁶, *Drosophila melanogaster* septins form
64 hexamers¹⁷ (**Figure 1A**), *Saccharomyces cerevisiae* septins form octamers¹⁸, and human septins
65 form both hexamers and octamers¹⁹ (**Figure 1A**). The ability of septin isoforms and splice variants
66 from the same subfamily to substitute each other in the complex and the (co-)existence of
67 differently sized hetero-oligomers have made it difficult to delineate the cellular functions of
68 different hetero-oligomeric complexes¹².

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 division²⁰⁻²². In dividing cells, septins cooperate with anillin²³⁻²⁵ and actin and myosin during
73 cytokinesis^{26,27}. At the late stages of cytokinesis, septins seem to regulate the endosomal sorting
74 complexes required for transport (ESCRT) system for midbody abscission²⁸. Additionally, there is
75 also evidence of septin located on the actin cortex and actin stress fibers of cells in interphase
76 cells²⁹⁻³¹. In specific cell types, septins also bind and regulate the microtubule cytoskeleton^{32,33}.

77

78 All of these features make septins a very interesting protein system to study, but also a
79 challenging one. The combination of a large number of septin subunits (13 genes in humans
80 without counting splice variants²) with the potential of septin subunits from the same subfamily
81 to substitute each other and form differently sized hetero-oligomers makes it difficult to draw a
82 conclusion on the cellular function of a specific septin by genetic manipulation. Furthermore, the
83 multiple interactions of septins make interpreting the effects of common research tools such as
84 drugs³⁴ directed at cytoskeletal or membrane components a hard task.

85

86 A way to overcome this situation is to complement research in cells with *in vitro* (cell-free)
87 reconstitution of septins. *In vitro* reconstitution allows for the isolation of a single type of septin

88 hetero-oligomers with a specific subunit composition and length^{18,35–37}. This complex can then
89 be studied in a controlled environment, either alone, to discover the basic structural and
90 physicochemical properties of septins^{38–40}, or in combination with desired partners such as model
91 biomembranes^{11,41,42}, actin filaments^{10,27}, or microtubules^{32,36}, to decipher the nature of their
92 interactions.

93

94 Therefore, a reliable method to purify different septin complexes efficiently is vital for septin
95 research. However, even using the same protocol, different purifications can give proteins with
96 different activity/functionality or even integrity. For commercially available proteins such as
97 enzymes, the functionality and enzymatic activity are carefully validated⁴³. Implementing careful
98 quality control for cytoskeletal or structural proteins such as septins can be challenging, but it is
99 essential to make experiments across labs comparable.

100

101 This paper describes a robust method to purify high-quality recombinant septins in their hetero-
102 oligomeric form based on the simultaneous expression of two vectors containing mono- or bi-
103 cistronic constructs (**Table 1**) in *Escherichia coli* cells. The method consists of a two-step affinity
104 chromatography approach to capture septin hetero-oligomers containing both a his₆-tagged
105 septin and a Strep-II-tagged septin (**Figure 1B,C**). This protocol, first described in Iv et al.¹⁰, has
106 been used to purify *Drosophila* septin hexamers^{11,27,35}, human septin hexamers¹⁰, and several
107 human septin octamers containing different native (isoform 1, 3, and 5)^{10,32} or mutated SEPT9
108 isoforms³². Furthermore, a description of a set of techniques to assess the quality of the purified
109 septins is given. First, the integrity and correct stoichiometry of the septin subunits is checked
110 using denaturing electrophoresis and transmission electron microscopy (TEM). Then, to check for
111 the presence of hetero-oligomers of the correct molecular mass and to test for the presence of
112 monomers or smaller oligomers indicative of complex instability is examined by native
113 electrophoresis and mass photometry *via* Interferometric scattering microscopy (iSCAT). Finally,
114 the last step consists of the assessment of the polymerizing activity of the septins using
115 fluorescence microscopy and TEM.

116

117 [Place **Figure 1** here]

118

119 **PROTOCOL:**

120

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

122

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

124

125 1.1.1. Select a combination of one pnEA and one pnCS plasmid⁴⁴ that will be used for expression.
126 Choose the combination depending on the desired subunit composition of the septin hetero-
127 oligomer^{10,35} and whether or not fluorescent tagging is required.

128

129 NOTE: C-terminally tagged monomeric superfolder GFP (msfGFP)-tagged SEPT2 (for human
130 septins) or msfGFP- or monomeric enhanced GFP (mEGFP)-Dsep2 (for *Drosophila* septins) is used
131 (**Table 1**).

132
133 1.1.2. Pipette 1 μL of each plasmid ($\sim 1 \text{ ng}/\mu\text{L}$) in 100 μL of competent BL21 *Escherichia coli* cells
134 and incubate on ice for 20 min.
135
136 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
137 min on ice.
138
139 1.1.4. Add 0.9 mL of lysogeny broth (LB) medium to the cell suspension and let the cells grow for
140 1 h at 37 $^{\circ}\text{C}$. Plate 100 μL of cells on warm LB-agar plates containing 100 $\mu\text{g}/\text{mL}$ of ampicillin and
141 100 $\mu\text{g}/\text{mL}$ spectinomycin and incubate overnight at 37 $^{\circ}\text{C}$.
142
143 1.2. Grow bacterial pre-culture
144
145 1.2.1. Fill a 250 mL Erlenmeyer flask with 100 mL of Terrific broth (TB) or LB medium containing
146 100 $\mu\text{g}/\text{mL}$ of ampicillin and 100 $\mu\text{g}/\text{mL}$ spectinomycin.
147
148 1.2.2. Pick out a single colony from the LB-agar plate with a sterile inoculation loop and transfer
149 it to fresh media from step 1.2.1.
150
151 1.2.3. Incubate at 37 $^{\circ}\text{C}$ in a rotary shaker incubator, either overnight (o/n) or for at least 6 h.
152
153 NOTE: From this culture, a glycerol stock can be prepared by mixing the bacterial suspension 1:1
154 with glycerol and stored at -80 $^{\circ}\text{C}$. This stock can be used in step 1.2.2 instead of a freshly
155 transformed colony.
156
157 1.3. Bacterial culture and protein expression induction
158
159 1.3.1. Transfer 100 mL of grown bacteria into 5 L of TB or LB containing 50 $\mu\text{g}/\text{mL}$ of ampicillin
160 and 50 $\mu\text{g}/\text{mL}$ spectinomycin.
161
162 1.3.2. Grow this culture at 37 $^{\circ}\text{C}$ in a shaker incubator until it reaches an optical density (OD)
163 measured at a wavelength of 600 nm in the range of 2-3 for unlabeled septins or 0.6-0.8 for
164 msfGFP/mEGFP-labelled septins and induce protein expression by adding a final concentration
165 of 0.5 mM IPTG. The lower OD for the labeled septins is to avoid reaching the death phase in their
166 longer expression time, as detailed in the next step.
167
168 1.3.3. Incubate the cells expressing unlabeled septin hetero-oligomers for 3 h at 37 $^{\circ}\text{C}$ or the cells
169 expressing msfGFP-labelled hetero-oligomers overnight at 17 $^{\circ}\text{C}$.
170
171 NOTE: The short protein expression time for unlabeled complexes, facilitated by the use of the
172 richer TB medium, is chosen to prevent protein degradation. The longer expression time
173 combined with lower temperature for labelled complexes is chosen to allow for correct folding
174 of the msfGFP tag.
175

176 1.4. Bacterial lysis and lysate clarification

177
178 NOTE: From this point onwards in the purification procedure, keep the protein-containing
179 solution on ice or at 4 °C at all times, to prevent proteolytic protein degradation or loss of activity.

180
181 1.4.1. Collect the cultured cells by centrifuging at 4000 x *g* for 20 min at 4 °C. Discard the
182 supernatant.

183
184 1.4.1.1. Optionally, snap-freeze the pellet in this step and store at -80 °C for around 6 months. If
185 this option is chosen, make sure to thaw the pellet on ice **before continuing**.

186
187 1.4.2. Dissolve the pellet in 100 mL of lysis buffer (**Table 2**) and lyse the cells. Choose one of the
188 two options below

189
190 1.4.2.1. Sonicate in 7 cycles of 30 s ON and 59 s OFF with a tip sonicator using 30% amplitude
191 (note that the settings are sonicator-dependent).

192
193 1.4.2.2. Break down the cells in the French press by passing them at least 3x.

194
195 1.4.3. Clarify the cell lysate by centrifuging at 20,000 x *g* for 30 min at 4 °C and keep the
196 supernatant. It is recommended to start with step 1.5.1 during this centrifugal step.

197
198 1.4.4. Optionally, take a sample for denaturing electrophoresis as described in section 2.

199
200 1.5. Affinity chromatography for His-tagged proteins

201
202 NOTE: This step yields complexes containing human SEPT2 or *Drosophila* Sep1 using a nickel
203 column (**Figure 1B**).

204
205 1.5.1. Equilibrate a pre-packed nickel sepharose high-performance chromatography column with
206 septin buffer (**Table 2**).

207
208 1.5.2. Load the clarified supernatant onto the column at 1 mL/min and wash the bound protein
209 with at least three column volumes of septin buffer.

210
211 1.5.3. Elute the septin complexes with 50% HisTrap elution buffer (**Table 2**), to yield an imidazole
212 concentration of 250 mM, at 1 mL/min while collecting 0.5 mL fractions.

213
214 1.5.4. Pick the fractions containing septin complexes as indicated by the optical absorbance of
215 the eluate at 280 nm monitored online with a fast protein liquid chromatography (FPLC) system
216 or after the purification with a microvolume spectrophotometer.

217
218 NOTE: Imidazole absorbs light at 280 nm. This probably explains why the protein peak does not
219 go back to zero absorbance after the septin elution (**Figure 2A**).

220

221 1.6. Affinity chromatography for Strep-II-tagged proteins

222

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

230

231 1.6.1. Equilibrate a pre-packed StrepTactin sepharose high-performance chromatography
232 column with septin buffer (**Table 2**).

233

234 1.6.2. Load the septin-containing fractions recovered from the nickel column at 1 mL/min and
235 wash the bound protein with at least three column volumes of septin buffer.

236

237 1.6.3. Elute the septin complexes with 100% StrepTrap elution buffer (**Table 2**), to yield a
238 concentration of 2.5mM desthiobiotin, at 1 mL/min while collecting 0.5 mL fractions.

239

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

241

242 1.6.4. 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 μ M, 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 °C.

264

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

268

269 **2. Quality control of purity and integrity of 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. Stoichiometric ratio of septin subunits *via* denaturing electrophoresis to check for the
276 formation of the septin hetero-oligomer with the correct components.

277

278 2.1.1. Mix 10 μL of the selected fractions with 10 μL 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 with InstantBlue to visualize
282 the results. Molecular weights of the individual septin proteins and septin hetero-oligomeric
283 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 800mL of anode buffer and 200mL of light blue cathode buffer the day before and
297 store them in the fridge. To prepare the anode buffer dilute 40mL of 20x running buffer with
298 760mL of type-I deionized water (I-water). To prepare the light blue cathode buffer dilute 10mL
299 of 20x running and 1mL of 20x cathode additive with 189mL of I-water.

300

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

304

305 2.2.3. Load the samples onto the gel and fill the system with the ice-cold anode and cathode
306 buffers.

307

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

312 2.3. Single-molecule mass distribution using mass photometry *via* interferometric scattering
313 microscopy
314

315 2.3.1. Wash #1.5 glass slides by sonicating them in an ultrasonic cleaner for 5 min in I-water, 5
316 min in isopropanol, and finally 5 min in I-water.
317

318 2.3.2. Dry two glass slides with a gentle stream of nitrogen gas and place a 7 μ L drop of 0.01%
319 Poly-lysine (PLL) solution on the center of one of the slides. Then, place the center of the other
320 slide on top of the PLL drop, orienting the two slides orthogonally for easy separation. Incubate
321 for 30 s.
322

323 2.3.2.1. Wash by immersing in a beaker with I-water once, and by directly applying a stream of I-
324 water twice. Then dry the two slides with a flow of nitrogen gas. These slides can be stored
325 afterward for around 6 weeks at room temperature in dry conditions.
326

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

329 2.3.3. Just before the experiment, cut a piece of 2x2, 3x2, or 3x3 gaskets (to yeild 4, 6, or 9
330 imaging chambers/slide, respectively) and stick it on the PLL-PEG treated part of a glass slide
331 while avoiding the glass slide and the gaskets to contact any dirty surface. Place the slide on a
332 light-duty wiper tissue and press on the gaskets with a pipette tip to stick them while there is still
333 the protecting plastic on the gaskets.
334

335 2.3.4. Warm septin buffer (**Table 2**) to room temperature and thaw the proteins in hand (keep
336 them on ice afterward).
337

338 NOTE: iSCAT shows the signal of some detergents and small molecules that resemble protein
339 signals⁴⁵. DTT is one of those small molecules and that is why it is not used for this experiment.
340 There is only a trace of DTT coming from the stored septin.
341

342 2.3.5. Place the slide with gaskets on the commercial mass photometry system containing 19 μ L
343 of septin buffer and focus the microscope using the autofocus option. Follow the manufacturer's
344 instructions to check if the found focus is correct. Optimize the frame rate and the exposure time
345 by pressing on **Optimize illumination** and refocus with the autofocus option with the new
346 settings. The standard 100x objective that is part of the setup is used.
347

348 2.3.6. Create or load a project folder to store the data using **File > New Project** or **File > Load**
349 **Project**.
350

351 2.3.7. Pipette 1 μL of sample on the 19 μL buffer drop used to focus and mix while minimizing the
352 movement of the slide by not touching anything while doing so. Then record a 6000-frame video
353 by pressing on **Record**.

354
355 2.3.7.1. For correct analysis, record the following samples: septin buffer, protein mass standard
356 for the calibration of the signal-to-mass ratio (if a recent calibration is available and the
357 environmental conditions have not changed, this sample can be skipped), and 250 nM of septin
358 complexes diluted in septin buffer without DTT (this gives a final concentration of ~ 12.5 nM).

359
360 2.3.8. Analyze the videos using the manufacturer's software to obtain the protein mass
361 distribution. Check for good quality data as follows.

362
363 2.3.8.1. If peaks of different septin hetero-oligomers sizes are overlapping too much or too many
364 events are detected (>3500 events for a 6000-frames video with the regular field of view of 128
365 pixels x 34 pixels spanning 10.8 μm x 2.9 μm), decrease the final septin concentration and
366 measure again.

367
368 2.3.8.2. If there are not enough counts of single molecules measured (at least 2500-3500 for a
369 6000-frame video with the regular field of view), increase the septin concentration and measure
370 again.

371
372 2.4. Direct imaging of septin complexes *via* negative stain transmission electron microscopy

373
374 2.4.1. Dilute samples to a concentration of about 50 nM in septin buffer and prepare the staining
375 solution (use either 2% uranyl formate or uranyl acetate in I-water).

376
377 NOTE: Uranyl formate must be prepared fresh.

378
379 2.4.2. Pipette 4 μL of diluted septins onto a glow discharged electron microscopy grid and
380 incubate for 30 s.

381
382 2.4.3. Remove most of the protein solution using a filter paper and wash the grid twice with
383 septin buffer and once with I-water to remove loosely adsorbed septins.

384
385 2.4.4. Stain with 2% uranyl acetate or uranyl formate solution in I-water for 1 min, absorb the
386 staining solution with a filter paper and air-dry the grid for a few minutes.

387
388 2.4.5. Screen the grid using a properly aligned transmission electron microscope to search for
389 regions of enhanced stain and collect about 100 images within these selected areas.

390
391 2.4.6. Collect images at a magnification of at least 50,000 to obtain a pixel size of about 2 \AA per
392 pixel and with a defocus varying from -1 to -2 μm . Use an acceleration voltage of 200 kV.
393 Preferentially use an automated procedure to collect the data, which will depend on the
394 acquisition software available.

395
396 2.4.7. Perform 2D image processing using dedicated software
397
398 2.4.7.1. Box out at least 2000 particles using a dedicated software⁴⁶.
399
400 2.4.7.2. Perform two-dimensional alignment and classification iteratively, until classes are
401 obtained without further improvement. The first alignment and classification step should be
402 reference-free to avoid any bias in the classification.
403
404 2.4.7.3. Use the averages obtained from the first reference-free classification as new references
405 to carry out an extra round of classification. Repeat this process iteratively until no further
406 improvement is achieved. Each class should be based on 50 to 100 picked particles and individual
407 subunits should be clearly visible. Different software tools can be used (Spider, Eman or Relion)⁴⁶⁻
408 ⁴⁸.

410 **3. Septin functional quality control *via* polymerization analysis.**

411
412 NOTE: The functionality quality control consists of a set of imaging techniques that allow for the
413 detection of of polymerized septin complexes. Below, unlabelled septins are referred as “dark”
414 septins and the buffer used for polymerizing unlabelled septins is referred as "dark" septin
415 polymerization buffer (SPB).

416 417 3.1. Septin bundle imaging *via* fluorescence microscopy

418
419 3.1.1. Prepare the 5x fluoSPB (**Table 2**) and a septin mix consisting of 90% dark septin and 10%
420 msfGFP-septin at six times higher concentration than the desired final concentration in septin
421 buffer + 1 mM DTT. A typical concentration for this assay is 300 nM, and, therefore, the
422 concentration is 1800 nM for this mix.

423
424 3.1.2. Polymerize the septin by mixing, in this specific order, I-water (enough to top up to the
425 final desired volume), 20% 5xfluoSPB (giving a final dilution of 1:5), 0.05 μ M PCD, and 16.67%
426 septin mix (giving a final dilution of 1:6). For 10 μ L, mix 6.23 μ L of I-water, 2 μ L of 5xfluoSPB, 0.1
427 μ L of PCD (with a stock of 5 μ M), and 1.67 μ L of septin mix. Incubate this mix for at least 30 min
428 at room temperature.

429
430 3.1.3. Add the samples to an imaging chamber washed with fluoSPB (**Table 2**) and image the
431 septin bundles. PLL-PEG passivated flow channels as described in^{10,32} work well for this
432 experiment.

433 434 3.2. Septin bundle imaging *via* negative stain transmission electron microscopy

435
436 3.2.1. Prepare the 5x darkSPB (**Table 2**) and a septin mix consisting of 100% dark septin at six
437 times higher concentration than the desired final concentration in septin buffer + 1 mM DTT. A

438 typical concentration for this assay is 300 nM, and, therefore, the concentration is 1800 nM for
439 this mix.

440
441 3.2.2. Polymerize the septin by mixing, in this specific order, I-water (enough to top up to the
442 final desired volume), 20% 5xdarkSPB, and 16.67% septin mix. For 5 μ L, mix 3.16 μ L of I-water, 1
443 μ L of 5xfluoSPB, and 0.83 μ L septin mix. Incubate this mix for at least 30 min at room
444 temperature.

445
446 3.2.3. Add 3-5 μ L of sample to a glow discharged electron microscopy grid and incubate for 1
447 min. Then, wash the grid twice with darkSPB (**Table 2**) by absorbing the liquid with a filter paper
448 and adding a drop of darkSPB buffer, wash once with I-water, incubate for \sim 30 s with 2% uranyl
449 acetate, blot the stain, and air-dry the sample for a few minutes.

450
451 **3.2.4.** Image the septin bundles at 120 kV and magnifications between 5,000x and 60,000x with
452 a defocus of between 1 and 2 μ m.

453
454 **REPRESENTATIVE RESULTS:**
455 As mentioned in the protocol, 5 L of *E. coli* cells co-transformed with the two septin expressing
456 plasmids were grown and the expression of septins was induced by adding IPTG. After 3 h, the
457 cells were collected by centrifugation, resuspended in lysis buffer, and lysed by sonication. The
458 lysate was then clarified by centrifugation and the clarified solution was applied to a HisTrap
459 column (**Figure 2A**). After the first purification, the septin-containing fractions were pooled and
460 applied onto a StrepTrap column (**Figure 2B**). This typically yields around 3-5 mL of \sim 1 μ M of
461 septin complex. Before pooling the septin-containing fractions, a denaturing gel can be used to
462 check for the integrity of the septin subunits and an equimolar stoichiometric ratio between the
463 different septin subunits forming the complex. (**Figure 3A**). If the gel shows similarly intense
464 bands corresponding to the molecular weights (**Table 3**) of the septin subunits, the protocol can
465 be continued. If not, it is recommended to start the protocol again. In the example shown for
466 human septin octamer with SEPT9_i1, **Figure 3A** clearly shows bands corresponding to SEPT9_i1,
467 SEPT6, SEPT7, and SEPT2 (in the order from top to bottom) with similar intensity; the 99%
468 confidence interval of the normalized intensity was 1.128 ± 0.048 for SEPT2, 1.092 ± 0.034 for
469 SEPT6, 1.108 ± 0.040 for SEPT7, and 1.067 ± 0.029 for SEPT9. If SEPT2 is tagged with msfGFP, it will
470 shift up, very close below SEPT9_i1. Depending on the electrophoresis system used and the
471 presence of the C-terminal TEV-Strep tag for SEPT7 (which makes it migrate more slowly than
472 untagged SEPT7), the SEPT7 and SEPT6 bands sometimes merge due to their comparable
473 molecular weights. The next step is to pool the fractions and dialyze them against septin buffer
474 with DTT. After the dialysis, if the concentration is too low ($<2 \mu$ M) or a higher concentration is
475 needed for the experiments, a concentration step can be included as described in the protocol.
476 Concentrations below 1 μ M usually indicate the bad functional quality of the septins. A final
477 septin complex concentration between 3.5 μ M and 7 μ M works well for most *in vitro* assays.
478 These concentrations are usually obtained when the volume after concentration reaches 0.5-1
479 mL.

480
481 [Place **Figure 2** here]

482
483 To continue with the quality control, a native electrophoresis, as described in the protocol, was
484 performed (**Figure 3B**). In the gels, a major band corresponding to the intact hetero-oligomers
485 and usually a minor band corresponding to trimers or tetramers can be observed. Human
486 hexamers are found a little bit above the 242 kDa marker band while octamers are found above
487 the 480 kDa band, above their calculated molecular mass. The location of these bands was
488 checked by western blot analysis of eukaryotic cell extracts³². Tagging with msfGFP couples each
489 SEPT2 with a msfGFP protein. This causes an increase in the molecular weight of septin complexes
490 of 53.4 kDa (26.7kDa per msGFP molecule). Nevertheless, on the native electrophoresis gel, the
491 apparent molecular weight of the msfGFP-tagged complexes is indistinguishable from that of the
492 untagged complexes.

493
494 A complementary technique to test whether the septin complexes are intact is mass photometry
495 by iSCAT microscopy. iSCAT monitors the light scattering of molecules landing on a glass slide
496 amplified by interference with reference light, typically the reflection of the laser on the bottom
497 of the glass slide. Then, a background subtraction approach is used to give contrast to the
498 particles. Due to this correction, the signal shows positive and negative values depending on
499 whether the particles land on the glass or move away from it⁴⁹. The detected signal is directly
500 proportional to the molecular weight of proteins⁵⁰. Therefore, a signal-to-mass calibration with
501 a mass standard can determine the mass of sample proteins. **Figure 3C** shows an example of
502 human septin octamers containing SEPT9_i1. Most of the detected single particles (~50%) are of
503 a molecular weight expected for complete octamers containing SEPT9_i1 (423 kDa) (**Figure 3C**).
504 There are also particles with masses between 150 and 300 kDa, but no clear peak is observed,
505 indicating the possible presence of other septin species in low abundance. Similarly, most of the
506 detected single particles for mEGFP-tagged *Drosophila* hexamers are of a molecular weight
507 expected for intact hexamers (361 kDa) (**Figure 3D**). An additional clear peak at 241 kDa indicates
508 the presence of stable tetramers containing two Peanut proteins, one DSep1, and one mEGFP-
509 DSep2. Finally, both the human and the fly septin complexes show a peak around 80 kDa, that
510 could be a mix of monomers and dimers, possibly amplified by a trace of DTT or any other small
511 molecule that aggregates showing a peak in the positive side of the plot⁴⁵.

512
513 [Place **Figure 3** here]

514
515 Given that both native gels and iSCAT provide ensemble-averaged information only, class
516 averaging of transmission electron microscopy images of single septin oligomers was used to
517 check the integrity and purity of the complexes by direct visualization. In TEM images of septin
518 complexes in septin buffer, rods of 24 nm (hexamers) or 32 nm (octamers) in length can be
519 observed. An example of a human septin octamer containing SEPT9_i1 can be seen in **Figure 3E**.
520 When class averaging them, each of the subunits can be observed and counted, as seen for the
521 msfGFP-tagged human octamer with SEPT9_i1 in **Figure 3F**. In case the oligomer is fluorescently
522 labeled, extra densities that correspond to the SEPT2-msfGFP can be observed at the end of the
523 rods (**Figure 3F**).

524

525 The combination of the above techniques proves that octamers (or hexamers) with the correct
526 stoichiometric ratio and high purity can be purified using the described protocol. Finally, the last
527 quality control check is for the functionality of the septin complexes in terms of their
528 polymerization ability. In the presence of low salt concentration (<150 mM KCl with the described
529 buffer⁹), if septins are not in the presence of other proteins or negatively charged lipid
530 membranes, they self-assemble into bundles⁹. Septins are prevented from polymerization by
531 keeping them in the storage buffer which has a high (300 mM) KCl concentration. The septin
532 hetero-oligomers are then diluted 1:6 volume ratio in a buffer of the same composition but
533 without KCl, to achieve a final KCl concentration of 50 mM. To do fluorescence imaging, this
534 buffer is complemented with an oxygen scavenging system, to protect from photobleaching, and
535 with a blinking suppressor. In TIRF microscopy, small clusters of proteins can be observed within
536 the shallow TIRF field (~100 nm; **Figure 4A,B**). On a confocal microscope, large clusters of
537 filamentous structures can be seen floating higher up in solution (**Figure 4C**). Finally, with TEM,
538 small bundles of septin (**Figure 4D**) corresponding to the clusters observed by TIRF and large
539 bundles (**Figure 4E**) corresponding to the structures observed by confocal microscopy can be
540 observed. The insets of **Figure 4D,E** reveal that both types of structures consist of long and thin
541 filaments that run in parallel, forming bundles with tapered ends. Together, the fluorescence and
542 TEM images prove that the purified septin complexes can polymerize into filaments, which in
543 turn self-assemble into bundles.

544

545 [Place **Figure 4** here]

546

547 **FIGURE AND TABLE LEGENDS:**

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

556

557 **Figure 2: Example chromatograms corresponding to the purification of dark human septin**
558 **octamers_9i1.** (A) HisTrap column chromatogram. After the septin elution peak, the absorbance
559 does not go back to zero, likely due to the presence of imidazole in the buffer. Pooled fraction
560 went from the start of the elution peak until the absorbance stabilized at around 250 mL. (B)
561 StrepTrap column chromatogram. Pooled fraction went from the start of the elution peak until
562 the absorbance went back to around 0 at 50 mL.

563

564 **Figure 3: Examples of results of the oligomer quality control.** (A) Example of denaturing gel
565 showing different fractions of the elution peak from the purification of dark human septin
566 octamers_9i1. (B) Example of native electrophoresis of different septin complexes. (C,D)
567 Different examples of histogram results of mass photometry at 12.5 nM of septin complexes: (C)
568 dark human septin octamers_9i1 and (D) DSep1-msfGFP *Drosophila* septin hexamers. Lines are

569 Gaussian fits. (E) TEM image of 25 nM dark human septin octamers_9i1 in septin buffer. Scale
570 bar = 200 nm. (F) Class average image of SEPT2-msfGFP human septin octamers_9i1. The msfGFP
571 tags are visible as fuzzy densities on the two ends. Scale bar = 10 nm. Panels E and F are adapted
572 from Iv et al.¹⁰ with permission.

573
574 **Figure 4: Examples of results of the polymerization ability quality control.** (A) TIRF image of 300
575 nM human septin hexamers (10% msfGFP-labelled hexamers) in fluoSPB. (B) TIRF image of 300
576 nM human septin octamers containing SEPT9_i1 (10% msfGFP-labelled octamers9_i1) in fluoSPB.
577 (C) Confocal maximum-intensity projections of Z-stacks across ~30 μm with 0.5 μm of spacing of
578 300 nM human septin octamers_9i3 in fluoSPB. (A-C) Scale bar = 10 μm and inverted grayscale.
579 (D,E) Example TEM images of small (D) and large (E) bundles of human septin octamers_9i1 in
580 darkSPB. Insets show regions where clear filaments running parallel within the bundle can be
581 observed. Scale bars = 500 nm. Panels C-E are adapted from Iv et al.¹⁰ with permission.

582
583 **Table 1: List of plasmids.** Plasmids to purify septin oligomers following this protocol. All plasmids
584 have been deposited in Addgene (first column).

585
586 **Table 2: List of buffers.** Buffer compositions used for the purification and quality control of septin
587 oligomers.

588
589 **Table 3: Molecular weights and extinction coefficients.** List of molecular weights (MW) and
590 optical extinction coefficients (ϵ) at a wavelength of 280 nm calculated with ProtParam based on
591 the sequences of the complex assuming a linear fusion of the septin subunits, the different septin
592 complexes, and unique septin subunits (only MW) that can be purified with the plasmids listed
593 in **Table 1**.

594 595 **DISCUSSION:**

596 The method described here allows for the robust purification and quality control of pre-formed
597 septin hetero-oligomers. Some issues of vital importance must be conducted for the correct
598 application of the method. First, during the elution steps in the chromatographic separations, it
599 is important to use the recommended (or lower) flow rate to minimize the dilution of the septin
600 complexes. Additionally, to maximize the recovery of protein during the final concentration step,
601 the concentrator column is oriented in such a way that the solution is not pushed against the
602 filter (when there is only a filter on one side). If the solution goes directly to the filter, the protein
603 sticks much more to it, substantially decreasing the final yield. It is also important to consider
604 that the concentration step is not always necessary. Picking fractions only from a narrow range
605 around the peak in the chromatogram usually gives a high enough stock concentration (>3000
606 nM) for many reconstitution applications (which usually operate between 10 and 300 nM).
607 Finally, for the quality control of the functionality of the septin complexes by fluorescence
608 microscopy, it is important to correctly passivate the surface of the microscopy slides, since
609 septin complexes avidly stick to glass. Passivation of the glass slides can be done either *via* PLL-
610 PEG functionalization or by the formation of neutral (100% DOPC) supported lipid bilayers^{11,32}.

611

612 Compared to the original purification protocol first described in Iv et al.¹⁰, there is a change in
613 the buffer compositions (**Table 2**): the concentration of MgCl₂ has been reduced from 5 to 2 mM
614 and the concentration and pH of Tris-HCl have been reduced from 50 to 20 mM and from 8 to
615 7.4, respectively. These changes were made to make the buffer conditions compatible with
616 studies of the interactions of human septins with lipid bilayers, actin filaments, and
617 microtubules^{10,11,32}. This is because the authors form supported lipid bilayers and polymerize
618 actin in the F-buffer, whose composition is identical to that of darkSPB, apart from the presence
619 of ATP in the F-buffer. The buffer change has not produced any changes in the quality or lifetime
620 of the purified septins compared to the original buffers.

621
622 This method of purification still has several limitations. First, different purification attempts can
623 vary in yield (0.5-1 mL of 2-5 μM of septin complexes) and functional quality, as checked by the
624 bundle formation ability of the purified septin complexes. That is why it is very important to
625 consistently perform the quality checks described in this paper. Controlling very well the times
626 of expression and the optical density of the bacterial culture can help mitigate the difference in
627 yield. Second, this purification pipeline cannot distinguish between trimers and hexamers, or
628 between tetramers and octamers (**Figure 1B**). However, the quality control experiments can be
629 used to prove that the majority of septin complexes are in their long oligomer form. In case an
630 even narrow oligomer size distribution is required, size exclusion chromatography can be
631 inserted in between steps 1.6 and 1.7 of the purification protocol. This optional step, however,
632 dramatically decreases the yield and it is not recommended unless it is strictly needed. A last,
633 more fundamental, limitation comes from the use of *E. coli* as an expression system for
634 recombinant septin complexes. Naturally, this system does not allow for post-translational
635 modifications (PTMs) which have been reported in animal cells, such as phosphorylation,
636 acetylation, and sumoylation^{6,51-53}. These posttranslational modifications could be added by
637 implementing a similar purification strategy in insect or human cells. Furthermore, this paper
638 only discussed the reconstitution of septins by themselves, but studies in cells indicate that
639 regulatory proteins such as proteins from the Borg family^{54,55} and anillin^{24,25,56} can have
640 substantial yet poorly understood effects on the assembly and functions of septins and are,
641 therefore, important to eventually incorporate in *in vitro* studies. Protocols for the purification
642 of Borg proteins and anillin have been reported^{54,57}.

643
644 The septin purification protocol reported here offers a standardized way to purify septins in their
645 oligomer form with the correct subunit stoichiometry, offering an important advance over many
646 earlier *in vitro* studies relying on single septin subunits. Even though some septins in specific
647 contexts can act as a single subunit², the current body of literature strongly suggests that, in
648 animal cells, septins mostly function in complexes^{9,58}. Therefore, the use of pre-formed hetero-
649 oligomers, such as the ones described in this paper and others^{10,11,18,32,35-37}, is of great importance
650 to study the structural and biophysical properties of septins *via in vitro* reconstitution to dissect
651 their functions in the cell. Furthermore, septins are self-assembling proteins with many
652 interaction partners, including the membrane and the cytoskeleton, which makes them of great
653 interest for bottom-up synthetic biology⁵⁹⁻⁶¹ and studies of protein-induced changes in
654 membrane biophysical properties such as curvature^{42,62,63}.

655

656 **ACKNOWLEDGEMENTS:**

657 We thank Cecilia de Agrela Pinto, Tomás de Garay, and Katharina Häußermann for their
658 assistance with mass photometry (iSCAT) experiments; Arjen Jakobi and Wiel Evers for their help
659 with TEM; Lucia Baldauf for her assistance with TIRF; Pascal Verdier-Pinard for his advice
660 concerning native electrophoresis; and Agata Szuba and Marjolein Vinkenoog for their help in
661 setting up the *Drosophila* septin purification efforts. This research received funding from the
662 Netherlands Organization for Scientific Research (NWO/OCW) through the ‘BaSyC—Building a
663 Synthetic Cell’ Gravitation grant (024.003.019) and from the Agence Nationale pour la Recherche
664 (ANR grant ANR-17-CE13-0014; SEPTIMORF).

665

666 **DISCLOSURES:**

667 The authors declare no competing or financial interests.

668

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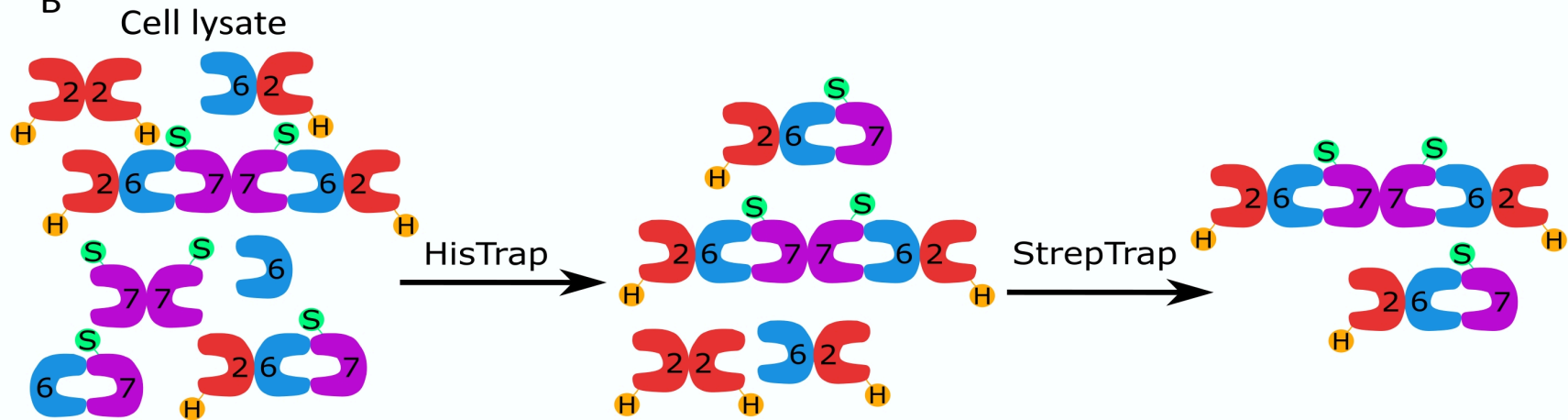
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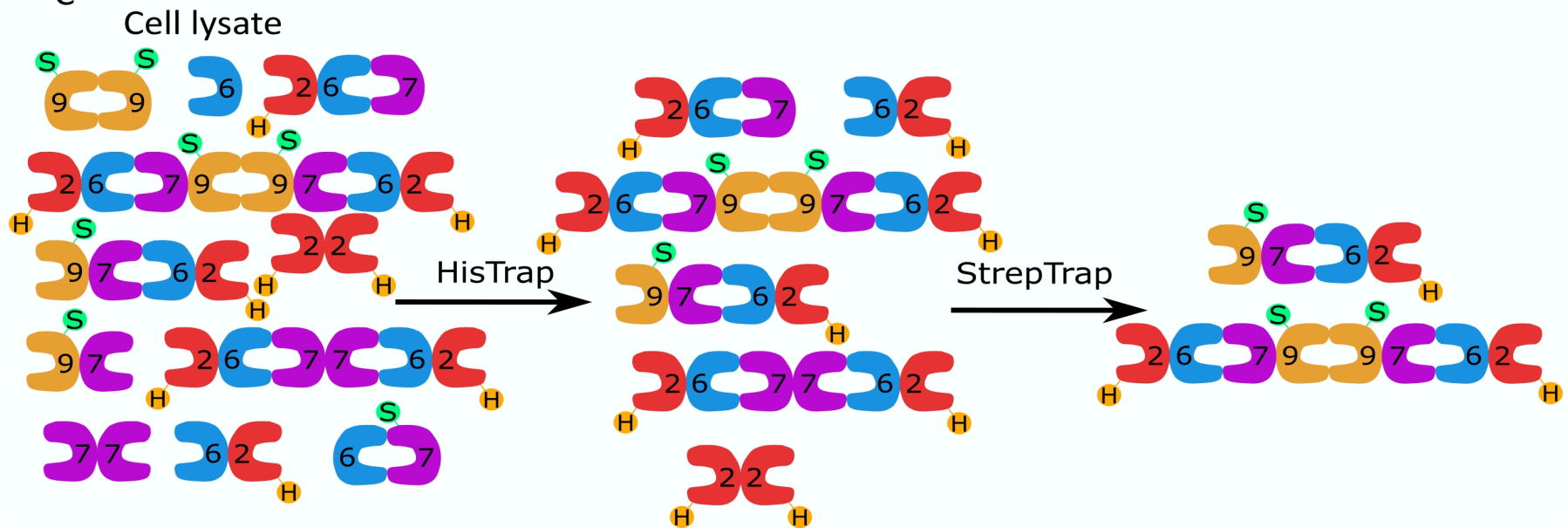
A

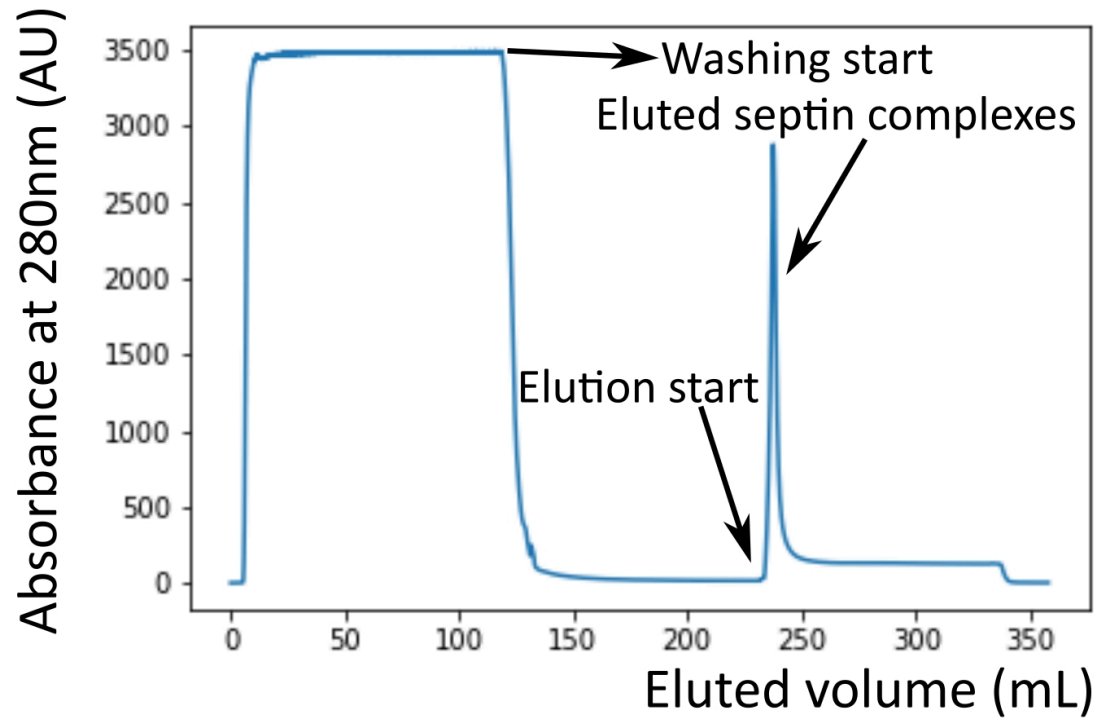


B



C



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