Chapter 10

Analyzing Phage–Host Protein–Protein Interactions Using *Strep*-tag® II Purifications

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Abstract

After injecting their genome into the bacterial host cell, bacteriophages need to convert the host metabolism toward efficient phage production. For this, specific proteins have evolved which interact with key host proteins to inhibit, activate or redirect the function of these proteins. Since 70% of the currently annotated phage genes are hypothetical proteins of unknown function, the identification and characterization of these phage proteins involved in host–phage protein–protein interactions remains challenging. Here, we describe a method to identify phage proteins involved in host–phage protein–protein interactions using a combination of affinity purifications and mass spectrometry analyses. A bacterial strain is engineered in which a bacterial target protein is fused to a *Strep*-tag® II at the C-terminal end. This strain is infected with a specific bacteriophage, followed by an affinity purification of the tagged protein which allows the copurification of all bacterial and phage specific interacting proteins. After SDS-PAGE analysis and an in-gel trypsin digestion, the purified interacting proteins are identified by mass spectrometry analysis. The identification of phage proteins involved in interactions provides first hints toward the elucidation of the biological function of these proteins.

Key words Phage–host protein–protein interactions, Affinity purifications, Bacteriophage, *Pseudomonas aeruginosa*, Mass spectrometry

1 Introduction

Upon infection of a susceptible bacterial cell, bacteriophages hijack the molecular machinery of their host to obtain efficient production of phage progeny. Specific mechanisms have evolved to achieve this and protein–protein interactions (PPI) between phage proteins and key host proteins play a crucial role in this. These PPIs serve to inhibit, activate, or redirect the function of the bacterial interaction partner [1]. Consequently, a number of studies state that the examination of these phage–host PPIs can provide a powerful tool in the search for new antibacterial targets in drug discovery [2, 3]. However, only a limited number of host-phage PPIs have

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already been studied, most of them involving interactions of the RNA polymerase of the model organism *Escherichia coli* and proteins of its phages, although emphasis has been placed on *Pseudomonas aeruginosa* in recent years [4–6].

One of the main challenges in phage biology is the functional elucidation of the in silico annotated phage genes. Due to the progress in high-throughput sequencing techniques, there is an increasing gap between the number of annotated phage genes and their functional annotation. Currently, about 70% of the annotated phage genes are hypothetical genes of unknown function (NCBI Entrez database). Many of them are small, early expressed phage genes which are believed to be involved in the specific host-phage PPIs described above [1].

Here, we describe a technique to identify and study host-phage PPIs by using affinity purifications coupled to mass spectrometry analyses. Key proteins of the host, in this case the opportunistic pathogen *Pseudomonas aeruginosa* [7, 8], are used as bait to pull-down interacting host and phage proteins. An advantage of using PPI techniques to study phage proteins is that their bacterial interaction partner provides a first direct clue toward the function of the phage protein. Indeed, it has become clear that proteins mediate their biological function through PPIs [9]. Moreover, the use of affinity purifications offers a wide screen for both direct and indirect interactions, in contrast to binary PPI techniques such as the yeast two-hybrid system [10]. To distinguish true and false positive interactions, we emphasise that secondary and complementary PPI assays need to be performed once an interaction is identified. However, when setting up a large-scale analysis involving several target host proteins and bacteriophages, the discrepancy between true and false hits is tremendously facilitated [11].

An overview of the different steps of the protocol is illustrated in Fig. 1. Once a target protein of the host is selected for analysis, a mutated *P. aeruginosa* strain is engineered which carries a genomic introduced affinity tag fused to its *C*-terminus. We selected the eight amino acid long *Strep*-tag® II, which is based on the streptavidin-biotin system [12]. Because of the small size, there is a very low chance of interference with the protein folding and function, and the protein complex can be purified in a one-step protocol [13]. The strain is constructed by "in vivo homologous recombination," using the λ Red recombination system [14] and a cassette containing the homologous fragments, a *Strep*-tag® II and a gentamicin resistance gene (*see* Fig. 2) (*see* Subheading 2.1). Prior to affinity purification, the viability of the strain and its sensitivity to phage infection are verified and compared to the wild type strain. Moreover, the detectability of the tagged protein is investigated (*see* Subheading 2.2). Subsequently, the strain is infected with a *P. aeruginosa* specific phage, the infection is stopped in the early stage of infection and an affinity purification is performed (*see*

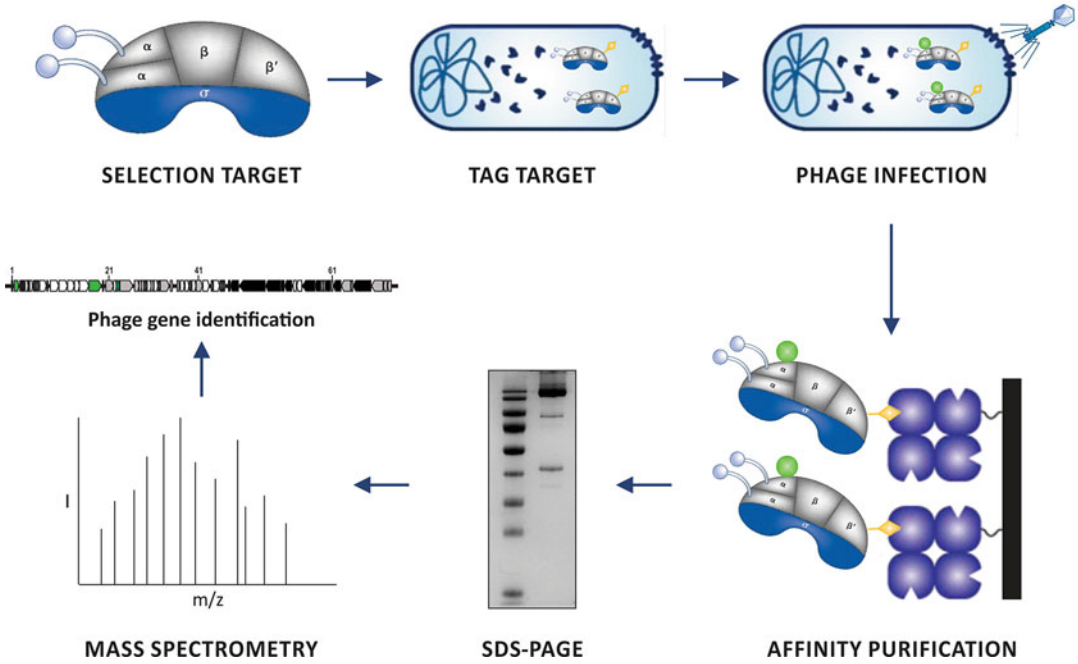

Fig. 1 Overview of the protocol used for the identification of interacting phage proteins. After selecting a bacterial target protein, a *Strep*-tag® II is fused at the C-terminal end of this target using in vivo homologous recombination. In the next step, the recombinant strain is infected with a specific bacteriophage and the infection cycle is stopped in the early phase of infection. The cells are lysed and an affinity purification is performed to purify the target protein and all interacting proteins. The eluted protein samples are loaded on SDS-PAGE. Finally, the samples are subject to an in-gel trypsin digestion and the resulting peptides are analysed by mass spectrometry, to identify interacting phage proteins

Subheading 2.3). The eluted fractions are subject to SDS-PAGE analysis (*see* Subheading 2.4), after which an in-gel trypsin digestion is performed and the samples are sent for mass spectrometry analyses to identify all purified proteins (*see* Subheading 2.5). By screening against a database that contains all host proteins and all "stop-to-stop" protein sequences in all six reading frames of the used phages, biases toward annotated genes are avoided and new, previously unannotated proteins might be identified (proteogenomics) [11, 15].

2 Materials

Prepare all solutions with ultrapure water and use analytical grade reagents. Prepare and store all reagents at room temperature (unless stated otherwise).

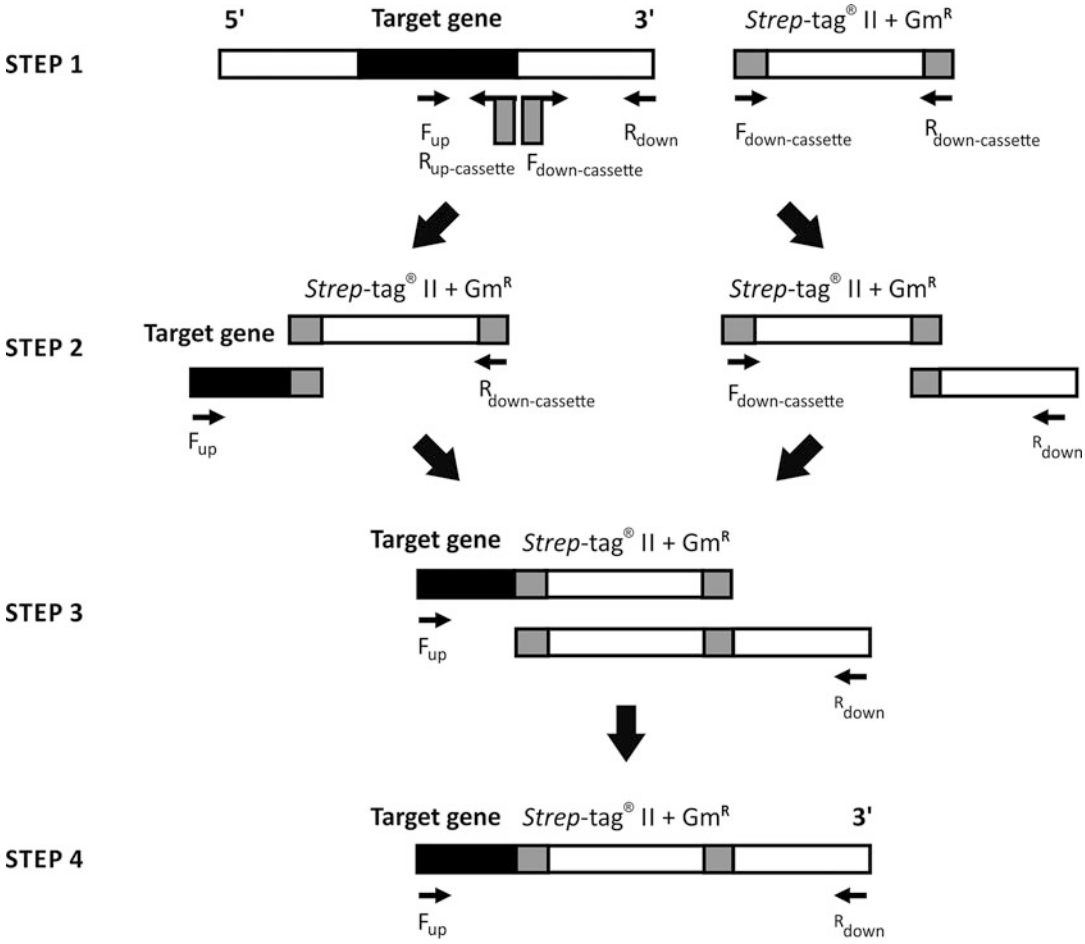

Fig. 2 Steps to produce the DNA construct for homologous recombination. In **step 1**, the separate fragments are amplified (C-terminal part of target gene without the stop codon, cassette containing *Strep*-tag® II + Gm^R gene and the 3′ region of the gene). In **step 2**, the two fragments are allowed to fuse and primers are added to get the two constructs which share the Gm^R cassette. In **step 3**, the fragments obtained in **step 2** are fused and primers are added. In **step 4**, the full DNA fragment is amplified using the outer primers

2.1 Construction of a C*-Terminal Strep-tag® II Fusion Protein in P. aeruginosa PAO1*

2.1.1 Construction of the Strep-tag® II Construct

1. 1 ng–1 μg/μl *P. aeruginosa* PAO1 genome (template).
2. 1 ng–1 μg/μl plasmid coding for gentamycin resistance (template).
3. DNA polymerase enzyme with corresponding PCR buffer (commercially available).
4. 10 mM dNTP solution.
5. Primers for sequencing: 5 μM working solution.
6. Primers for construct design: 20 μM working solution.
7. GeneJet PCR Purification kit (Thermo Fisher Scientific; https://www.thermofisher.com/).
8. GeneJet Gel Extraction kit (Thermo Fisher Scientific).

9. TOPO TA cloning kit for sequencing (Thermo Fisher Scientific).
10. Agarose.
11. Ethidium bromide (50 μg/ml).
12. 6× Loading buffer: 40% (w/v) sucrose, 0.1% (w/v) bromophenol blue.
13. TAE running buffer: 40 mM Tris (pH 7.2), 0.5 mM sodium acetate and 50 mM ethylene-diaminetetraacetic acid (EDTA).
14. DNA size concentration ladder (e.g., GeneRuler DNA ladder mix, Thermo Fisher Scientific).
15. PCR machine (T3000 Thermocycler, Biometra; http://www.biosciences.ie/biometra).

2.1.2 In Vivo Recombination

1. *P. aeruginosa* PAO1 strain containing the pUC18-RedS plasmid (coding for the lambda-Red recombination proteins [14]).
2. Autoclaved Lysogeny Broth (LB): 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract.
3. Autoclaved LB solid: 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar.
4. 1000× stock of carbenicillin (200 mg/ml).
5. 1000× stock of gentamycin (30 mg/ml).
6. 20% (w/v) L-arabinose.
7. 300 mM sucrose.
8. 100% glycerol.
9. Primers for sequencing: 5 μM working solution.
10. DNA polymerase enzyme with corresponding PCR buffer.
11. 10 mM dNTP solution.
12. GeneJet PCR purification kit (Thermo Fisher Scientific).
13. Spectrophotometer (LKB Novaspec® II, Pharmacia Biotech; http://www3.gehealthcare.com/).
14. Electroporator (Bio-Rad Pulser) and 0.2 cm electroporation cuvettes (Bio-Rad laboratories).
15. PCR machine (T3000 Thermocycler, Biometra).

2.2 Verification of the Constructed Strains

2.2.1 Effect on the Bacterial Viability and the Infectivity of the Phage

1. *P. aeruginosa* PAO1 strain containing the *target*::*Strep*II fusion (made in Subheading 3.1) and the wild type *P. aeruginosa* PAO1 strain.
2. Pure stock of selected phage (>10^{10} PFU/ml), stored in phage buffer at 4 °C (*see* **Note 1**).
3. Autoclaved LB.
4. Autoclaved LB solid.

5. Autoclaved LB soft: 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.7% (w/v) agar.
6. Phage buffer: 10 mM Tris (pH 7.5), 100 mM $MgSO_4$, 150 mM NaCl.
7. Spectrophotometer (LKB Novaspec® II, Pharmacia Biotech).

2.2.2 The Production of the Strep-tag® II-Fused Protein

1. *P. aeruginosa* PAO1 *target*::*Strep*II strain and the wild type *P. aeruginosa* PAO1 strain.
2. 50 ml autoclaved LB containing 30 μg/ml gentamicin (using a 1000× stock of 30 mg/ml).
3. Filtered (0.22 μm) TE buffer: 50 mM Tris (pH 8.0), 2 mM EDTA.
4. Cooled transfer buffer: 25 mM Tris, 192 mM Glycine, 20% (v/v) Ethanol (*see* **Note 2**).
5. PBST buffer: 140 mM NaCl, 10 mM KCl, 10 mM Na_2PO4, 1.8 mM KH_2PO_4, 0.1% (v/v) Tween, pH 7.5 (*see* **Note 3**).
6. Blocking solution: PBST + 5% (w/v) Powder milk.
7. Ultrapure H_2O.
8. A protein carrying a *Strep*-tag® II (positive control).
9. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).
10. Hen Egg White Lysozyme (HEWL, Sigma Aldrich; https://www.sigmaaldrich.com/).
11. Pefabloc® SC (4-(2-aminoethyl)-benzene-sulfonyl fluoride, aebsf, aminoethyl-benzene-sulfonyl fluoride, 4-2-, proteinase k inhibitor; https://www.sigmaaldrich.com/).
12. Benzonase® nuclease (EMD Millipore Corporation; http://www.emdmillipore.com/).
13. Prestained reference ladder (e.g., the PageRuler Prestained Protein Ladder (Thermo Fisher Scientific)).
14. Monoclonal anti-*Strep*-tag® II antibodies conjugated to horseradish peroxidase (HRP, IBA).
15. Whatman paper (Sigma-Aldrich).
16. Transparant paper.
17. Nitrocellulose membrane (Hybond-C Extra, Ge Healthcare).
18. Amersham Hyperfilm ECL (18 × 24 cm) (GE Healthcare).
19. Hypercassette Blue Std Depth 18 × 24 cm (GE Healthcare).
20. Mini Trans-Blot® Cell (Bio Rad): Gel Holder Cassette, Foam Pads, Trans-Blot Central Core, Bio-Ice Cooling Unit and Mini-PROTEAN® Tetra Cell Systems (electrophoresis chamber).

21. Sonicator (Sonics Ultra cell; https://www.sonics.com/).
22. Heating block (95 °C).
23. WT17 mini tumbling table (Biometra).

2.3 Affinity Purifications

1. *P. aeruginosa* PAO1 *target*::*Strep*II strain and the wild type *P. aeruginosa* PAO1 strain.
2. 600 ml autoclaved LB containing 30 μg/ml gentamicin.
3. Pure stock of selected phage (>10^{10} PFU/ml), stored in phage buffer at 4 °C (*see* **Note 1**).
4. Resuspension buffer: 10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) NP-40 (*see* **Note 4**).
5. Wash buffer: 100 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, or *Strep*-tag® Washing Buffer (IBA) (*see* **Note 4**).
6. Elution buffer: 100 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, or dilute 10× *Strep*-tag® II Elution (Buffer E, IBA).
7. Regeneration buffer: dilute 10× *Strep*-tag® Regeneration Buffer (IBA).
8. Hen Egg White Lysozyme (HEWL, Sigma Aldrich).
9. Pefabloc® SC (Merck).
10. Benzonase® nuclease (EMD Millipore Corporation).
11. 10× BugBuster® Protein extraction reagent (http://www.emdmillipore.com/).
12. *Strep*-Tactin® Sepharose beads (Sigma-Aldrich).
13. A 10 ml Bio-Rad Poly-Prep® Chromatography column (Bio-Rad Laboratories; http://www.bio-rad.com/).
14. An ice-cold collection tube (300–600 ml) (stored at −80 °C, at the start of the procedure).
15. An icy water bath.

2.4 SDS-PAGE

1. SDS-PAGE 4× loading buffer: 200 mM Tris (pH 6.8), 8 mM EDTA, 40% (v/v) glycerol, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue.
2. Separation gel 12%: Tris–SDS buffer pH 8.8 (1.5 mM Tris (pH 8.8), 0.4% (w/v) SDS), 12% (v/v) 37.5:1 acrylamide–bisacrylamide gel, 0.01% (v/v) APS (ammonium persulfate), 0.001% (v/v) TEMED (N,N,N′,N′ tetramethylethylenediamine).
3. Stacking gel 4%: Tris–SDS buffer pH 6.8 (1.5 mM Tris (pH 6.8), 0.4% (w/v) SDS), 4% (v/v) acrylamide–bisacrylamide gel, 0.01% (v/v) APS, 0.001% (v/v) TEMED.

4. Running buffer: 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS.
5. Isopropanol.
6. Standard reagents for Coomassie blue staining (e.g., GelCode Blue Safe (Thermo Fisher Scientific) or the more sensitive Imperial Protein Stain (Thermo Fisher Scientific)).
7. Heating block (95 °C).
8. Standard 1D-gel electrophoresis unit (e.g., Mini-PROTEAN® Tetra Cell Systems, BioRad).

2.5 Mass Spectrometry Analyses

1. 133 mM NH_4HCO_3: 1.05 g NH_4HCO_3/100 ml ultrapure H_2O.
2. 100 mM NH_4HCO_3: 0.79 g NH_4HCO_3/100 ml ultrapure H_2O.
3. 50 mM NH_4HCO_3: 25 ml 100 mM NH_4HCO_3 + 25 ml ultrapure H_2O.
4. 20 mM NH_4HCO_3: 10 ml 100 mM NH_4HCO_3 + 40 ml ultrapure H_2O.
5. 55 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3: 0.01 g IAA/ml 100 mM NH_4HCO_3 (prepare shortly before use) (*see* **Note 5**).
6. 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3: 0.0015 g DTT/ml 100 mM NH_4HCO_3 (prepare shortly before use).
7. 50 mM acetic acid: dilute 286 µl acetic acid in ultrapure H_2O to 100 ml.
8. Trypsin Gold (Promega): 20 µg lyophilized trypsin/ml 50 mM acetic acid (store at −80 °C).
9. Trypsin digestion buffer: 150 µl trypsin (20 µg/ml) + 90 µl 133 mM NH_4HCO_3 (prepare shortly before use).
10. 5% formic acid in 50% acetonitrile.
11. 25 mM NH_4HCO_3 in 50% acetonitrile: 195.65 mg NH_4HCO_3/100 ml ultrapure H_2O + 100 ml acetonitrile.
12. Vacuum centrifuge (Martin Christ Gefriertrocknungsanlagen GmbH; Osterode am Harz, Germany; https://www.martinchrist.de/).
13. Oven at 37 °C.
14. Sonicator bath (Branson 2210, https://www.bransonic.com/).
15. Water bath at 56 °C.
16. Icy water bath.

17. Mass spectrometry facility: Group of Prof. Jean-Paul Noben, Biomedical Research Institute and Transnational University Limburg, Hasselt University, 3950 Diepenbeek, Belgium.
Equipment: Easy-nLC 1000 liquid chromatograph (Thermo Fisher Scientific) on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) via a Nanospray Flex ion source (Thermo Fisher Scientific) using sleeved 30 μm ID stainless steel emitters
Software: Proteome Discoverer software v.1.3 (Thermo Fisher Scientific) with build-in Sequest and interfaced with an in-house Mascot v.2.4 server (Matrix Science).

3 Methods

3.1 Construction of a C-Terminal Strep-tag® II Fusion Protein in P. aeruginosa PAO1

3.1.1 Construction of the Strep-tag® II Construct

Once a target protein of *P. aeruginosa* is selected, a DNA construct which will be used for homologous recombination is made using an "overlap-extension" PCR. This construct contains the C-terminal part of the target protein fused to a *Strep*-tag® II, followed by a gentamicin resistance (Gm^R) gene and the fragment downstream of the target gene. The principle for constructing this *Strep*-tag® II-containing DNA fragment is shown in Fig. 2.

1. Design the six primers which are needed to generate the three fragments (*see* **Note 6**). These fragments are a cassette, which contains the *Strep*-tag® II sequence at the 5′ end followed by the Gm^R gene, the 3′ part (approx. 300 bp) of the target gene and the downstream 300 bp.
2. Amplify the three fragments using standard PCR. Use the *P. aeruginosa* PAO1 genome as template for the 3′ fragment of the gene and the downstream fragment. Use a vector containing the Gm^R gene as template for the cassette.
3. Check the correct size of all three fragments by gel electrophoresis and purify the product with a PCR purification kit (*see* **Note 7**).
4. Combine 20 ng of the cassette with an equimolar amount of the 3′ part of the gene. Use this mixture as the template for an "overlap extension" PCR without adding primers. After 5 cycles, add the primers (F_{up} and $R_{down\text{-}cassette}$) to amplify this fragment and perform a standard PCR for 30 cycles (*see* **Note 8**).
5. Combine 20 ng of the cassette with an equimolar amount of the downstream fragment and perform an "overlap extension" PCR as described in **step 4**. In this case, the primer couple $F_{down\text{-}cassette}$ and R_{down} is used to amplify the fragment.
6. Repeat **step 3** for both fragments.

7. Combine 20 ng of both fragments and use this mixture as the template for an "overlap extension" PCR. After 5 cycles, add the primers F_{up} and R_{down} to amplify the entire construct.
8. Repeat **step 3** for this construct.
9. Use two primers situated in the middle of the cassette to sequence the construct and check if the annealing sites between the fragments contain no mutations (*see* **Note 9**).
10. If the construct is mutation free, clone the fragment (approx. 100 ng) into the pCR4-TOPO vector using the "TOPO TA cloning kit for sequencing" following the instruction manual.
11. Check the entire construct for mutations by sequence analysis (*see* **Note 10**).
12. Use 10–20 ng of the correct plasmid as a template for a standard PCR to gain sufficient amounts of the DNA construct (*see* **Note 11**).
13. Repeat **step 3**.

3.1.2 In Vivo Recombination

Once the DNA construct is ready it has to be transformed to *P. aeruginosa*. First, fresh electrocompetent *P. aeruginosa* cells are prepared using the method by Choi et al. [16]. Next, the construct is transformed to these cells using electroporation, after which homologous recombination occurs and the correct mutants are selected.

1. Inoculate 6 ml of LB/Cb^{200} with 120 µl overnight culture of *P. aeruginosa* PAO1 cells containing the pUC18-RedS plasmid.
2. At an optical density at 600 nm (OD_{600nm}) of 0.4, add 60 µl of 300 mM L-arabinose (final concentration of 0.2%) to induce the Red operon.
3. Divide the culture over four Eppendorf tubes (1.5 ml/tube) after 2.5 h of induction.
4. Spin the cultures (2 min, 13,000 rpm (16,000 × *g*)).
5. Remove the supernatant and dissolve the pellet in 1 ml of 300 mM sucrose (*see* **Note 12**).
6. Repeat subsequently **step 4**, **step 5** and **step 4**.
7. Remove the supernatant and collect the pellets into one tube by dissolving them in a final volume of 100 µl 300 mM sucrose (*see* **Note 13**).
8. Mix the 100 µl cells with 500–1000 ng of the construct and transfer them to a 0.2 cm electroporation cuvette (*see* **Note 14**).
9. Electroporate the cells with a pulse of 2.5 kV (*see* **Note 15**).
10. Add 500 µl of preheated (37 °C) LB to the electroporation cuvette and transfer the entire volume to a glass tube.

11. Shake the cells for 2 h at 37 °C to allow recombination to occur.
12. Plate 50 µl, 200 µl and the rest of the cells on selective medium (LB/Gm^{30}) and incubate overnight at 37 °C.
13. Pick the colonies, dissolve each of them in a small volume LB and streak them on LB /Gm^{30}. Incubate a second time overnight at 37 °C.
14. Pick a few single colonies and dissolve them in 100 µl LB/Gm^{30}. Perform a PCR on 5 µl of the culture and use DNA gel electrophoresis to confirm the correct insertion of the construct. Use primers that are situated 100 bp upstream and downstream of the insert to ensure insertion at the correct location in the genome (*see* **Note 16**).
15. Purify the PCR product and check the sequence for mutations by DNA sequencing analysis.
16. If the sequence is correct, store a 20% glycerol stock of the strain at −80 °C.

3.2 Verification of the Constructed Strains

3.2.1 Effect on the Bacterial Viability and the Infectivity of the Phage

Once a correct strain is constructed, the effect of the insert on the viability of the bacterial cells and the infectivity by *P. aeruginosa*-specific phages is tested. Neither of these parameters should experience an effect compared to a wild type *P. aeruginosa* strain. First the viability of the mutant is analysed.

1. Inoculate 4 ml of LB/Gm^{30} with 40 µl of an overnight culture of *P. aeruginosa* PAO1 *target*::*Strep*II and 4 ml of LB with 40 µl of an overnight culture of the wild type *P. aeruginosa* PAO1 strain.
2. Measure the OD_{600nm} every 20 min during 5 h, for both cultures.
3. Plot the OD_{600nm} in function of time and compare both curves. No differences should be present.

   Next, the effect on phage infection has to be investigated. For this, the "efficiency of plating" (EOP) is determined, using the double-agar method.
4. Mix 4 ml LB Soft with 200 µl of an overnight culture of *P. aeruginosa* PAO1 *target*::S*trep*II and 100 µl of a dilution of the phage (*see* **Note 17**).
5. Pour the mix on top of an LB agar plate (*see* **Note 18**).
6. Repeat **steps 4** and **5** by using the wild type *P. aeruginosa* PAO1 strain.
7. Incubate the plates overnight at 37 °C.
8. Count the number of plaques formed and determine the "plaque forming units" (PFU)/ml. Calculate the EOP as the ratio of the PFU/ml on the constructed strain to the PFU/ml on the wild type strain. The EOP should approximately be 1.

3.2.2 Production of the Strep-tag® II-Fused Protein

After verifying the viability and infectivity of the engineered strain, the presence of the tagged protein under physiological conditions is investigated (*see* **Note 19**). Therefore, a Western blot is performed on the cell lysate of the constructed cells (without phage infection). If the protein is produced, a signal should be detected when using monoclonal anti-*Strep*-tag® II antibodies which target the *Strep*-tag® II.

1. Inoculate 50 ml of LB/Gm^{30} in a 200 ml flask with 1 ml of an overnight culture of the engineered *P. aeruginosa* strain *target*::*Strep*II. As a negative control, inoculate 50 ml LB with an overnight culture of wild type *P. aeruginosa* PAO1 cells and follow the same procedure.
2. Grow the cells at 37 °C to an OD_{600nm} of 0.3 (*see* **Note 20**), transfer them to a 50 ml-tube and collect the cells by centrifugation (30 min, 4600 × *g*, 4 °C).
3. Discard the supernatant, dissolve the cell pellet in 500 µl TE buffer and transfer the sample to a 1.5 ml Eppendorf tube.
4. To lyse the cells, the sample is first subjected to one freeze-thaw cycle (*see* **Note 21**).
5. Subsequently, incubate the sample for 15 min at room temperature while gently agitating, after the addition of 10 µl of 5 mg/ml HEWL, 10 µl of 100 mM Pefabloc® SC, and 1 µl Benzonase® nuclease.
6. Sonicate the sample 8 times 5 s (amplitude 40%) and add 166 µl 4× loading buffer.
7. Boil the sample for 5 min at 95 °C (*see* **Note 22**).
8. Load 15–20 µl of the sample on a polyacrylamide gel and subject it to SDS-PAGE as described in 'Subheading 3.4 (*see* **Note 23**). Load 5 µl of the prestained reference ladder next to the sample (*see* **Note 24**). As a negative control, load 15–20 µl of the cell lysate of the wild type cells. As a positive control, load a fraction of a protein carrying a *Strep*-tag® II.
9. Prepare a Western blot "sandwich": Soak the foam pads in transfer buffer and put them on each side of the holder. Soak two Whatman papers (size of the foam pads) in transfer buffer and put them on each side of the holder. Soak the nitrocellulose membrane (size of the gel) in transfer buffer and put it on the side which will be connected with the positive pool of the power source. Soak the gel in transfer buffer and put it on the negative side of the holder. Close the holder (*see* **Note 25**–**27**).
10. Place the holder in a tank filled with cooled transfer buffer and run an electrical field of 100 V (350 mA) over it during 1 h–1 h 30 min (*see* **Notes 28** and **29**).

11. Place the membrane in a small box (approximately the size of the membrane) with the protein side facing up and block it by incubating the membrane with 50 ml blocking solution for 1 h at room temperature, while gently shaking.
12. Discard the blocking solution and rinse the membrane with PBST to remove the residues of blocking solution.
13. Incubate the membrane at room temperature for 1 h with 10 ml PBST to which 2 μl monoclonal anti-*Strep*-tag® II antibodies was added (1:5000 dilution), while gently agitating (*see* **Note 30**).
14. Discard the solution and wash the membrane by incubating it three times with 10 ml PBST for 3 min while agitating and rinse the membrane with water.
15. Mix detection solutions A and B to a 1:1 ratio and drop 2 ml onto the protein side of the membrane (*see* **Note 31**). Incubate for 2 min at room temperature.
16. Drain off the detection solution and dry the membrane by gently shaking (*see* **Note 32**).
17. Place the membrane between to transparent papers and place it in the audioradiography cassette with the protein side of the membrane facing up.
18. Bring the cassette to the dark room, place a sheet of X-ray film on the membrane (*see* **Note 33**) and close the cassette.
19. Allow exposure of the chemiluminescent reaction on the film (3–20 min) (*see* **Note 34**).
20. Develop the film by placing it in developing solution while gently shaking.
21. Once a good signal is visible, rinse the film with water and place the film in fixation solution until the film becomes completely transparent.
22. Rinse the film with water, place it in a rack and let it dry.
23. Repeat **steps 18–22** two to three times to optimize the results.

3.3 Affinity Purifications

To search for protein–protein interactions between bacteria and their phages, the target proteins are purified by affinity purification. Therefore, the engineered strains are infected with phages and the infection cycle is stopped at the early stage of infection, since it is believed that most host-phage protein-protein interactions occur at this stage [1, 11]. To not disturb the interactions, a mild lysis of the cells is performed, followed by a pull-down of the target protein/complex and all its interaction partners.

1. Inoculate 600 ml LB/Gm^{30} in a 2 l flask with 8 ml of an overnight culture of the engineered *P. aeruginosa* strain *target*::*Strep*II.

2. Grow the cells at 37 °C to an OD_{600nm} of 0.3, infect them with a *P. aeruginosa* specific phage (MOI 5-10) and incubate at 37 °C (*see* **Note 35**).
3. Stop infection at the early stage of infection by chilling the culture in an icy water bath during 5–10 min (*see* **Note 36**).
4. Transfer the culture to an ice-cold tube and spin the culture (4600 × *g*, 45 min, 4 °C) (*see* **Note 37**).
5. Discard the supernatant and resuspend the cell pellet in 8 ml resuspension buffer supplemented with 100 µl of 100 mM Pefabloc® SC and 500 µl of 20 mg/ml HEWL (*see* **Note 21**).
6. Subject the sample to one freeze-thaw cycle.
7. Add 10 µl Benzonase® nuclease and 800 µl 10× BugBuster® Protein extraction reagent and incubate the sample for 10–20 min at room temperature while gently agitating (*see* **Note 38**).
8. Spin the sample in ice-cold Eppendorf tubes (30 min, 16,000 × *g*, 4 °C), collect the supernatant and place on ice.
9. Prepare the affinity purification column: Add 1 ml *Strep*-Tactin® Sepharose beads to a 10 ml Bio-Rad Poly-Prep® Chromatography column. Wash the beads two times with 2 ml of wash buffer (*see* **Note 39**).
10. Load the supernatant on the column, collect the flow through (FT) and store at 4 °C.
11. Wash the beads five times with 1 ml wash buffer. Collect the wash fractions in separate Eppendorf tubes (W1-5) and store at 4 °C.
12. Elute the proteins with elution buffer in six fractions of 500 µl. Collect the elution fractions in separate Eppendorf tubes (E1-6) and store at 4 °C.
13. Regenerate the column by adding three times 5 ml of regeneration buffer and twice 4 ml of wash buffer. Close the column, add 2 ml wash buffer and store the column at 4 °C.
14. Concentrate the elution fractions by ultrafiltration (Amicon Ultra-0.5 ml Centrifugal filter, 3 kDa).

3.4 SDS-PAGE

The eluted fractions are subsequently subjected to SDS-PAGE. The one-dimensional separation of the proteins present in the samples, allows a first analysis of the composition of the eluted fractions. Moreover, gel electrophoresis removes low molecular weight impurities, including detergents and buffer components, which are often not compatible with downstream mass spectrometry analysis.

1. Prepare a 12% SDS-PAGE gel: Pour the separation gel mixture between the two glace plates of the Mini-PROTEAN® Tetra Cell Systems (*see* **Note 40**). Add a small layer of isopropanol

and wait until the gel has solidified. Remove the isopropanol and pour the stacking gel mixture (*see* **Note 40**). Place the comb insight and wait until the gel has solidified.

2. Place the gel in the holder and subsequently place the holder in the tank. Fill the tank with running buffer.
3. Suspend aliquots of 10–15 µl proteins in SDS-PAGE 4× loading buffer and denature by heating them at 95 °C for 5 min.
4. Load the protein samples in the wells (after removal of the comb) and run an electric field of 200 V until the electrophoresis front reaches the bottom of the gel (*see* **Note 41**).
5. Remove the gel from the glace plates, place it in a box and wash the gel with water for 15–30 min.
6. Stain the SDS-PAGE gel for 0.5–2 h with a MS-compatible standard Coomassie stain like GelCode Blue Safe Stain (Thermo Fisher Scientific).
7. Wash the gel overnight with water, to reduce the background stain.

3.5 Mass Spectrometry Analyses

Lastly, the composition of the samples has to be identified. Therefore, gel pieces are sliced from the SDS-PAGE gel and subjected to an in-gel trypsin digestion. Afterward, the obtained peptides are analysed by LC-MS/MS analyses. During the experiment, gloves must be worn at all times, and contact with skin, hair, and clothes should be avoided (*see* **Note 42**). Moreover, keratin-free materials should be used.

1. Excise protein bands (8–13 spots in total) from the gel using a 1000 µl micropipette after widening the opening of the tip with a scalpel (*see* **Note 43**–**45**).
2. Transfer each gel piece into a separate 1.5 ml Eppendorf tube.
3. Remove the residual water, submerge each gel piece in 100 µl 25 mM NH_4HCO_3 in 50% acetonitrile, and incubate for 10 min at room temperature.
4. Remove the liquid, and repeat **step 3** until the Coomassie blue is completely removed from the gel pieces (approx. 3 times).
5. Dry the pieces in a vacuum centrifuge for 10–15 min at 40 °C (*see* **Note 46**).
6. Submerge the pieces in 30 µl 10 mM DTT in 100 mM NH_4HCO_3 to reduce all disulfide bonds (reduction) and incubate for 1 h at 56 °C.
7. Cool the samples to room temperature and remove the liquid.
8. Submerge the pieces in 30 µl 55 mM IAA in 100 mM NH_4HCO_3 to modify cysteine residues and prevent reformation of disulfide bonds (alkylation).

9. Incubate for 45 min in the dark, shake every 10 min using a vortex. Remove the liquid afterward.
10. Add 100 µl 100 mM NH_4HCO_3, incubate for 10 min, and remove the liquid (hydration).
11. Add 100 µl acetonitrile, incubate for 10 min, and remove the liquid (dehydration).
12. Repeat **steps 10** and **11**.
13. Dry the pieces in a vacuum centrifuge for 10–15 min at 40 °C.
14. Submerge the pieces in 10 µl trypsin digestion buffer, and incubate for 45 min in an icy water bath.
15. Add 30 µl 50 mM NH_4HCO_3, and incubate overnight at 37 °C.
16. Collect the supernatants containing the tryptic peptides in new Eppendorf tubes, one for each gel piece.
17. Submerge the pieces in 20 µl 20 mM NH_4HCO_3, sonicate 20 min in a sonicator bath and collect the supernatants in the corresponding tubes (*see* **Note 47**).
18. Submerge the pieces in 50 µl 5% formic acid in 50% acetonitrile, sonicate 20 min in a sonicator bath and collect the supernatants in the corresponding tubes (*see* **Note 47**).
19. Repeat **step 18**.
20. Store the collected supernatants at −20 °C until mass spectrometry analyses can be performed (*see* **Note 48**).
21. To identify the peptides present in the samples, send the samples to a mass spectrometry facility for analysis. In this case, an Easy-nLC 1000 liquid chromatograph (Thermo Fisher Scientific), which is on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific), is used at the Biomedical Research Institute and Transnational University Limburg (Hasselt University, Belgium) [17].
22. RAW data are analyzed with the Proteome Discoverer software version 1.3 (Thermo Fisher Scientific) with build-in Sequest and interfaced with an in-house Mascot v.2.4 server (Matrix Science). MS/MS spectra were searched against a database containing all *P. aeruginosa* PAO1 proteins and all "stop-to-stop" protein sequences in all six frames of all phages.

4 Notes

1. The phage stock does not need to be ultrapure (e.g., cesium chloride centrifugation); instead a PEG-precipitated stock can be used.

2. The transfer buffer is stored at 4 °C and can be reused a few times.
3. A 10× PBS buffer without Tween can be made and stored at room temperature. Tween is freshly added to the PBS at the moment of use.
4. The buffer is stored at 4 °C.
5. Light sensitive: store in a dark place.
6. In total, six different primers have to be developed (*see* Fig. 2). Two primers ($F_{down\text{-}cassette}$ and $R_{down\text{-}cassette}$) to amplify the fragment containing the *Strep*-tag® II-tag and the Gm^R gene. $F_{down\text{-}cassette}$ starts with the *Strep*-tag® II-tag sequence followed by a stop codon. $R_{down\text{-}cassette}$ comprises the C-terminal part of the Gm^R gene. Two primers (F_{up} and $R_{up\text{-}cassette}$) to amplify the C-terminal part of the target gene (approx. 300 bp). $R_{up\text{-}cassette}$ contains the C-terminal sequence of the target gene (without stop codon!) fused to the 5′-region of the fragment containing the *Strep*-tag® II-tag and the Gm^R gene. Likewise, both fragments will share 18–25 bp homology. Equally, two primers ($F_{down\text{-}cassette}$ and R_{down}) are designed to amplify the region downstream of the target gene (approx. 300 bp). In this case, $F_{down\text{-}cassette}$ shares 18–25 bp homology with the 3′-region of the fragment containing the *Strep*-tag® II-tag and the Gm^R gene.
7. If there are multiple bands, use the gel excision kit to select the fragment of the correct size.
8. For the "overlap extension" PCR it is important to use equimolar amounts of the fragments that you want to fuse, which corresponds to an equal number of DNA molecules. Therefore, you have to take into account the size of the fragments. For example, 10 ng of a 1000 bp fragment is equimolar to 1 ng of a 100 bp fragment.
9. Design and use primers which are situated in the Gm^R cassette and located at a distance of 200–300 bp to the overlap regions.
10. Use all six primers used for the design of the construct to ensure full sequence coverage of the construct.
11. It is sometimes necessary to repeat the amplification to get sufficient amounts of construct, which is around 1 μg of DNA.
12. Be careful when removing the supernatant, since the pellet loses its consistency after the multiple washing steps.
13. First dissolve pellet of one tube in 100 μl 300 mM sucrose and then transfer solution to second tube.
14. Never add more than 3 μl of DNA construct due to the risk of arcing caused by high salt concentrations. When the

concentration of the DNA construct is to low, first apply an ethanol precipitation on the sample to increase the final concentration.

15. The time constant should be between 4.8–5.1 ms. Increasing the number of washing steps can increase this value.
16. If the PCR on the cells fails, it is recommended to first perform a genome extraction on an overnight culture of the cells. The attained genomic DNA can then be used as template which will yield better results.
17. The dilution of the phages should contain about 100 of PFU/ml. This amount depends on the size of the plaques formed, since the plaques formed on the plate should be countable. If necessary, several dilutions can be plated.
18. Ensure that the soft agar forms an even layer on top of the plate, which helps for the counting of plaques.
19. The detectability of the tagged proteins is verified under the same conditions that will be used for the affinity purifications.
20. An OD_{600nm} of 0.3 was used, since this is the OD_{600nm} at which the cells will be infected with the phages prior to the affinity purifications in Subheading 3.3.
21. At this point, the cells can be stored at −80 °C.
22. At this point, the sample can be stored at −20 °C.
23. Depending on the size of the target protein, the percentage of the gel might be adjusted: 8% for high molecular weight proteins, to 15% for low molecular weight proteins.
24. A prestained ladder is chosen, since it will be visible on the membrane after blotting. Likewise, the ladder can be used to verify that the proteins are successfully transferred to the membrane during the Western blot.
25. During the Western blot procedure it is important to wear gloves.
26. It is advised to cut one corner of the membrane to recognize the front and back side after transfer of the proteins.
27. After closing the "sandwich," air bubbles between the gel and the membrane should be removed by rolling a rod over it.
28. To keep the temperature low, the tank can be placed on ice or a Cooling Unit can be placed inside the tank.
29. The time of transfer depends on the size of the protein. For proteins with a high molecular weight, a longer transfer time is needed. The transfer can be verified by the presence of the prestained ladder.

30. The dilution of antibody that should be used and time of incubation depends on the brand and the antibody, and might be optimized prior to the experiment.
31. The detection solutions should always be kept on ice.
32. To take the membrane, a pincer should be used.
33. Cut one corner of the membrane and place it in the same direction as the corner of the membrane.
34. The time of exposure should be optimized each time. Therefore, 2 or 3 films can be used.
35. The "multiplicity of infection" (MOI) is the ratio of the amount of the phages (PFU) versus the amount of bacterial cells (CFU, "colony forming units") at the time point of infection. The desired MOI should be optimized for each phage prior to the large-scale affinity purification. Therefore, the amount of bacterial cells (CFU/ml) 5 min after phage infection should be reduced to less than 5% of the CFU/ml before infection, to accomplish a successful and synchronic infection.
36. The early stage of phage infection is estimated approximately as 1/3e of the length of the infection cycle.
37. To ensure that the infection cycle is stopped in the early phage of infection, it is very important that the sample stays cool until the cells are lysed.
38. The cells are lysed when the sample turns from a turbid to a clear solution.
39. All buffers should have the same temperature as the column to avoid the formation of air bubbles. To be sure, the purification can be done at 4 °C, however, the procedure can also be performed on the bench at room temperature.
40. APS and TEMED are added just before pouring the gel. Invert a few times to obtain a well-mixed solution.
41. Take care that the electrophoresis front (potentially containing small phage proteins) does not run off the gel.
42. To avoid keratin contamination, gloves and keratin-free materials should be used. Moreover, it might help to perform all manipulations under a hood.
43. If the protein bands are not clearly visibly, it might help to collect all elution fractions in which proteins are present, concentrate them by ultrafiltration and perform a new SDS-PAGE analysis prior to the in-gel digestion.
44. Take a picture before and after isolation of the protein bands to visualize the localisation of the picked spots on the SDS-PAGE gel.

45. As an alternative to manually slice the gel, a number of automated spot pickers are available as well.
46. Dry gel pieces become white and loosen from the wall of the Eppendorf tube. The dried gel pieces can be stored at −20 °C for a few months until further analysis is performed.
47. All supernatants originating from the same gel piece are collected in one Eppendorf tube.
48. Do not discard the extracted gel pieces, but store the gel pieces at −20 °C until MS analyses are performed. If the digestion fails, it can be repeated with the same gel pieces [18].

References

1. Roucourt B, Lavigne R (2009) The role of interactions between phage and bacterial proteins within the infected cell: a diverse and puzzling interactome. Environ Microbiol 11:2789–2805
2. Liu J, Dehbi M, Moeck G et al (2004) Antimicrobial drug discovery through bacteriophage genomics. Nat Biotechnol 22:185–191
3. Yano ST, Rothman-Denes LB (2011) A phage-encoded inhibitor of Escherichia coli DNA replication targets the DNA polymerase clamp loader. Mol Microbiol 79:1325–1338
4. Nechaev S, Severinov K (2003) Bacteriophage-induced modifications of host RNA polymerase. Annu Rev Microbiol 57:301–322
5. Häuser R, Blasche S, Dokland T et al (2012) Bacteriophage protein-protein interactions. Adv Virus Res 83:219–298
6. De Smet J, Hendrix H, Blasdel BG et al (2017) Pseudomonas predators: understanding and exploiting phage-host interactions. Nat Rev Microbiol 61:517–530
7. Stover CK, Pham XQ, Erwin AL et al (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406:959–964
8. Gellatly SL, Hancock REW (2013) *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis 67:159–173
9. Wodak S, Vlasblom J, Turinsky A et al (2013) Protein-protein interaction networks: puzzling riches. Curr Opin Struct Biol 23:941–953
10. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340:245–246
11. Van den Bossche A, Ceyssens PJ, De Smet J et al (2014) Systematic identification of hypothetical bacteriophage proteins targeting key protein complexes of *Pseudomonas aeruginosa*. J Proteome Res 13:4446–4456
12. Korndörfer IP, Skerra A (2002) Improved affinity of engineered streptavidin for the Strep-tag II peptide is due to a fixed open conformation of the lid-like loop at the binding site. Protein Sci 11:883–893
13. Schmidt TGM, Skerra A (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. Nat Protoc 2:1528–1535
14. Lesic B, Rahme LG (2008) Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa*. BMC Mol Biol 9:20
15. Armengaud J, Trapp J, Pible O (2014) Non-model organisms, a species endangered by proteogenomics. J Proteome 105:5–18
16. Choi KH, Kumar A, Schweizer HP (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64:391–397
17. Ceyssens PJ, Minakhin L, Van den Bossche A et al (2014) Development of giant bacteriophage ϕKZ is independent of the host transcription apparatus. J Virol 88:10501–10510
18. Shevchenko A, Tomas JH, Olsen J et al (2007) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860