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1 **Protein-protein interaction: Tandem Affinity Purification in bacteria**

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3

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9 Running head: TAP in bacteria

10

## 11 **Summary**

12 The discovery of protein-protein interaction networks can unveil protein complex(es) forming  
13 cellular machinerie(s) or can reveal component proteins of a specific cellular pathway.  
14 Deciphering protein-protein interaction networks therefore participates to a better  
15 understanding of how the cell functions. Here, we describe the protocol to perform Tandem  
16 Affinity Purification (TAP) in bacteria that enables the identification of the partners of a bait  
17 protein under native conditions. This method consists in two sequential steps of affinity  
18 purification, using two different tags. For that purpose, the bait protein is translationally fused  
19 to the TAP-tag, which consists in a calmodulin binding peptide (CBP) and two IgG binding  
20 domains of *Staphylococcus aureus* protein A (ProtA) that are separated by the Tobacco Etch  
21 Virus (TEV) protease cleavage site. After the first round of purification based on the binding  
22 of ProtA to IgG coated beads, TEV protease cleavage releases CBP-tagged bait-protein along  
23 with its partners for a second round of purification on calmodulin affinity resin and leaves  
24 behind protein contaminants bound to IgG. Creating the TAP-tag translational fusion at the  
25 chromosomal locus allows detection of protein interactions occurring in physiological  
26 conditions.

27

## 28 **Keywords**

29 Protein–protein interaction, protein complex, affinity purification, TAP, CBP, ProtA, TEV,  
30 *E. coli*, *Salmonella*

31

32

## 33 **1. Introduction**

34 At the end of the nineties, mass spectrometry combined with genome sequencing rendered  
35 possible the rapid and systematic identification of all the proteins present in a purified sample.  
36 However, a protocol amenable to standardized and systematic purification of protein  
37 complexes without any prior knowledge was missing. In 1999, the laboratory of B. Séraphin  
38 in EMBL proposed such a generic procedure for the identification of protein complexes in  
39 yeast [1]. This permitted the subsequent description of the full interactome of yeast [2, 3].  
40 This method has since been used in a variety of organisms. We have first described its use in  
41 bacteria [4], and it was soon after used to obtain the first interactome of *E. coli* [5].

42 One general principle of the TAP method is to use 2 successive steps of affinity  
43 purification to lower as much as possible the amount of contaminants, together with an  
44 elution preserving the interactions (without changing much the buffer chemical properties)  
45 between these 2 steps. Specifically, the original TAP tag consists of 2 repeats of the IgG  
46 binding domain of Protein A from *Staphylococcus aureus*, and a Calmodulin Binding Peptide,  
47 separated by a TEV protease cleavage site (Fig. 1). However, it has to be noted that any  
48 combination of affinity tags is potentially usable. Published examples are the GS-TAP  
49 (Protein G and Strep tag), the SPA tag (CBP and 3Flag), the SF-TAP (Strep-tag II and Flag  
50 tag), or the HB tag (6Histidine and Biotin) (see [6] for specific references). The second  
51 general principle of the TAP procedure is to use physiological expression of the recombinant  
52 tagged protein. This needs to be adapted to each organism of interest. For *E. coli* and closely  
53 related bacteria, Lambda Red based recombination [7] combined with specific dedicated SPA  
54 and TAP cassettes [8], makes it very easy to introduce the tag at the 3' extremity of the gene  
55 on the chromosome to obtain the physiological production of a recombinant protein tagged at

56 its C-terminus (Fig. 2). If more convenient, however, TAP tag translational fusion can also be  
57 expressed from a plasmid (Fig. 3).

58 We present here the TAP protocol that has been successfully used in our institute to purify  
59 protein complexes of *E. coli*, *Salmonella*, and *Bacillus subtilis* [4, 9, 10, and unpublished  
60 results]. A detailed protocol for the SPA purification has been published before [11]. In order  
61 to isolate a protein complex by TAP, a strain producing a recombinant bait protein tagged  
62 with the TAP tag has to be constructed first (Fig. 1, step 1). Then, a soluble extract is prepared  
63 from a sufficient volume of bacteria (about 500 ml). The complex is enriched by a first step of  
64 affinity chromatography on IgG beads (Fig. 1, step 5). After washes, TEV protease is added,  
65 which cleaves the specific site located between the CBP and ProtA domains, resulting in the  
66 elution of the specifically bound material (Fig. 1, step 6). This material is purified a second  
67 time by affinity of the CBP tag with Calmodulin beads (Fig. 1, step 7). After washes, the  
68 purified complex is eluted by adding EGTA that chelates the calcium required for the  
69 CBP/Calmodulin interaction (Fig. 1, step 8). The totality of the purified material is analyzed  
70 on SDS-PAGE. Bands detected by Coomassie blue or Silver staining are cut from the gel and  
71 analyzed by mass spectrometry.

72 This consists in the basic TAP procedure. It has to be noted that this procedure can be  
73 amenable to adaptation or improvements depending on the specific needs. For example, the  
74 extensive washes and the duration of the procedure only allows for the recovery of relatively  
75 stable complexes. For the detection of more transient or unstable interactions, a cross-linking  
76 procedure can be applied before purification [12]. This might be helpful also for the  
77 purification of membrane complexes, where modifications have to be made in the protocol for  
78 solubilization of the membranes [3]. Finally, it is possible to play with the 2 tags to gain  
79 information on the organization of the complexes. Indeed, in some cases, one bait protein  
80 might participate to the formation of several types of complexes. To purify one specific type

81 of complex it is therefore possible to put the two tags on 2 distinct proteins that are both  
82 members of the desired type of complex (split tag method [9, 13]). Alternatively, it is possible  
83 to perform the subtraction method that consists in eliminating the unwanted complex(es)  
84 during the first purification step, by leaving it, for example, on IgG beads thanks to a partner  
85 protein of the bait that belongs to the unwanted complex and bears a non cleavable protA tag.  
86 The desired complex, made of untagged partner proteins, will elute with the bait after TEV  
87 protease cleavage [13, 14].

88 To our knowledge, the TAP procedure has not been used so much for the  
89 characterization of secretion systems in bacteria, certainly due to the difficulty of working  
90 with integral envelope components. However, it has proved to be powerful to identify the  
91 target of effectors of *Legionella* T4SS or *Pseudomonas* T6SS in eukaryotic host cells [15, 16].  
92 In addition, as mentioned above, it is amenable to several improvements that might permit to  
93 identify unsuspected partners of the secretion machineries in the bacterium.

94

## 95 **2. Materials**

### 96 2.1 Media, stock solutions and reagents

- 97 1. 2YT: 16 g Yeast Extract, 10 g Tryptone, 10 g NaCl, make up to 1 L with distilled  
98 water. Autoclave and store at room temperature.
- 99 2. LB: 5 g Yeast Extract, 10 g Bactotryptone, 10 g NaCl, make up to 1 L with distilled  
100 water. Autoclave and store at room temperature.
- 101 3. PBS: 8 g NaCl, 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 2.9 g Na<sub>2</sub>HPO<sub>4</sub>, make up to 1 L with  
102 distilled water. Autoclave and store at room temperature.

- 103 4. 1 M Tris pH 8 stock solution: Dissolve 121 g of Tris base in 800 ml of distilled water,  
104 make up to 1 L with distilled water once the pH has been adjusted to 8 with HCl. Autoclave  
105 and store at room temperature.
- 106 5. 5 M NaCl stock solution: Dissolve 292 g NaCl in 1 L of distilled water. Autoclave and  
107 store at room temperature.
- 108 6. 10 % Nonidet P-40 (NP-40 or Igepal): Mix 10 ml NP-40 to 90 ml of distilled water,  
109 0.2  $\mu\text{m}$  filter and store at room temperature (note 1).
- 110 7. 0.5 M EDTA ( $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$ ): Dissolve 18.6 g of EDTA to 80 ml of distilled  
111 water, make up to 100 ml with distilled water once the pH has been adjusted to 8 with  
112 10 N NaOH (note 2). Autoclave and store at room temperature.
- 113 8. 1 M DTT: Dissolve 1.54 g in 10 ml distilled water. Store at  $-20^\circ\text{C}$ .
- 114 9. 1 M magnesium acetate ( $\text{Mg}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$ ): Dissolve 10.7 g magnesium acetate  
115 in 50 ml distilled water, 0.2  $\mu\text{m}$  filter and store at room temperature.
- 116 10. 1 M imidazole: Dissolve 3.4 g imidazole to 50 ml distilled water, 0.2  $\mu\text{m}$  filter and  
117 store at  $-20^\circ\text{C}$ .
- 118 11. 1 M  $\text{CaCl}_2$ : Dissolve 11.1 g to 100 ml distilled water. Autoclave and store at room  
119 temperature.
- 120 12. 1 M EGTA: Dissolve 19 g to 40 ml distilled water, make up to 50 ml with distilled  
121 water once the pH has been adjusted to 8 with 10 N NaOH (note 2), 0.2  $\mu\text{m}$  filter and store at  
122  $4^\circ\text{C}$ .
- 123 13. 0.1 M PMSF: Dissolve 87.1 mg PMSF to 5 ml isopropanol. Prepare 1 ml aliquots and  
124 store at  $-20^\circ\text{C}$  (note 3)

- 125 14. 16 mg/ml sodium deoxycholate: Dissolve 160 mg sodium deoxycholate to 10 ml water  
126 and 0.2  $\mu$ m filter. Store at room temperature.
- 127 15.  $\beta$ -mercaptoethanol (stock is at 14.3 M)
- 128 16. Liquid Trichloroacetic acid (stock is 100 %)
- 129 17. Acetone
- 130 18. Ethanol
- 131 19. Peroxidase Anti-Peroxidase antibody (PAP) (Sigma, # P1291)
- 132 20. AcTEV<sup>TM</sup> protease (Invitrogen, # 12575015)
- 133 21. IgG sepharose 6 fast flow (GE Healthcare, # 17-0969-01)
- 134 22. Calmodulin affinity resin (Agilent, # 214303)
- 135 23. Disposable chromatography columns (Bio-Rad, # 7311550)

136

137 2.2 Buffers

- 138 1. Protein A binding buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40

139 Approximately 50 ml will be required per sample per experiment.

140 Prepare 500 ml containing 5 ml of 1M Tris-HCl pH 8, 15 ml of 5 M NaCl, 5 ml of  
141 10% NP-40 and 475 ml of distilled water. Store at 4°C.

- 142 2. TEV cleavage buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,  
143 0.5 mM EDTA, 1 mM DTT



144 Approximately 1.5 ml will be required per sample per experiment.

145 Prepare 100 ml containing 1 ml of 1M Tris-HCl pH 8, 3 ml of 5M NaCl, 1 ml of 10% NP-40,

146 100  $\mu$ l of 0.5 M EDTA, 100  $\mu$ l of 1 M DTT (note 4) and 95 ml of distilled water. Store at 4°C.

147 3. Calmodulin binding buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,

148 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol

149 Approximately 40 ml will be required per sample per experiment.

150 Prepare 500 ml containing 5 ml of 1M Tris-HCl pH 8, 15 ml of 5M NaCl, 5 ml of 10% NP-40,

151 500  $\mu$ l of 1 M magnesium acetate, 500  $\mu$ l of 1 M imidazole, 1 ml of 1 M CaCl<sub>2</sub>, 348.5  $\mu$ l of

152 14.3 M  $\beta$ -mercaptoethanol (note 5) and 473 ml of distilled water. Store at 4°C.

153 4. Calmodulin elution buffer : 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP-40,

154 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10 mM  $\beta$ -mercaptoethanol

155 Approximately 1 ml will be required per sample per experiment.

156 Prepare 100 ml containing 1 ml of 1M Tris-HCl pH 8, 3 ml of 5M NaCl, 1 ml of 10% NP-40,

157 100  $\mu$ l of 1 M magnesium acetate, 100  $\mu$ l of 1 M imidazole, 200  $\mu$ l of 1 M EGTA, 69,7  $\mu$ l of

158 14.3 M  $\beta$ -mercaptoethanol (note 5) and 94.5 ml of distilled water. Store at 4°C.

159 5. TCA Washing Buffer: Mix 70 ml acetone, 20 ml ethanol, 5 ml 1 M Tris pH 8 and 5 ml

160 distilled water. Store at 4°C.

161

## 162 2.3 Equipment

163 1. Centrifuge tubes and rotor compatible with spinning volumes of 250 ml, 10 ml and

164 50 ml, at approximately 5 000 rpm, 15 000 rpm and 6 000 rpm, respectively.

- 165 2. Sonicator, French press or Cell disruptor
- 166 3. Disposable chromatography columns of 10 ml
- 167 4. Wheel

168

### 169 **3. Methods**

#### 170 3.1 Preamble

##### 171 *Construction of the TAP-tagged protein translational fusion*

172 A translational fusion between the protein of interest and TAP tag, either at the chromosomal  
173 locus or on an appropriate plasmid has to be constructed. Translational fusion at the  
174 chromosomal locus will allow a physiological expression, while constructing the translational  
175 fusion on a plasmid may be more amenable.

176 A C-terminal TAP tag translational fusion can be introduced at the chromosomal locus  
177 using the  $\lambda$ red recombination system [7]. To prepare the appropriate PCR product, use pJL72  
178 plasmid as template (this latter harbors a cassette made of the TAP tag and the kanamycine  
179 resistance gene, Fig. 2A) [8], design a forward primer that contains, in the 5'-end, the 45  
180 nucleotides that are immediately upstream the stop codon of the gene of interest, followed by  
181 the sequence 5'-TCCATGGAAAAGAGAAG-3' (this sequence will hybrid to the CBP tag,  
182 Fig. 2B) and design a reverse primer that contains at its 5'-end the reverse complement 45  
183 nucleotides that are immediately downstream the stop codon of the gene of interest, followed  
184 by the sequence 5'-CATATGAATATCCTCCTTAG-3' (Fig. 2A).

185 Alternatively, the sequence corresponding to the open reading frame of the gene of interest  
186 can be cloned in the plasmid pEB587 [17] (Fig. 3A), which allows a N-terminal TAP tag  
187 translational fusion (Fig. 3B) under the control of the PBAD arabinose inducible promoter.

188

189 *Verifying the expression of the TAP-tag translational fusion by western blot*

190 Prepare a cytoplasmic or a crude protein extract (note 6), load 10  $\mu\text{g}$  of protein extract (or  
191 proteins corresponding to a bacterial sample of 0.3  $\text{OD}_{600}$  unit) on a SDS-PAGE and proceed  
192 to transfer and western blot to verify production of the hybrid protein (note 7).

193 A one step western blot can be performed using PAP antibody (note 8) and using an  
194 appropriate substrate to detect horseradish peroxidase activity. In our experience, the  
195 detection of the tagged protein in crude extracts using this PAP antibody is mandatory for a  
196 successful TAP purification.

197

### 198 3.2 Protein cytoplasmic extract

199 1. Day 1 - Inoculate 10 ml of 2YT media with a bacterial colony and grow overnight at  
200 37°C with shaking (note 6).

201 2. Day 2 - Dilute culture 1:100 in 500 ml LB and grow 5h30 at 37°C with shaking until  
202  $\text{OD}_{600} \approx 2-3$ .

203 3. Pellet bacteria by centrifugation 20 min 5 000 rpm at 4°C.

204 4. Wash once with cold PBS, transfer to 50ml centrifuge tubes, centrifuge again 10 min  
205 6 000 rpm at 4°C, discard supernatant and freeze bacterial pellet with liquid nitrogen.

206 Keep frozen bacterial pellet at -80°C until you are ready to prepare cytosolic protein extract  
207 and proceed to Tandem Affinity Purification.

208 5. Day 3 - Resuspend frozen bacterial pellet with 10 ml of Protein A binding buffer  
209 containing 0.5 mM PMSF (note 3).

210 6. Use sonication, French Press or Cell disruptor to break bacterial cells (note 9).

211 7. Centrifuge 30 min 15 000 rpm at 4°C and save supernatant, which is the cytoplasmic  
212 protein extract.

213

### 214 3.3 Tandem Affinity Purification

215 From here, carry out all procedures with gloves to avoid contamination of your sample(s)  
216 with keratine.

217 1. Put 200  $\mu$ l of IgG sepharose beads in a disposable chromatography column and wash  
218 by gravity with 5 ml of Protein A binding buffer.

219 2. *Binding of the Protein A tag to IgG sepharose beads.* After washing the beads, close  
220 the bottom of the chromatography column and, using a pipette, transfer 9 ml of the  
221 cytoplasmic protein extract. Close the top of the column and put on a wheel for 2 hours at 4°C.

222 3. Remove first the top plug of the column and then the bottom one. Leave the unbound  
223 material flow by gravity and discard.

224 4. Wash the IgG beads still in the column by gravity, with 3 times 10 ml of Protein A  
225 binding buffer.

- 226 5. *TEV protease cleavage.* Close the bottom of the column, fill it with 1 ml of TEV  
227 cleavage buffer and 100 units of AcTEV™ protease. Close the top of the column and put on a  
228 wheel at room temperature for one hour.
- 229 6. Remove the top and bottom plugs and recover elution by gravity. Add an additional  
230 200  $\mu$ l of TEV cleavage buffer in the column in order to recover as much material as possible  
231 from the sides of column.
- 232 7. Add 3 ml of calmodulin binding buffer and 3  $\mu$ l of 1M CaCl<sub>2</sub> (note 10) to the elution  
233 fraction.
- 234 8. *Binding by the CBP tag part on calmodulin affinity resin.* In a new disposable  
235 chromatography column, put 200  $\mu$ l of calmodulin affinity resin, and wash it with 5 ml of  
236 Calmodulin binding buffer. Then, close the bottom of column.
- 237 9. Add the 4.2 ml elution fraction (obtained at steps 6 and 7). Close the top of column  
238 and put on wheel for 1 hour at 4°C.
- 239 10. Remove first the top plug of the column and then the bottom one. Leave the unbound  
240 material to flow by gravity and discard.
- 241 11. Wash calmodulin affinity resin by gravity, with 3 times 10ml of calmodulin binding  
242 buffer
- 243 12. *Elution.* Elute with 5 times 200  $\mu$ l of Calmodulin elution Buffer.
- 244 13. Pool fraction 2, 3 and 4 and proceed to TCA precipitation of elution fractions (1),  
245 (pooled 2, 3 and 4), and (5).
- 246

247            3.4 TCA precipitation

248    1. To each of the eluted protein samples, add 1/100th of 16 mg/ml sodium deoxycholate.

249    Vortex and leave on ice 30 min.

250    2.     Add TCA to 10% final. Vortex and leave on ice 30 min.

251    3.     Centrifuge 15 min 15 000 rpm at 4°C.

252    4.     Wash twice with TCA wash buffer.

253    5.     Leave the pellet to dry on bench and resuspend in 20  $\mu$ l protein loading buffer 1x.

254

255            3.5 Analysis by SDS-PAGE and mass spectrometry

256    1.     Load the totality of the samples on SDS-PAGE (note 11) and stain with Coomassie  
257    Blue.

258    2.     Unstained and then rinse with distilled water.

259    3.     Cuts bands to identify partner proteins by mass spectrometry (note 12).

260

261            **4. Notes**

262    Note 1: Gently agitate the solution for complete dissolution of NP-40 if necessary.

263    Note 2: EDTA and EGTA may not be soluble until pH had been adjusted to 8 with  
264    10 N NaOH.

265 **Note 3:** PMSF crystallizes at  $-20^{\circ}\text{C}$ , therefore heat PMSF aliquot to  $37^{\circ}\text{C}$  to redissolve PMSF  
266 before use. We use PMSF as generic protease inhibitor but protease inhibitor cocktail can be  
267 used alternatively.

268 **Note 4:** Add DTT to the volume of buffer you will need when starting the experiment. DTT  
269 is necessary for TEV activity.

270 **Note 5:** Add  $\beta$ -mercaptoethanol to the volume of buffer you will need when starting the  
271 experiment.

272 **Note 6:** Also plan to prepare a protein extract of an untagged strain as negative control of the  
273 experiment.

274 **Note 7:** Translational TAP tag fusion adds 20 kDa to the mass of the protein of interest;  
275 3 kDa corresponds to the CBP tag and 15 kDa to the Protein A tag.

276 **Note 8:** Immunoglobulins will bind the protein A fragment of TAP tag.

277 **Note 9:** French Press or Cell disruptor might be more gentle to preserve protein complexes.

278 **Note 10:** Addition of extra  $\text{CaCl}_2$  is required to quench EDTA that was previously necessary  
279 for TEV protease activity.

280 **Note 11:** Usually a 12% SDS-PAGE allows visualization of low and high molecular weigh  
281 proteins.

282 **Note 12:** Use one blade per band.

283

284 **5. References**

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331

## Guidelines

### 1. Construction

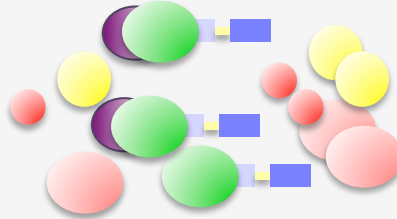


### 2. Check Expression of the TAP-tagged bait protein by Western blot with PAP antibody



### 3. Production

### 4. Cytoplasmic protein extract



**Add:**

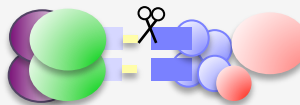
### 5. Protein A binding

partner bait contaminants



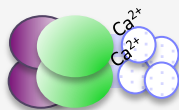
IgG beads

### 6. TEV cleavage



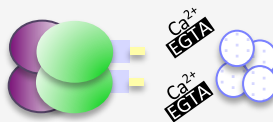
TEV protease

### 7. CBP binding



Calmodulin beads  
Ca<sup>2+</sup>

### 8. Elution + TCA precipitation



EGTA

### 9. SDS-PAGE and staining

Cut band and analyze by Mass spectrometry

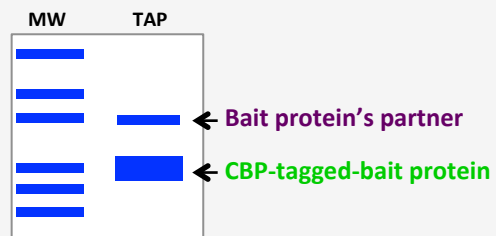


Fig. 1. Guidelines for the overall Tandem Affinity Purification procedure

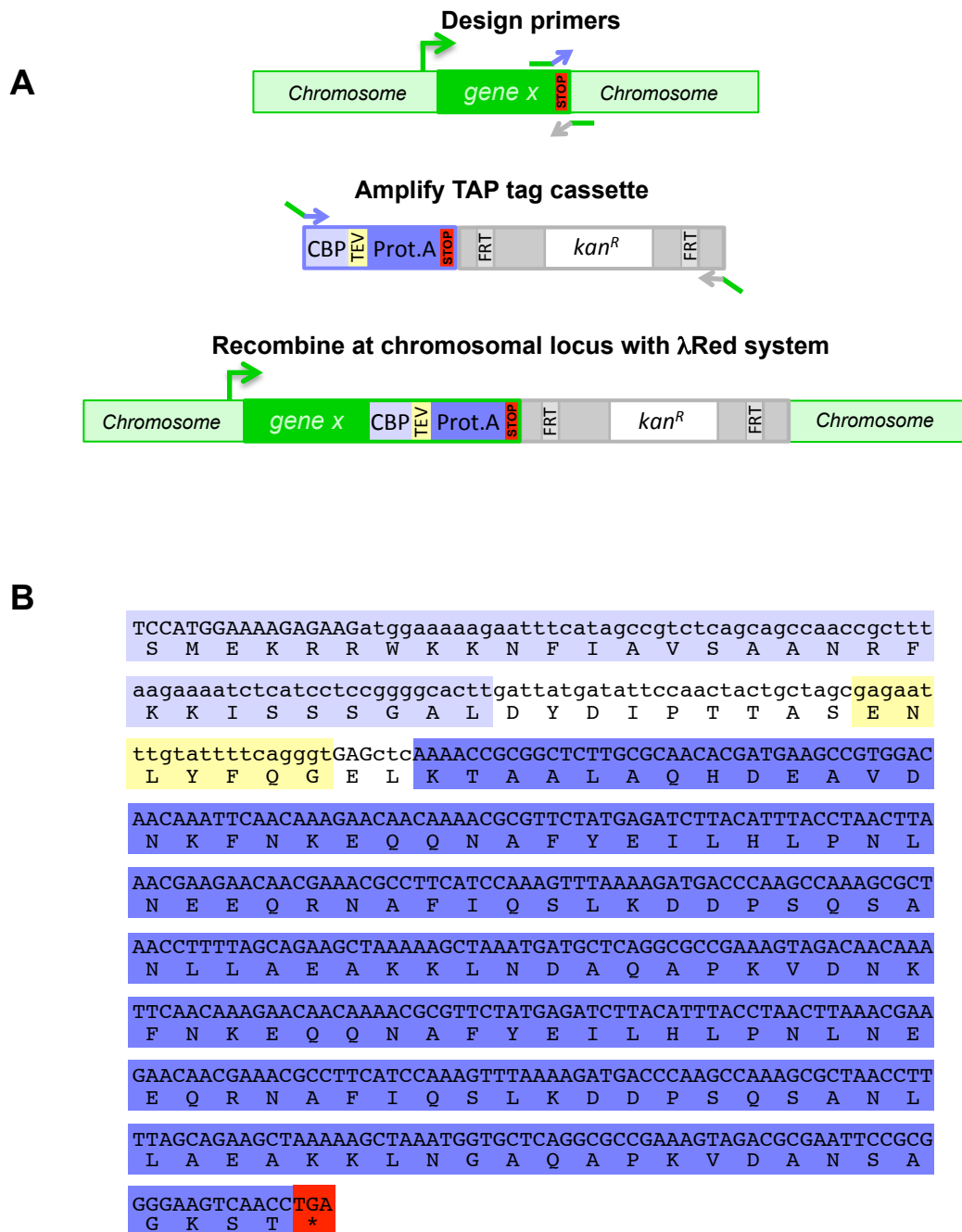
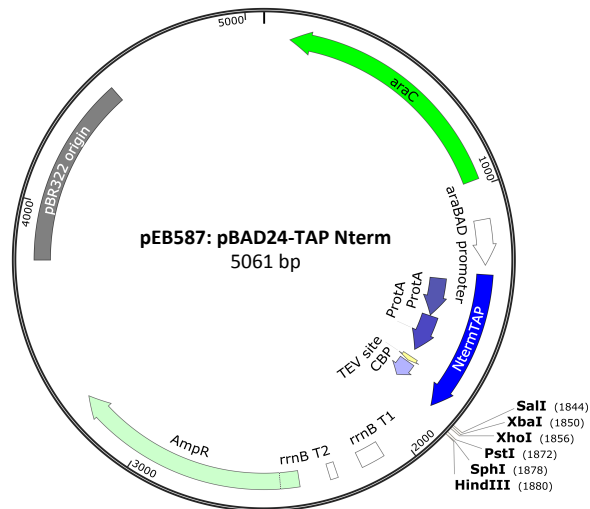


Fig 2. Scheme of the procedure for creation of a C-terminal TAP-tag translational fusion at the chromosomal locus (**A**) and the corresponding nucleotidic and protein TAP-tag sequences (**B**). CBP sequence is in clear purple; upper cases at the beginning of the nucleotidic CBP sequence corresponds to the primer sequence mentioned in 3.1, TEV protease cleavage site is in yellow and Protein A sequence is in dark purple with the stop codon in red.

**A**



**B**

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atggcaggcCTTGCGCAACACGATGAAGCCGTGGACAACAAATTC AACAAAGAACAACAA
M A G L A Q H D E A V D N K F N K E Q Q
AACGCGTTCATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTC
N A F Y E I L H L P N L N E E Q R N A F
ATCCAAAGTTTAAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAG
I Q S L K D D P S Q S A N L L A E A K K
CTAAATGATGCTCAGGCGCCGAAAGTAGACAACAAATTC AACAAAGAACAACAAAACGCG
L N D A Q A P K V D N K F N K E Q Q N A
TTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTCATCCAA
F Y E I L H L P N L N E E Q R N A F I Q
AGTTTAAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAAT
S L K D D P S Q S A N L L A E A K K L N
GGTGCTCAGGCGCCGAAAGTAGACGCGAATgtgatatacctacaactgctcttgaaaat
G A Q A P K V D A N C D I P T T A S E N
ttatattttcaaggtgaactaaagagaagatggaaaaagaatttcatagccgctcagca
L Y F Q G E L K R R W K K N F I A V S A
gccaacgctttaagaaaatctcatcctccggggcacttgtcgaatcgactCTAGActcg
A N R F K K I S S S G A L V E S T L D S
agttcgcacCTGCAGgcatgcAAGCTTg
S S T C R H A S L
  
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Fig 3. Map of plasmid pEB587 used to create a N-terminal TAP-tag translational fusion under the control of the  $P_{BAD}$  arabinose inducible promoter (A) and the corresponding nucleotidic and protein TAP-tag sequences (B). Protein A sequence is in dark purple, TEV protease cleavage site is in yellow, CBP sequence is in clear purple, the multicloning site is underlined and restriction enzyme sites are indicated.