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

2 Julie P.M. Viala and Emmanuelle Bouveret

3

4 Aix-Marseille University - CNRS - UMR7255 - Institut de Microbiologie de la Méditerranée,

5 Laboratoire d'Ingénierie des Systèmes Macromoléculaires, 31 Chemin Joseph Aiguier, 13009

6 Marseille, FRANCE

7

8 Correspondence: jviala@imm.cnrs.fr

9 Running head: TAP in bacteria

10

11 **Summary**

12 The discovery of protein-protein interaction networks can lead to the unveiling of protein
13 complex(es) forming cellular machinerie(s) or reveal component proteins of a specific cellular
14 pathway. Deciphering protein-protein interaction networks therefore contributes to a deeper
15 understanding of how cells function. Here we describe the protocol to perform Tandem
16 Affinity Purification (TAP) in bacteria, which enables the identification of the partners of a
17 bait 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 of a calmodulin binding peptide (CBP) and two
20 immunoglobulin G (IgG) binding domains of *Staphylococcus aureus* protein A (ProtA) that
21 are separated by the tobacco etch virus (TEV) protease cleavage site. After the first round of
22 purification based on the binding of ProtA to IgG coated beads, TEV protease cleavage
23 releases CBP-tagged bait-protein along with its partners for a second round of purification on
24 calmodulin affinity resin and leaves behind protein contaminants bound to IgG. Creating the
25 TAP-tag translational fusion at the chromosomal locus allows detection of protein interactions
26 occurring in physiological conditions.

27

28 **Keywords**

29 Protein–protein interaction, Protein complex, Affinity purification, Tandem affinity
30 purification (TAP), Calmodulin binding peptide (CBP), Protein A (ProtA), Tobacco etch virus
31 (TEV), *Escherichia coli*, *Salmonella*

32

33

34 **1. Introduction**

35 At the end of the 1990s, mass spectrometry combined with genome sequencing rendered
36 possible the rapid and systematic identification of all the proteins present in a purified sample.
37 However, a protocol amenable to standardized and systematic purification of protein
38 complexes without any prior knowledge was missing. In 1999, the laboratory of B. Séraphin
39 at the European Molecular Biology Laboratory in Heidelberg, Germany, proposed such a
40 generic procedure for the identification of protein complexes in yeast (**1**). This permitted the
41 subsequent description of the full interactome of yeast (**2, 3**). This method has since been used
42 in a variety of organisms. We first described its use in bacteria (**4**), and soon after this, it was
43 used to obtain the first interactome of *Escherichia coli* (**5**).

44 One general principle of the tandem affinity purification (TAP) method involves the use
45 of two successive steps of affinity purification to lower as much as possible the amount of
46 contaminants, together with an elution preserving the interactions (without significantly
47 changing the buffer's chemical properties) between these two steps. Specifically, the original
48 TAP tag consists of two repeats of the immunoglobulin G (IgG) binding domain of Protein A
49 (Prot A) from *Staphylococcus aureus* and a calmodulin binding peptide (CBP), separated by a
50 TEV protease cleavage site (**Fig. 1**). However, it must be noted that any combination of
51 affinity tags is potentially usable. Published examples are the GS-TAP (Protein G and Strep
52 tag), the sequential peptide affinity (SPA) tag (CBP and 3Flag), the SF-TAP (Strep-tag II and
53 Flag tag), and the HB tag (6Histidine and Biotin) (see (**6**) for specific references). The second
54 general principle of the TAP procedure is to use physiological expression of the recombinant
55 tagged protein. This needs to be adapted to each organism of interest. For *E. coli* and closely
56 related bacteria, Lambda Red based recombination (**7**) combined with specific dedicated SPA

57 and TAP cassettes (**8**), makes it very easy to introduce the tag at the 3' extremity of the gene
58 on the chromosome to obtain the physiological production of a recombinant protein tagged at
59 its C-terminus (**Fig. 2**). If more convenient, however, TAP tag translational fusion can also be
60 expressed from a plasmid (**Fig. 3**).

61 We present here the TAP protocol that has been successfully used in our institute to purify
62 protein complexes of *E. coli*, *Salmonella*, and *Bacillus subtilis* (**4, 9, 10, 11**). A detailed
63 protocol for the SPA purification has been published before (**12**). To isolate a protein complex
64 by TAP, a strain producing a recombinant bait protein tagged with the TAP must be
65 constructed first (**Fig. 1**, step 1). Then, a soluble extract is prepared from a sufficient volume
66 of bacteria (about 500 mL). The complex is enriched by a first step of affinity
67 chromatography on IgG beads (**Fig. 1**, step 5). After washes, TEV protease is added, which
68 cleaves the specific site located between the CBP and ProtA domains, resulting in the elution
69 of the specifically bound material (**Fig. 1**, step 6). This material is purified a second time by
70 affinity of the CBP tag with calmodulin beads (**Fig. 1**, step 7). After washes, the purified
71 complex is eluted by adding EGTA that chelates the calcium required for the
72 CBP/Calmodulin interaction (**Fig. 1**, step 8). The totality of the purified material is analyzed
73 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Bands detected
74 by coomassie blue or silver staining are cut from the gel and analyzed by mass spectrometry.

75 This is the basic TAP procedure. Note that the procedure can be amenable to
76 adaptation or improvements depending on the specific needs. For example, the extensive
77 washes and the duration of the procedure only allows for the recovery of relatively stable
78 complexes. For the detection of more transient or unstable interactions, a cross-linking
79 procedure can be applied before purification (**13-15**). This might be helpful also for the
80 purification of membrane complexes, where modifications have to be made in the protocol for
81 solubilization of the membranes (**3**). Finally, it is possible to play with the two tags to gain

82 information on the organization of the complexes. Indeed, in some cases, one bait protein
83 might participate in the formation of several types of complexes. To purify one specific type
84 of complex, it is therefore possible to put the two tags on two distinct proteins that are both
85 members of the desired type of complex (split tag method **(9, 16)**). Alternatively, it is possible
86 to perform the subtraction method that consists in eliminating the unwanted complex(es)
87 during the first purification step by leaving it, for example, on IgG beads thanks to a partner
88 protein of the bait that belongs to the unwanted complex and bears a noncleavable ProtA tag.
89 The desired complex, made of untagged partner proteins, will elute with the bait after TEV
90 protease cleavage **(16, 17)**.

91 To our knowledge, the TAP procedure has not been used very much for the
92 characterization of secretion systems in bacteria, certainly owing to the difficulty of working
93 with integral envelope components. However, we used it successfully to reveal a protein-
94 protein interaction involved in the post-translational maturation of the translocon of
95 *Salmonella* T3SS (10). And the TAP procedure has proved to be powerful in identifying the
96 target of effectors of *Yersinia* T3SS (18, 19), *Legionella* T4SS (14, 20) or *Pseudomonas* T6SS
97 in eukaryotic host cells **(21)**. In addition, as mentioned earlier, it is amenable to several
98 improvements that might make it possible to identify unsuspected partners of the secretion
99 machineries in the bacterium.

100

101 **2. Materials**

102 2.1. Creation of a TAP-tag translational fusion and verification of production of hybrid
103 protein by western blot

104 1. Bacterial strain with translational fusion between protein of interest and TAP tag at
105 chromosomal locus (*see Note 1*)

106 2. Alternatively, translational fusion between protein of interest and TAP tag on an
107 appropriate plasmid (*see Note 2*)

108 3. Yeast extract and tryptone media (2YT): 16 g yeast extract, 10 g tryptone, 10 g NaCl, make
109 up to 1 L with distilled water. Autoclave and store at room temperature.

110 4. Lysogeny broth (LB): 5 g yeast extract, 10 g bactotryptone, 10 g NaCl, make up to 1 L with
111 distilled water. Autoclave and store at room temperature.

112 5. Minigel caster system and SDS PAGE apparatus

113 6. Transblot apparatus for western blot

114 7. Peroxidase Anti-Peroxidase antibody (PAP) (Sigma).

115

116 2.2 Protein cytoplasmic extract(PBS): 8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄,
117 make up to 1 L with distilled water. Autoclave and store at room temperature.

118 2. 10 % Nonidet P-40 (NP-40 or Igepal): Mix 10 mL NP-40 to 90 mL of distilled water, pass
119 through a 0.2 µm filter and store at room temperature (*see Note 3*).

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

121 Approximately 50 mL will be required per sample per experiment. Prepare 500 mL

122 containing 5 mL of 1M Tris-HCl, pH 8, 15 mL of 5 M NaCl, 5 mL of 10% NP-40 and
123 475 mL of distilled water. Store at 4°C.

124 4. 0.1 M phenylmethylsulfonyl fluoride (PMSF): Dissolve 87.1 mg PMSF in 5 mL
125 isopropanol. Prepare 1 mL aliquots and store at -20°C (*see Note 4*)

126 5. Liquid nitrogen

127 6. Sonicator, French press or Cell disruptor

128 7. Centrifuge tubes and rotor compatible with spinning volumes of 250 mL, 10 mL and
129 50 mL, at approximately 5, 000xg and 25, 000xg, respectively.

130 2.3. Tandem Affinity purification

131 1. IgG sepharose 6 fast flow (GE Healthcare).

132 2. Protein A binding buffer: see subheading 2.2

133 3. 0.5 M EDTA ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$): Dissolve 18.6 g of EDTA in 80 mL of distilled
134 water, make up to 100 mL with distilled water once the pH has been adjusted to 8 with
135 10 N NaOH (*see Note 5*). Autoclave and store at room temperature.

136 4. TEV cleavage buffer : 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% NP-40,
137 0.5 mM EDTA, 1 mM dithiothreitol (DTT) (*see Note 6*)

138 5. AcTEVTM protease (Invitrogen)

139 6. Calmodulin binding buffer : 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% NP-40,
140 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, 10 mM β-mercaptoethanol.
141 Approximately 40 mL will be required per sample per experiment. Prepare 500 mL
142 containing 5 mL of 1M Tris-HCl, pH 8, 15 mL of 5M NaCl, 5 mL of 10% NP-40, 500 μl

143 1 M magnesium acetate, 500 μ l 1 M imidazole, 1 mL 1 M CaCl₂, 348.5 μ l
144 14.3 M β -mercaptoethanol (*see Note 7*) and 473 mL of distilled water. Store at 4°C.

145 7. 1 M CaCl₂: Dissolve 11.1 g to 100 mL distilled water. Autoclave and store at room
146 temperature.

147 8. Calmodulin affinity resin (Agilent).

148 9. 1 M EGTA: Dissolve 19 g in 40 mL distilled water, make up to 50 mL with distilled water
149 once the pH has been adjusted to 8 with 10 N NaOH (*see Note 5*), 0.2 μ m filter and store at
150 4°C.

151 10. Calmodulin elution buffer : 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% NP-40,
152 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10 mM β -mercaptoethanol.
153 Approximately 1 mL will be required per sample per experiment. Prepare 100 mL containing
154 1 mL of 1M Tris-HCl, pH 8, 3 mL 5M NaCl, 1 mL 10% NP-40, 100 μ l
155 1 M magnesium acetate, 100 μ l 1 M imidazole, 200 μ l 1 M EGTA, 69,7 μ l
156 14.3 M β -mercaptoethanol (*see Note 7*) and 94.5 mL of distilled water. Store at 4°C.

157 11. Disposable chromatography columns of 10 mL with narrow bottom, for example, Poly-
158 prep chromatography column from Biorad

159 12. Rotating wheel

160 13. Centrifuge tubes and rotor compatible with spinning volumes of 10 mL at approximately
161 25, 000xg.

162

163 2.4. Trichloroacetic Acid Precipitation

164 1. 16 mg/mL sodium deoxycholate: Dissolve 160 mg of sodium deoxycholate in 10 mL water.
165 Pass through a 0.2 µm filter and store at room temperature.

166 2. Liquid trichloroacetic acid (TCA) (stock is 100 %)

167 3. TCA washing buffer: Mix 70 mL acetone, 20 mL ethanol, 5 mL 1 M Tris-HCl, pH 8 and
168 5 mL of distilled water. Store at 4°C.

169 4. SDS PAGE Loading buffer

170

171

172 **3. Methods**

173 A translational fusion between the protein of interest and TAP tag, either at the chromosomal
174 locus or on an appropriate plasmid must be constructed (*see Note 1 and 2*). Translational
175 fusion at the chromosomal locus will allow a physiological expression, while constructing the
176 translational fusion on a plasmid may be more amenable.

177

178 3.1. Verification of expression of TAP-tag translational fusion by western blot

179 1. Prepare a cytoplasmic or a crude protein extract (*see Note 8*)

180 2. Load 10 µg of protein extract (or proteins corresponding to a bacterial sample of
181 0.3 OD₆₀₀ unit) on a SDS-PAGE and proceed to transfer and western blot to verify production
182 of hybrid protein (*see Note 9*).

183 3. Perform a one step western blot using PAP antibody (*see Note 10*) and using an
184 appropriate substrate to detect horseradish peroxidase activity (*see Note 11*)

185

186 3.2 Protein cytoplasmic extract

187 1. Day 1 - Inoculate 10 mL of 2YT media with a bacterial colony and grow overnight at
188 37°C with shaking (*see Note 8*).

189 2. Day 2 - Dilute culture 100 fold in 500 mL LB and grow 5h30 at 37°C with shaking
190 until $OD_{600} \approx 2-3$.

191 3. Pellet bacteria by centrifugation 20 min 5000xg at 4°C.

192 4. Wash once with cold PBS, transfer to 50 mL centrifuge tubes, centrifuge again for 10
193 min 5000xg at 4°C, discard supernatant and freeze bacterial pellets with liquid nitrogen.

194 Maintain frozen bacterial pellet at -80°C until you are ready to prepare cytosolic protein
195 extract and proceed to Tandem Affinity Purification.

196 5. Day 3 - Resuspend frozen bacterial pellets with 10 mL of Protein A binding buffer
197 containing 0.5 mM PMSF (*see Note 4*).

198 6. Use sonication, French Press or Cell disruptor to break bacterial cells (*see Note 12*).

199 7. Centrifuge for 30 min 25,000xg at 4°C and save supernatant, which is the cytoplasmic
200 protein extract.

201

202 3.3 Tandem Affinity Purification

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

- 205 1. Put 200 μ l of IgG sepharose beads in a disposable chromatography column and wash
206 by gravity with 5 mL of Protein A binding buffer.
- 207 2. *Binding of Protein A tag to IgG sepharose beads.* After washing the beads, close the
208 bottom of the chromatography column and, using a pipette, transfer 9 mL of the cytoplasmic
209 protein extract. Close the top of the column and put on a wheel for 2 hours at 4°C.
- 210 3. Remove first the top plug of the column and then the bottom one. Leave the unbound
211 material flow by gravity and discard.
- 212 4. Wash three times the IgG beads with 10 mL of Protein A binding buffer.
- 213 5. *TEV protease cleavage.* Close bottom of column, fill it with 1 mL of TEV cleavage
214 buffer and 100 units of AcTEV™ protease. Close top of column and put on wheel at room
215 temperature for 1 h.
- 216 6. Remove top and bottom plugs and recover elution fraction by gravity. Add additional
217 200 μ L of TEV cleavage buffer in column to recover as much material as possible from the
218 sides of column.
- 219 7. Add 3 mL calmodulin binding buffer and 3 μ l of 1M CaCl₂ (*see Note 13*) to elution
220 fraction.
- 221 8. *Binding by the CBP tag part on calmodulin affinity resin.* Put 200 μ l of calmodulin
222 affinity resin in a new disposable chromatography column, wash it with 5 mL of Calmodulin
223 binding buffer. Then, close bottom of column.
- 224 9. Add the 4.2 mL of the elution fraction (obtained at steps 6 and 7). Close top of column
225 and put on wheel for 1 hour at 4°C.

- 226 10. Remove first the top plug of the column and then the bottom one. Leave unbound
227 material to flow by gravity and discard.
- 228 11. Wash three times calmodulin affinity resin with 10 mL calmodulin binding buffer.
- 229 12. *Elution.* Elute with five times 200 μ L of Calmodulin elution Buffer.
- 230 13. Pool fraction 2, 3 and 4 and proceed to TCA precipitation of elution fraction 1, pooled
231 fractions 2, 3 and 4, and fraction 5.

232

233 3.4 TCA precipitation

- 234 1. To each of the eluted protein samples, add 1/100th of 16 mg/mL sodium deoxycholate.
235 Vortex and leave on ice 30 min.
- 236 2. Add TCA to 10% final. Vortex and leave on ice 30 min.
- 237 3. Centrifuge 15 min 15,000xg at 4°C.
- 238 4. Wash twice with TCA washing buffer.
- 239 5. Leave pellets to dry on bench and resuspend in 20 μ L protein loading buffer 1x.

240

241 3.5 Analysis by SDS-PAGE and mass spectrometry

- 242 1. Load totality of samples on SDS-PAGE (see **Note 14**) and stain with Coomassie Blue.
- 243 2. Unstain and then rinse with distilled water.
- 244 3. Cuts bands to identify partner proteins by mass spectrometry (*see Note 15*).

245

246 4. Notes

- 247 1. A C-terminal TAP tag translational fusion can be introduced at the chromosomal locus
248 using the λ red recombination system (7). To prepare the appropriate polymerase chain
249 reaction (PCR) product, use pJL72 plasmid as template (this latter harbors a cassette made of
250 the TAP tag and the kanamycin resistance gene, **Fig. 2A**) (8), design a forward primer that
251 contains, in the 5'-end, the 45 nucleotides that are immediately upstream the stop codon of the
252 gene of interest, followed by the sequence 5'-TCCATGGAAAAGAGAAG-3' (this sequence
253 will hybrid to the CBP tag, **Fig. 2B**) and design a reverse primer that contains at its 5'-end the
254 reverse complement 45 nucleotides that are immediately downstream the stop codon of the
255 gene of interest, followed by the sequence 5'-CATATGAATATCCTCCTTAG-3' (**Fig. 2A**).
- 256 2. Alternatively, the sequence corresponding to the open reading frame of the gene of interest
257 can be cloned in the plasmid pEB587 (17) (**Fig. 3A**), which allows a N-terminal TAP tag
258 translational fusion (**Fig. 3B**) under the control of the arabinose inducible promoter P_{BAD}.
- 259 3. Gently agitate the solution for complete dissolution of NP-40 if necessary.
- 260 4. PMSF crystallizes at -20°C, so heat the PMSF aliquot to 37°C to redissolve the PMSF
261 before use. We use PMSF as generic protease inhibitor but protease inhibitor cocktail can be
262 used as well.
- 263 5. EDTA and EGTA may not be soluble until pH had been adjusted to 8 with 10 N NaOH.
- 264 6. Add DTT to the volume of buffer you will need when starting the experiment. DTT is
265 necessary for TEV activity.
- 266 7. Add β -mercaptoethanol to the volume of buffer you will need when starting the
267 experiment.

268 8. Also plan to prepare a protein extract of an untagged strain as negative control of the
269 experiment. Or, use a strain that produce a TAP tag but uncoupled from the bait protein as
270 negative control.

271 9. Translational TAP tag fusion adds 20 kDa to the mass of the protein of interest; 3 kDa
272 corresponds to the CBP tag and 15 kDa to the Protein A tag.

273 10. Immunoglobulins will bind the protein A fragment of TAP tag.

274 11. In our experience, the detection of the tagged protein in crude extracts using this PAP
275 antibody is mandatory for a successful TAP purification.

276 12. French Press or Cell disruptor might be more gentle to preserve protein complexes.

277 13. Addition of extra CaCl_2 is required to quench EDTA that was previously necessary for
278 TEV protease activity.

279 14. Usually a 12% SDS-PAGE allows visualization of low and high molecular weight
280 proteins. Alternatively, proteins can just be sandwiched in the stacking gel and all together
281 analyzed by mass spectrometry. Plan a biological triplicate for the sample of interest and its
282 negative control.

283 15. Use one blade per band if proteins have been separated through the running gel.

284

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346

347 **Figure Captions**

348 **Fig. 1. Guidelines for overall TAP procedure**

349 **Fig 2. Creation of a C-terminal TAP-tag translational fusion at chromosomal locus**

350 Scheme of the TAP-tag translational fusion at the chromosomal locus (A) and corresponding
351 nucleotidic and protein TAP-tag sequences (B). CBP sequence is in clear purple; uppercases
352 at the beginning of the nucleotidic CBP sequence correspond to the primer sequence

353 mentioned in subheading 3.1, TEV protease cleavage site is in yellow and Protein A sequence
354 is in dark purple with stop codon in red.

355

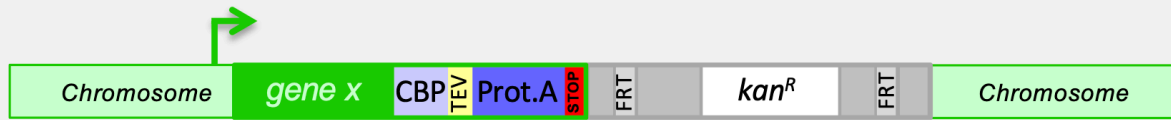
356 **Fig 3. Creation of a plasmidic N-terminal TAP-tag translational fusion**

357 Map of plasmid pEB587 used to create a N-terminal TAP-tag translational fusion under the
358 control of the P_{BAD} arabinose inducible promoter (**A**) and the corresponding nucleotidic and
359 protein TAP-tag sequences (**B**). Protein A sequence is in dark purple, TEV protease cleavage
360 site is in yellow, CBP sequence is in clear purple, the multicloning site is underlined and
361 restriction enzyme sites are indicated.

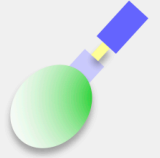
362

Guidelines

1. Construction

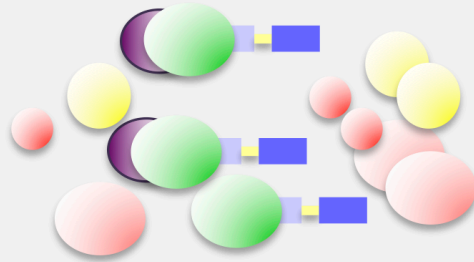


2. Check production of the TAP-tagged bait protein by Western blot with PAP antibody



3. Larger scale production

4. Cytoplasmic protein extract



Add:

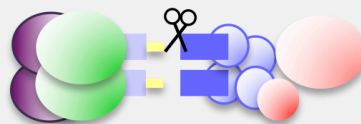
5. Protein A binding

partner bait contaminants



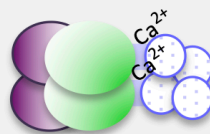
 IgG beads


6. TEV cleavage



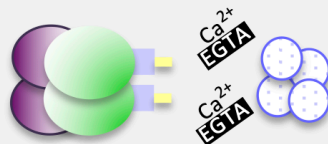
 TEV protease

7. CBP binding



 Calmodulin beads
Ca²⁺

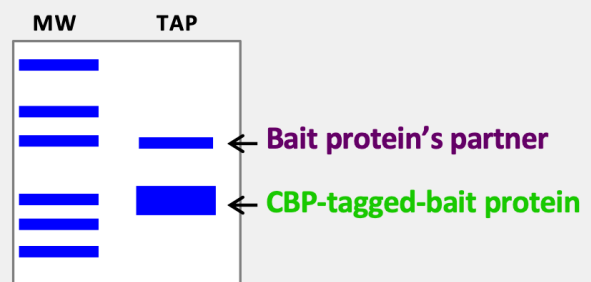
8. Elution + TCA precipitation

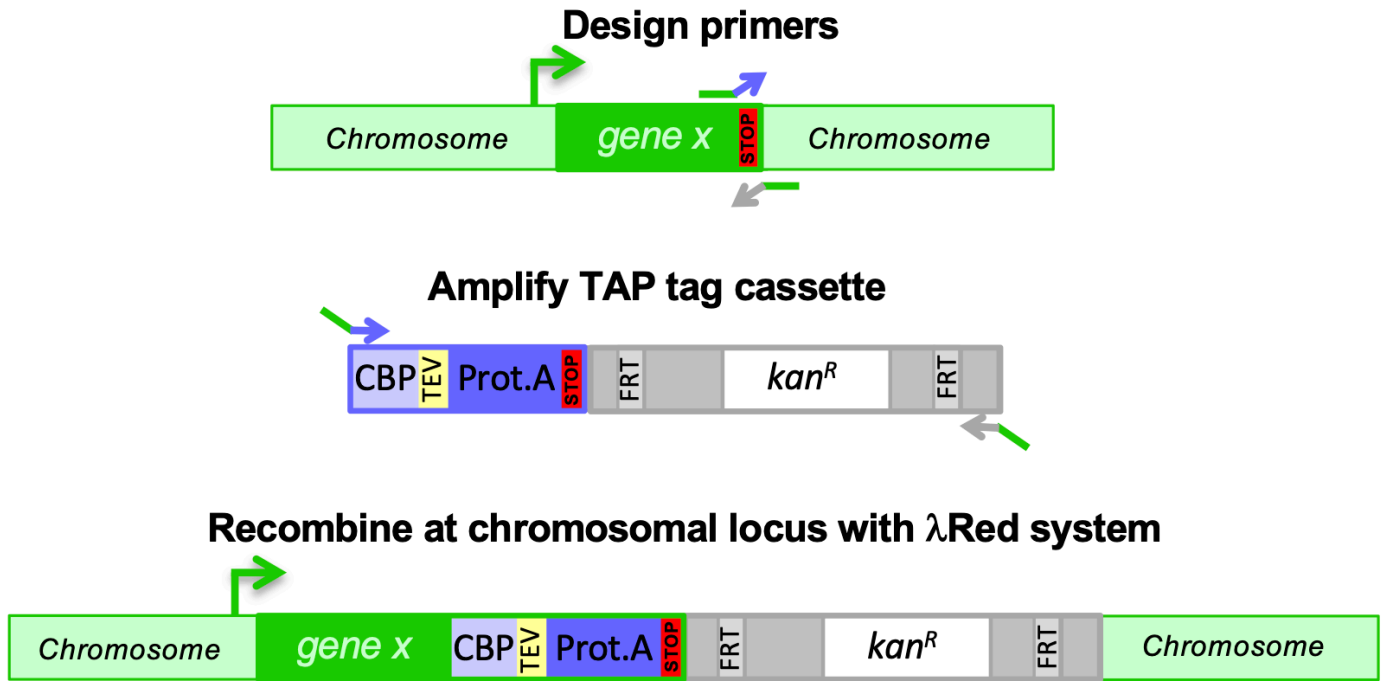


EGTA

9. Visualization by SDS-PAGE

Identification by Mass spectrometry



a**b**

TCCATGGAAAAGAGAAGatggaaaaagaatttcatagccgtctcagcagccaaccgcttt
 S M E K R R W K K N F I A V S A A N R F

aagaaaatctcatcctccggggcacttgattatgatattccaactactgctagcgagaat
 K K I S S S G A L D Y D I P T T A S E N

ttgtatthttcaggggtGAGctcAAAACCGCGGCTCTTGCGCAACACGATGAAGCCGTGGAC
 L Y F Q G E L K T A A L A Q H D E A V D

AACAAATTC AACAAAGAACAACAAAACGCGTTCTATGAGATCTTACATTTACCTAACTTA
 N K F N K E Q Q N A F Y E I L H L P N L

AACGAAGAACAACGAAACGCCTTCATCCAAAGTTTAAAAGATGACCCAAGCCAAAGCGCT
 N E E Q R N A F I Q S L K D D P S Q S A

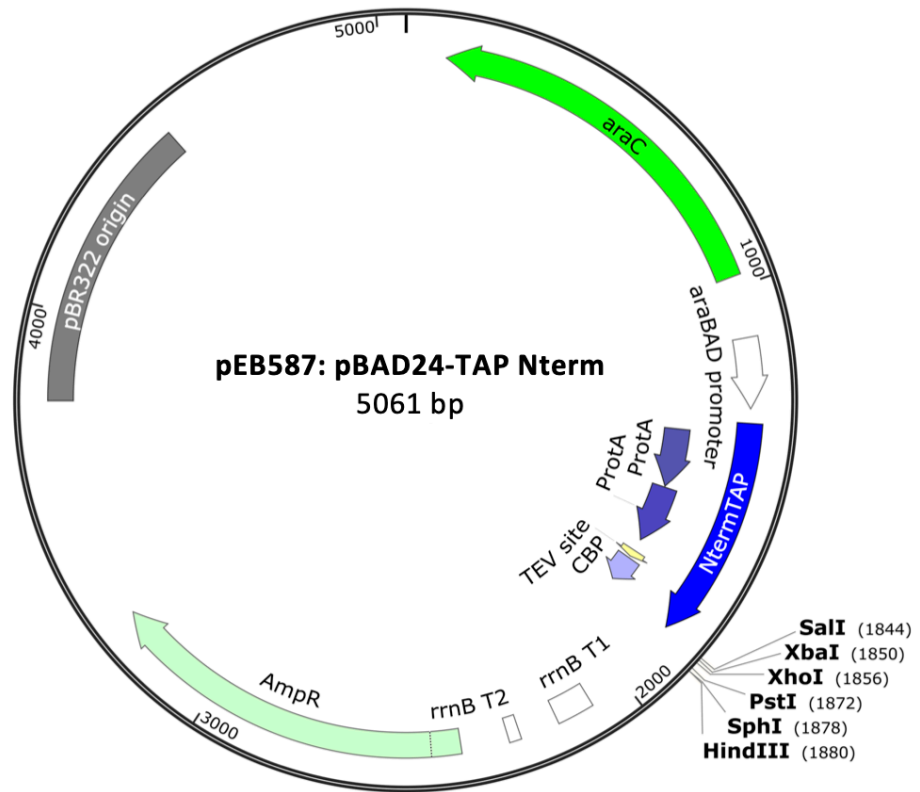
AACCTTTTAGCAGAAGCTAAAAAGCTAAATGATGCTCAGGCGCCGAAAGTAGACAACAAA
 N L L A E A K K L N D A Q A P K V D N K

TTCAACAAGAACAACAACAAAACGCGTTCTATGAGATCTTACATTTACCTAACTTAAACGAA
 F N K E Q Q N A F Y E I L H L P N L N E

GAACAACGAAACGCCTTCATCCAAAGTTTAAAAGATGACCCAAGCCAAAGCGCTAACCTT
 E Q R N A F I Q S L K D D P S Q S A N L

TTAGCAGAAGCTAAAAAGCTAAATGGTGCTCAGGCGCCGAAAGTAGACGCGAATTCCGCG
 L A E A K K L N G A Q A P K V D A N S A

GGGAAGTCAACC TGA
 G K S T *

a**b**

atggcaggcCTTGCGCAACACGATGAAGCCGTGGACAACAAATTCAACAAAGAACAACAA
M A G L A Q H D E A V D N K F N K E Q Q

AACGCGTTCTATGAGATCTTACATTTACCTAACTTAAACGAAGAACAACGAAACGCCTTC
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

CTAAATGATGCTCAGGCGCCGAAAGTAGACAACAAATTCAACAAAGAACAACAAAACGCG
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

GGTGCTCAGGCGCCGAAAGTAGACGCGAATtgtgatatacctacaactgcttctgaaaat
G A Q A P K V D A N C D I P T T A S E N

ttatattttcaaggtgaactaaagagaagatggaaaaagaatttcatagccgtctcagca
L Y F Q G E L K R R W K K N F I A V S A

gccaaccgctttaagaaaatctcatcctccggggcacttgctcgagtgcacTCTAGActcg
A N R F K K I S S S G A L V E S T L D S

agttcgacCTGCAGgcatgcaAAGCTTg
S S T C R H A S L

Restriction sites:
PstI **SphI** **HindIII** **SalI** **XbaI** **XhoI**