Chapter 16

Selection of Peptides Interfering with Protein–Protein Interaction

Annette Gaida, Urs B. Hagemann, Dinah Mattay, Christina Räuber, Kristian M. Müller, and Katja M. Arndt

Abstract

Cell physiology depends on a fine-tuned network of protein–protein interactions, and misguided interactions are often associated with various diseases. Consequently, peptides, which are able to specifically interfere with such adventitious interactions, are of high interest for analytical as well as medical purposes. One of the most abundant protein interaction domains is the coiled-coil motif, and thus provides a premier target. Coiled coils, which consist of two or more α-helices wrapped around each other, have one of the simplest interaction interfaces, yet they are able to confer highly specific homo- and heterotypic interactions involved in virtually any cellular process. While there are several ways to generate interfering peptides, the combination of library design with a powerful selection system seems to be one of the most effective and promising approaches. This chapter guides through all steps of such a process, starting with library options and cloning, detailing suitable selection techniques and ending with purification for further down-stream characterization. Such generated peptides will function as versatile tools to interfere with the natural function of their targets thereby illuminating their down-stream signaling and, in general, promoting understanding of factors leading to specificity and stability in protein–protein interactions. Furthermore, peptides interfering with medically relevant proteins might become important diagnostics and therapeutics.

Key words: Activator protein-1, affinity chromatography, coiled coil, leucine zipper, library design, phage display, protein design, protein engineering, protein-fragment complementation assay, selection technology, semi-rational design, bacterial surface display.

1. Introduction

Peptide aptamers are short peptides generated to interfere with protein interactions. Our lab focuses on the generation of peptides that are able to interfere with protein–protein interactions primarily mediated by coiled coil sequences. Another application

Annette Gaida, Urs B. Hagemann, Dinah Mattay, and Christina Räuber contributed equally and are listed alphabetically.
is the targeting of ligand receptor interaction. In contrast to the classic view of peptide aptamers, which are usually inserted into a protein scaffold, these peptides are “stand-alone”, and we call them interfering peptides (iPEP).

Approximately 3–5% of amino acids in proteins are estimated to adopt coiled-coil folds (1). Coiled coils have one of the simplest dimerization interfaces, yet they can mediate highly selective protein associations (2, 3) and play key roles in virtually every physiological process. Furthermore, they are widely used in the protein engineering field (4, 5). Coiled coils consist of two or more α-helices wrapped around each other in a left-handed supercoil (2, 3, 6). Their sequences are characterized by a conserved seven-residue repeat, \((abcdefg)_n\). The first (a) and fourth (d) position, which form the dimerization interface between the helices, are generally occupied by hydrophobic amino acids and only a very restricted repertoire of polar and charged residues (6, 7). The other amino acids in the repeat are mostly polar or charged and can form inter- and intrahelical interactions that contribute to the stability and specificity of complex formation. Proline is largely excluded to preserve the helical architecture.

We specifically target coiled-coil domains of proteins relevant to biology or medicine with small helical peptide probes. Important targets include the oncoproteins c-Jun, c-Fos, and c-Myc. We use a semi-rational design approach in combination with different selection systems to generate such peptides (6). Libraries are designed using mixed codons at protein interface positions, mainly heptad positions a, d, e and g (8–10). Selection is carried out using phage display (11) or a protein-fragment complementation assay (PCA) based on the enzymatic activity of murine dihydrofolate reductase (8, 12). The latter assay was also modified to include competitive and negative design aspects (13). Other intracellular selection assays such as the Ras-recruitment system have also been tested (14). Another approach is the display on cell surfaces, which is also discussed in this chapter (15, 16). Such a semi-rational approach helps define the fundamental principles guiding protein–protein interaction while at the same time generating tight-binding peptides targeted to scientifically or medically relevant proteins. Such peptides are ideal for signaling studies and are used as effective tools to explore and manipulate function in vivo using proteomics approaches.

This chapter briefly summarizes different libraries that can be used for the generation of peptide aptamers (Section 3.1) and focuses on the subsequent selection of suitable peptides. We provide detailed protocols accompanied with a short theoretical introduction for selection methods in vitro (Section 3.2), inside cells (Section 3.3) and on the surface of cells (Section 3.4). Furthermore, we briefly touch the selection of D-peptide aptamers...
Section 3.2). The chapter ends with one general example for purifying selected aptamers (Section 3.5).

2. Materials

2.1. Library Construction

2.1.1. Fill-in Reaction

1. Overlapping oligonucleotides (e.g., from Microsynth, Operon).
4. dNTPs (deoxynucleosidetriphosphates; 10 mM; Genaxxon).

2.2. Phage Display

2. Roller mixer.
3. 4 ml Immunotubes (e.g., Nunc).
4. E. coli ER 2738 (see Note 1).
5. Phage library (e.g., NEB C7C from New England Biolabs or [11]) or individually designed phagemid library (examples in the lab are coiled coil libraries or a mutated EGF library in phagemid vector pAK100 [17]).
6. Helper phage (e.g., M13KO7, VCSM13, Stratagene; see manufacturers information for amplification of helper phage).
7. Glycerol, autoclaved.
8. LB-medium: 10 g Bacto-Tryptone, 5 g Yeast–Extract, 5 g NaCl, add ddH2O to 1 l.
9. LB-agar (1.5%): 15 g Bacto-Agar per liter LB-medium.
10. Tetracycline (Tet) 1,000x stock solution: 20 mg/ml in ddH2O, sterile filtered through 0.2 μm.
11. Kanamycin (Kan) 1,000x stock solution: 70 mg/ml in ddH2O, sterile filtered through 0.2 μm.
12. PEG6000-NaCl: 2.5 M NaCl, 20% (w/v) poly ethylene glycol (PEG)-6000.
13. TBS: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl.
14. Top agar (0.7%): 0.35 g Agar per 50 ml LB medium.
15. IPTG/X-Gal stock solution: 1.25 g IPTG (isopropyl-β-D-thiogalactoside), 1 g X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), dissolved in 25 ml dimethylformamide (wrap in aluminum foil) (see Note 2).
16. IPTG/X-Gal plates: 15 g agar per liter LB-medium, use IPTG/X-Gal stock solution 1:1,000 (wrap in aluminum foil).
17. Coating buffer: 0.1 M NaHCO₃.
18. Blocking buffer: TBS containing 4% (w/v) BSA (see Note 3).
19. Washing buffer: TBS, 0.05% (v/v) Tween (see Note 3).
20. Elution buffer: 0.1 M glycine–HCl (pH 2.2), 100 mM NaCl.

2.3. DHFR Protein-Fragment Complementation Assay

2. Double yeast-trypton (DYT) medium: 16 g trypton, 10 g yeast extract, 5 g NaCl, add ddH₂O to 11.
3. Enriched DYT medium: DYT medium, 0.5% (w/v) glucose, 2.5 mM KCl, 10 mM MgCl₂.
4. M9 minimal medium: 0.1 mM CaCl₂, 0.048 mM Na₂HPO₄, 0.022 mM KH₂PO₄, 8.6 μM NaCl, 0.019 mM NH₄Cl, 2 mM MgSO₄, 0.4% glucose (see Note 4).
5. Ampicillin (Amp) 1,000x stock solution: 100 mg/ml in EtOH, sterile filtered through 0.2 μm.
6. Chloramphenicol (Cm) 1,000x stock solution: 25 mg/ml in EtOH, sterile filtered through 0.2 μm.
7. Kanamycin (Kan) 1,000x stock solution: 50 mg/ml in ddH₂O, sterile filtered through 0.2 μm.
8. Trimethoprim (TMP) 500x stock solution: 500 μg/ml in MeOH, sterile filtered through 0.2 μm.
9. IPTG 1,000x stock solution: 1 M in ddH₂O, sterile filtered through 0.2 μm.

2.4. Bacterial Surface Display

2. 96-well microtiter plate.
3. 137-mm diameter Nitrocellulose membrane filters.
4. E. coli GI826 (Invitrogen).
5. FliTrx™ Peptide display library with randomized dodecamer peptide (or pFliTrx™ peptide display vector with multiple cloning site; Invitrogen).
6. Target molecule (conjugated to carrier protein).
7. Casamino acids (with low tryptophan content).
9. DNase I.
10. Lysozyme.
11. 10X M9 salts: dissolve 60 g Na₂HPO₄, 30 g KH₂PO₄, 5 g NaCl and 10 g NH₄Cl in 900 ml deionized water, adjust pH to 7.4 with 10 M NaOH and add water to 1 l (final volume). Autoclave solution.
12. 1 M MgCl₂: dissolve 20.33 g in 100 ml deionized water and autoclave the solution.

13. Ampicillin (Amp) 1,000x stock solution: dissolve 1 g ampicillin in 10 ml deionized water and pass solution through sterile filter.

14. 20% (w/v) Glucose: dissolve 20 g glucose in 100 ml deionized water and pass solution through sterile filter.

15. 10 mg/ml L-Tryptophan: dissolve 100 mg L-tryptophan in 10 ml deionized water, pass the solution through sterile filter and store it in the dark at 4°C.

16. 5 M NaCl: dissolve 29.22 g in 100 ml deionized water. Autoclave solution.

17. 1 M Tris: dissolve 121.1 g Tris in 900 ml deionized water, adjust pH to 7.4 with 37% HCl and add water to 1 l (final volume).

18. IMC Medium: mix 2 g casamino acids (low amounts of tryptophan!) with 875 ml water and autoclave mixture. Add 100 ml 10x M9 salts, 1 ml MgCl₂, 25 ml 20% glucose and 1 ml 100 mg/ml ampicillin aseptically (store at 4°C).

19. IMC medium for induction: add aseptically 10 mg/ml L-tryptophan to the stock solution of IMC-medium (final concentration: 100 µg/ml L-tryptophan).

20. RM medium: Mix 20 g casamino acids, 10 ml 100% glycerol and 890 ml deionized water and autoclave solution. When solution is cooled down add aseptically 100 ml 10x M9 salts, 0.5 ml 1 M MgCl₂ and 100 mg/ml ampicillin.

21. RMG-Amp plates: dissolve 20 g casamino acids, 15 g agar in 875 ml deionized water. Autoclave solution. Add 100 ml 10x M9 salts, 25 ml 20% glucose, 1 ml MgCl₂ and 1 ml 100 mg/ml ampicillin when solution is cooled to ca. 55°C (before preparing plates).

22. RMG-Amp-Trp plates: dissolve 20 g casamino acids, 15 g agar in 855 ml deionized water. Autoclave solution. Add 100 ml 10x M9 salts, 25 ml 20% glucose, 1 ml MgCl₂, 1 ml 100 mg/ml ampicillin and 20 ml 10 mg/ml L-tryptophan when solution is cooled to ca. 55°C (before preparing plates).

23. TS-buffer: mix 30 ml 5 M NaCl with 50 ml 1 M Tris–HCl (pH 7.5) and 920 ml deionized water.

24. Lysis-buffer: mix 99.5 ml TS-buffer with 1 mg non-fat dry milk, 0.5 ml 1 M MgCl₂ and add 2 mg lysozyme and 100 U DNase I.

2.5. Purification of Peptide Aptamers

1. GSH matrix: 1 ml-GSTrap (GE Healthcare) or GSH coupled to sepharose beads (GE Healthcare).
2. Reversed phase column: Jupiter Proteo column, 4 μm particle size, 90 Å pore size, 250 × 10 mm (Phenomenex).
3. Syringe filters: 0.45 μm PVDF filters (Roth).
4. Centrifuge: e.g., Sorvall RC5B with SS34 rotor.
5. Peristaltic pump or HPLC system coupled to UV detector.
6. GST binding buffer: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl (pH 7.3).
7. Elution buffer: 50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione (GSH).
8. FXa buffer: 20 mM Tris–HCl, pH 8, 50 mM NaCl, 1 mM CaCl₂.
9. RP-HPLC: Acetonitrile (ACN, HPLC-grade, Roth), 0.1% trifluoric acid (TFA, Roth) and ddH₂O, 0.1% TFA.
10. FXa enzyme: 1 U/μg protein (Qiagen).

3. Methods

3.1. Library Construction

Libraries allow for simultaneous testing of a high number of variants in one screen or selection. There are several possibilities to find the best adapted library for selection of peptide aptamers regardless of the selection method chosen. Definitely the easiest is to purchase a complete library from a company, but in some instances, a more target specific designed library is required. In this case, residue positions in the peptide sequence are randomized via degenerated codon usage and the oligonucleotides have to be synthesized with mixed bases at the randomized positions (6, 9). Randomization can be tailored by the mixture of nucleotides as well as their ratio. Shorter libraries (up to about 80 bases) can be generated with one library oligonucleotide. The complementary strand is generated in a Klenow fill-in reaction from a short oligonucleotide pairing with the 3’ end of the library oligonucleotide. Longer libraries can be generated by two overlapping library oligonucleotides but as a consequence require a non-randomized stretch as hybridization region for the two oligonucleotides. The double strand is similarly generated in a Klenow fill-in reaction.

A different strategy to generate libraries is by error-prone PCR and/or DNA shuffling, which has been detailed in another publication of this series (18) and elsewhere (19).

After generation of the double-stranded library fragment, it can be ligated into the adequate vector and transformed into bacteria to collect clones. Either chemical transformation or
electroporation can be chosen, the latter harboring the benefit of higher transformation efficiencies, but in this case the DNA needs to be desalted. A two- to tenfold overrepresentation of the library is desirable to ensure a complete representation of the library. For assessing the library quality after transformation, a simple calculation can be performed to determine the number of variants not represented in the collected pool \( E \), which should be as small as possible:

\[
E = n \cdot (1 - \frac{1}{n})^m,
\]

where \( n \) is the theoretical library size and \( m \) is the number of collected clones \((20)\).

In order to verify the expected distribution of the randomized positions, the DNA of individual clones should be sequenced.

### 3.1.1. Overlapping DNA Fragments

To get double-stranded fragments for long genetic libraries it may be necessary to align two overlapping single-stranded fragments and fill in the overhangs by reaction with Klenow fragment \((8, 21)\). The overlapping part should ideally harbor an annealing temperature between 50 and 60°C. It should be considered to introduce appropriate restriction sites at both termini of the library to ensure further cloning steps into the adequate vector.

1. Mix the following ingredients for the fill-in reaction in a total volume of 20 µl:
   - 25 pmol of each oligonucleotide
   - 2 µl Klenow buffer
   - 2 µl dNTPs
   - add ddH\(_2\)O to 20 µl
2. Incubate the mixture at 94°C for 3 min.
3. Slowly decrease the temperature from 94 to 37°C at a rate of 0.5°C/s \((\text{see Note 5})\).
4. At 37°C, add 0.5 µl Klenow fragment and incubate for 1 h.
5. The fill-in product should be purified via an agarose gel. It then can be digested by restriction endonucleases and cloned into an adequate vector.

### 3.2. Phage Display

Phage Display is a technique to display peptides or proteins on the surface of filamentous phages by directly coupling the genotype with its phenotype \((\text{see Fig. 16.1A})\). Phage display was invented by George Smith in 1985 \((22)\) and has evolved to one of the most used techniques for in vitro screening ranging from small peptides to proteins, like antibodies Fab fragments, binding to virtually any desired target \((23, 24)\). Filamentous phages \(\phi 1\), M13 and \(\phi d\), which all infect \(E. coli\) are usually used for phage display. They are referred to as Ff phages, and their genomes are to 98% identical to each other \((25)\). As there is a vast amount of information
Fig. 16.1. (A) Simplified illustration of polyvalent phage display, showing the phage plasmid, containing a signal sequence (ss), all phage genes and the gene of interest fused to a coat protein (here genell); and monovalent phagemid display, showing the phagemid vector with a signal sequence (ss), the gene of interest fused to a coat protein (here genell) and an additional selection marker (here chloramphenicol, Cm). (B) First round of phage amplification, precipitation and panning, starting with P0, the unselected library pool.
available in the literature (25–27), the principles of phage display will be explained only briefly. Variations of phage display such as Prosise (28) and selectively infective phages (SIP) (29, 30) have also been reported but are less frequently used and are thus not detailed in this chapter.

In phage display, peptide or protein libraries (see Section 3.1) can be fused to the coat proteins of phages (mostly geneIII protein), which are displayed on the surface of the phage particle. All the proteins needed for cell infection and assembly of phage particles are encoded in the single-stranded phage genome. By fusing the gene of interest to a gene that encodes for one of the coat proteins (mostly geneIII), the protein of interest will be displayed on the phage particle upon expression with the coat protein (see Fig. 16.1A). In phage display with geneIII-fusion, the number of displayed peptides or proteins should be equivalent to the number of geneIII proteins, resulting in a so-called polyvalent display (Fig. 16.1A), which causes an avidity effect. If this is not desired, a variant of the classical phage display, the so-called phagemid display can be performed. In this technique a phagemid vector is used that only encodes the fusion gene, e.g., geneIII, and no other phage genes. In addition to the Ff origin, phagemid vectors have an E. coli origin for replication and an antibiotic cassette to allow propagation in E. coli. To generate phages, superinfection of phagemid-carrying E. coli, with a helper phage, an Ff phage encoding for all phage genes, but with a compromised phage origin is needed. As a consequence, the helper phage itself is packed inefficiently, and generated phages almost exclusively have the phagemid vector packaged. After infection, the resulting phages display wild type geneIII proteins, assembled from structural proteins encoded by the helper phage and a reduced amount of the geneIII-fusion protein encoded by the phagemid vector. Depending on expression level, this statistically results in a monovalent display (see Fig. 16.1A) of the peptide or protein of interest and allows screening for high affinity.

While phage display is usually used for selection of small peptides from five to thirty amino acids, phagemid display can be used to select whole antibodies or proteins. Although phage display is faster, the advantage of the phagemid system is the monovalent display, allowing one to select for peptides with a high affinity. However, due to the high level of displayed wild-type proteins, the display level of the fused library is often rather low (25). One round of phage display always consists of the panning on the target, including washing and elution steps, and the subsequent amplification and purification of the phages (Fig. 16.1B). The first round is referred to as panning round 1. To select for specific binders, several panning rounds (always repeating the steps mentioned above), are accomplished subsequently. Usually, four panning rounds are sufficient to enrich for the best binders in the pool.
For simplicity, phages derived from the panning are referred to as \(E\) (for Elution) and the number of the panning round, that is, phages eluted in panning round 1 are referred to as \(E_1\) and so on. Phages derived from the amplification are referred to as \(P\) (for Panning), with \(P_0\) being the unselected pool of the initial amplification round.

A deviation from the common used strategy in phage display is the so-called mirror image phage display (see Fig. 16.2)\(^{31, 32}\). This technique enables the generation of \(\alpha\)-peptides binding to targets of interest and it relies on the mirror symmetry properties of naturally occurring \(\alpha\)-amino acids in comparison to their enantiomeric \(\beta\)-counterparts. Peptides, which only contain \(\beta\)-amino acids are less susceptible to proteolytic degradation \(^{33}\) and might therefore be advantageous in the design of therapeutical peptides. The difference to the commonly used phage display lies within the target of interest, which is synthesized in \(\alpha\)-amino acids. After selection from an \(\alpha\)-peptide library, the identified binder, an \(\alpha\)-peptide, is converted into \(\beta\)-amino acids, resulting in a \(\beta\)-peptide binding to the native \(\alpha\)-target.

### 3.2.1. Library Preparation

1. Store library stock according to manufacturer’s information.
2. Before starting the first panning round, amplify the phages from the original stock and keep them frozen at \(-20^\circ\text{C}\).
3. Perform the amplification protocol as described in Section 3.2.2.
### 3.2.1.2. Self-Made Libraries

1. For phage display, ligate the generated library (see Section 2.1) into, e.g., MKE13 vector, available from NEB, by using appropriate restriction sites (EagI and Acc65I or KpnI for this vector) (see Note 6).

2. For phagemid display, ligate the generated library (see Section 2.1) into, e.g., pAK100 (17) by using appropriate restriction sites.

3. Transform the library into *E. coli* ER 2738 cells (NEB) and plate them on LB-agar plates containing 20 µg/ml tetracycline (see Note 1). For phagemid display the plates also need to contain the selection marker of the phagemid vector (see Note 7).

4. At the next morning, carefully pool the cells by adding some LB medium and scratching them from the plate using, e.g., a Drigalski spatula, dilute them in LB-medium and gently shake them for about 5 min at 37°C to ensure homogeneous cell suspension.

5. Optionally, glycerol stocks can be made from the pool by mixing cell culture with autoclaved glycerol in a ratio of about 2:1. Glycerol stocks are stored at –80°C.

6. Dilute the culture with LB-medium to an OD₆₀₀ below 0.3.

7. Perform the amplification protocol as described in Section 3.2.2, starting at Step 5 for phage display or Step 3 for phagemid display.

### 3.2.2. Phage Amplification

**WARNING:** Phages are highly infectious for bacterial cells. Work responsible and accurate to avoid cross-contamination (see Note 8)!

#### 3.2.2.1. Phage Display

1. Inoculate 40 ml of LB-medium containing 20 µg/ml tetracycline with an overnight culture of *E. coli* ER 2738 cells to an OD₆₀₀ of 0.2 (see Note 1).

2. Use a 500 ml Erlenmeyer flask without baffles (see Note 9).

3. Grow the culture at 37°C on an orbital shaker to an OD₆₀₀ of 0.5.

4. Infect the cells with $1 \times 10^{11}$ phages of the library. Directly after infection leave the culture for 15 min without shaking at 37°C (see Note 10).

5. Let the cultures grow on the shaker for about 4–6 h (see Note 11) and continue with Section 3.2.3.

#### 3.2.2.2. Phagemid Display

1. Inoculate an overnight culture of ER 2738 cells, which harbor the phagemid library to an OD₆₀₀ of 0.2 (see Note 1). Make sure to add the respective antibiotics.

2. Use a 500 ml Erlenmeyer flask without baffles (see Note 9).
3. Grow the culture at 37°C on an orbital shaker to an OD_{600} of 0.5.

4. Add helper phages at a ratio of 1:20 (bacteria to phages) and leave the culture without shaking for 15 min at 37°C (see Note 10).

5. Put the culture onto a shaker, let it grow for about 60–70 min and add kanamycin at a concentration of 70 µg/ml.

6. Let the culture grow for 4–6 h (see Note 11) and continue with Section 3.2.3.

3.2.3. Phage Precipitation

1. Transfer the culture into 50 ml polypropylene (PP)-tubes and spin down at 5,000 g, 4°C for 10–30 min. The longer the centrifugation step, the cleaner the phage pellet will be in the end.

2. After centrifugation, transfer the supernatant, which now contains the phages, into a fresh 50 ml PP-tube and add 1/6 of the volume of PEG6000-NaCl to precipitate the phages. Invert the tube carefully to allow mixing. If the amplification was successful, the phages might already be seen as a cloudy suspension.

3. Leave the precipitation at 4°C overnight. A white fluffy precipitation should be visible next morning.

4. Spin down the precipitation for 45 min at 4°C and 5,000 g.

5. Discard the supernatant and resuspend the pellet in 1 ml ice-cold TBS. Transfer the phages into a 1.5 ml reaction tube and spin down for 10 min at 15,000 g. Transfer the supernatant to a new reaction tube, add 1/6 of the volume of PEG6000-NaCl and incubate on ice for at least 1 h for a second precipitation step.

6. Spin down for 45 min, at 4°C and 15,000 g. Discard the supernatant and resuspend the pellet in 1 ml ice-cold TBS. Spin down for additional 10 min to remove all traces of bacteria and transfer the supernatant to a new reaction tube.

7. Determine the phage concentration either at OD_{260} in a UV/Vis spectrophotometer (see Note 12) or by plating phage dilutions on IPTG/X-Gal plates (only for phage display, see Section 3.2.4).

3.2.4. Phage Titering

Phage titering with plaques is used for phage display only. When using phages encoding an inducible α-complementation of lacZ, e.g., M13 derivatives, a more convenient blue/white screening is possible, as described below. For phagemid display, *E. coli* ER2738 cells can be infected with phages and plated on LB-agar plates containing the respective selection encoded on the
phagemid vector, and the phage titer is determined by counting colony-forming units (cfu).

1. Inoculate 10 ml of LB-medium containing 20 µg/ml tetracycline with an overnight culture of *E. coli* ER 2738 cells to an OD<sub>600</sub> of 0.2 and let them grow to an OD<sub>600</sub> of 0.5 (*see Note 1*).

2. Preheat a water bath to 40°C and put as many test tubes as needed, that is one for each dilution, into the water bath.

3. Boil top agar until it liquefies and pipette 4 ml into each test tube.

4. Let IPTG/X-Gal plates adjust to a temperature of 37°C

5. Prepare phage dilutions in TBS to a final volume of 10 µl: For amplified phages start at a higher dilution, e.g., 1:1 × 10<sup>10</sup>, 1:1 × 10<sup>12</sup>, 1:1 × 10<sup>14</sup>, for eluted phages start with lower dilution, e.g., 1:1 × 10<sup>4</sup>, 1:1 × 10<sup>6</sup>, 1:1 × 10<sup>8</sup>, as the number of phages will be lower.

6. To each dilution add 90 µl of the *E. coli* ER 2738 culture, mix gently and incubate for 5–10 min at 37°C without shaking.

7. Pipette each dilution to one of the prepared reaction tubes containing the top agar and mix by vortexing very carefully.

8. Immediately pour the top agar onto the prewarmed IPTG/X-gal agar plates and spread the top agar evenly by tilting the plate carefully.

9. Proceed the same way with the other dilutions.

10. Wait until the top agar has hardened and incubate the plates at 37°C. Wrap the plates in aluminum foil as X-Gal is light sensitive.

11. At the next morning blue plaques can be seen in the bacterial lawn and the titer of phages can be determined (*see Note 13*).

### 3.2.5. Phage Panning

1. Immobilize the target of interest in 1.5 ml coating buffer in an immunotube or well of a microtiter plate. The protein or peptide concentration should be between 3 and 100 µg/ml. Incubate under gentle agitation at 4°C over night on a roller mixer.

2. Remove the supernatant of the immunotube next morning and wash 3 × with TBS. Use as much buffer as needed to fill up the immunotube completely.

3. Incubate the immunotube with blocking buffer by filling the immunotube completely. Incubate for 2 h at room temperature under gentle agitation.

4. Inoculate 40 ml of LB-medium containing 20 µg/ml tetracycline with an overnight culture of *E. coli* ER 2738 cells to an OD<sub>600</sub> of 0.2 (*see Note 1*).

5. Wash the immunotube 3–5 x with washing buffer. Use as much buffer as needed to fill the tube completely.
6. Add $1 \times 10^{11}$ phages in 1 ml washing buffer containing 1% (w/v) BSA (see Note 3) and incubate for 50 min at room temperature under gentle agitation.

7. Remove the phages carefully by pipetting to avoid contamination and wash the tube $10 \times$ with washing buffer by discarding the supernatant and subsequently filling the entire tube with washing buffer (see Note 14).

8. Add 1 ml elution buffer into the tube and incubate for 10–30 min at room temperature under gentle agitation. Transfer the eluate into a fresh 1.5 ml reaction tube and neutralize with neutralization buffer immediately (see Note 15).

9. Infect the *E. coli* ER 2738 culture with the eluted phages at an OD$_{600}$ of 0.5. Keep a small rest of the eluted phages (e.g., 10 μl to determine the phage titer or concentration; see Section 3.2.3, Step 7 or Section 3.2.4).

10. After infection, leave the culture for 15 min at 37°C without shaking. Proceed as described in Section 3.2.2.

11. Repeat the whole procedure until a dominating sequence has settled in the library pool (see Note 16).

### 3.2.6. Evaluation and Characterization

#### 3.2.6.1. Sequencing

In the case of phage display single stranded or double stranded DNA can be prepared for sequencing. In the case of phagemid display, double stranded DNA is used for sequencing.

1. Infect an *E. coli* ER 2738 culture with phages from panning rounds to be investigated.

2. Let the culture grow for approximately 5 h (see Note 17).

3. Perform a standard DNA preparation and sequence the DNA. Note that either single phage clones or phage pools can be sequenced. In the pool sequences, the distribution of randomized codons and hence amino acids can be followed during the selection process. With increasing selection rounds, one favored sequence should dominate the pool. When using defined libraries with certain positions randomized to a set of amino acids (as opposed to libraries generated by error-prone PCR or DNA shuffling), it is even possible to quantify mixed codons and relate these to the amino acid distribution to assess the kinetics of selection (8, 10).

#### 3.2.6.2. Phage ELISA

1. Prepare phage pools or single phages as described in Sections 3.2.2 and 3.2.3. Single phages can be generated by inoculating an *E. coli* ER 2738 culture with a single plaque, picked from an IPTG/X-Gal plate. Grow culture over night and proceed as described in Section 3.2.2.1. For phagemid display, inoculate LB-medium containing the respective
concentrations with a single colony of an agarose plate, containing the phagemid pool. Grow culture over night and proceed as described in Section 3.2.2.2.

2. Immobilize a 96-well microtiter plate with the target that was used for selection.

3. Perform an ELISA with $1 \times 10^{12}$ phages per well.

4. Detect with an anti-phage antibody (e.g., anti-M13 antibody for using a phage display library generated in an M13-vector) (see Note 18).

An increase of phages binding to the target should be seen from panning round 1 to the last round. As control, unspecific binding should be tested as well.

3.3. DHFR Protein-Fragment Complementation Assay

The Protein-fragment complementation assay (PCA) described in this chapter is carried out in bacterial cells. However, applications of PCA have also been reported for mammalian cells (34) and plant cells (35). The assay relies on the principle that the survival of cells simultaneously expressing complementary fragments of the enzyme murine dihydrofolate reductase (mDHFR) is dependent on the correct folding and interaction of these fragments (12). The mDHFR is genetically dissected into two rationally designed fragments (mDHFR1 and mDHFR2, see Note 19), each of which is fused to a gene of interest or target gene, and reconstitution of enzymatic activity is obtained through interaction of these fusion proteins (see Fig. 16.3). The endogeneous procaryotic DHFR is specifically inhibited by trimethoprim, preventing biosynthesis of purines, thymidylate, methionine, and pantothenate, and therefore cell division. In this manner, only cells in which interacting proteins provide proper folding of mDHFR can survive. The most efficient interacting proteins lead to a higher growth rate for this clone, which can be enriched in repeated turns of the growth competition assay in liquid culture.

Non-specific and unstable library members are removed during the assay, leaving those with strong interaction capacities (8). The assay is very sensitive, since a few numbers of molecules of reassembled mDHFR are sufficient for bacterial survival (34). Consequently, the assay is qualified to detect proteins that are expressed at extremely low levels or even for screening of weak interactions. Furthermore, it is also an excellent strategy for a large-scale library-versus-library selection (9). Additionally, we have further developed the assay to select not only for interaction affinity but also for specificity by simultaneous expression of homologues peptides, which compete with protein libraries for an interaction with the target molecule (13). Library members binding to their target, and promoting cell growth, must outcompete competitor interactions with the target (i.e., competition) and evade binding to
the competitors (i.e., negative design). We term this a competitive and negative design initiative (CANDI).

1. Clone one vector encoding the fusion protein X-mDHFR1 and a second vector containing the fusion protein Y-mDHFR2, where X and Y are genes for specific target proteins, peptides or libraries (see Note 20). For the CANDI procedure, a third vector ending the competing peptide is used (see Note 21).

2. Transform the vectors (200 ng each) into BL21 gold cells (see Note 22). After electroporation of 100 µl cells, immediately add 1 ml enriched DYT medium, shake for 1 h at 37°C.

3. To reduce carryover of complex nutrients, wash the cells with M9 medium by gently pelleting the cells, removing the supernatant and resuspending the cells in M9 medium.

4. Plate cells in appropriate dilution series to facilitate counting and incubate at 37°C for 24–48 h:
   a. 1, 5, and 10 µl on LB-agar (+ Amp, Cm, Kan) (see Notes 23 and 24).
   b. 1, 5, and 10 µl on M9-agar (+ Amp, Cm, Kan, TMP, IPTG).

---

Fig. 16.3. Principle of the DHFR protein-fragment complementation assay (PCA). Protein X (in this case an α-helical peptide) is fused to mDHFR1 on a plasmid containing the gene for Ampicillin (Amp) resistance, protein Y is fused to mDHFR2 on a plasmid with the Chloramphenicol (Cm) resistance gene. Only cells which are cotransformed with both plasmids and have interacting partners can rebuild active mDHFR and survive on minimal medium plates containing trimethoprim. Further steps include sequencing and the growth competition assay.
c. Remaining amount on M9 agar (+ Amp, Cm, Kan, TMP, IPTG).

For the CANDI procedure Tet needs to be added as selection marker (see Note 24).

5. Count the colonies from the dilution series on the 1, 5, and 10 μl plates to determine their theoretical number from the LB plates and their actual number from the M9 plates. When the required theoretical number of colonies (overrepresentation of each clone of the library tenfold) is reached, combine the colonies by pooling them into ~10 ml of M9 medium and shake at 37°C for 5 min to disrupt cell clumps.

6. Prepare glycerol stocks with 30% (v/v) glycerol and store at –80°C. For sequence analysis, DNA from library pools or single colonies can be prepared and sequenced (see Note 25).

7. Inoculate 100 ml M9 medium (+ Amp, Cm, Kan, TMP, IPTG, and Tet in the case of CANDI) to a start OD 600 of 0.0001 and grow them at 37°C until an OD 600 of 0.2–0.5 is reached (see Notes 26 and 27).

8. Repeat Steps 6 and 7 until a unique sequence has settled.

3.4. Bacterial Surface Display

Parallel to phage display another display technology emerged during the last 20 years: Bacterial surface display enables the presentation of peptides and proteins on the cell surface of gram-negative and gram-positive bacteria. Like phage display, this technology provides physical linkage of genotype and phenotype. By selection of the target specific peptide displayed on the cell surface, also whole bacteria and their genetic information are obtained. A great variety of different display systems and their applications (from biocatalysts to vaccine delivery) has been published (15). Although phage display is the more common technology for screening of peptide libraries, there was a successful approach by Lu et al. in 1995 (16). Their so called “FLITRX peptide library” is commercially available from Invitrogen and will be described here.

Lu et al. (16) introduced a randomized oligonucleotide encoding for a dodecamer peptide [(XNN)12; N (any nucleotide); X (any nucleotide with G:A:C:T = 7:7:7:3] into the trxA (thioredoxin) gene. Expression of this construct results in modified thioredoxin molecules harboring a conformationally constrained peptide library in their active site loop. Thioredoxin is a cytoplasmic protein. For cell surface display the fusion construct was inserted into a non-essential region of the flagella fliC gene. The obtained FLITRX gene (fliC + trxA+DNA-sequence for peptide library) is under control of the P1l promoter at the pFliTrx vector. The bacterial chromosome of E. coli GI826 contains a bacteriophage λ cI repressor gene controlled by a trp (tryptophan) promoter.
In the absence of tryptophan, the λ cI repressor gene is expressed and the repressor can bind to the operator region upstream of the PL promoter and inhibits expression of FLITRX gene. By adding tryptophan to the culture medium, cI repressor expression is blocked and FLITRX expression is induced. As a result, E. coli lacking wild type fliC carried thousands of FLITRX proteins at their flagella. In this way the peptide library is presented to the extracellular environment.

The following procedure (see Fig. 16.4) describes the selection of target-specific peptides with the “FliTrx™ Random Peptide Display Library” (Invitrogen). It is also possible to clone an individually designed peptide library via provided multiple cloning sites into the pFliTrx™ vector (Invitrogen) and to perform a selection procedure.

Characterization of the selection can be performed by pool sequencing (see Section 3.2.6). Lu et al. described a screening on nitrocellulose membranes (16, 36) for a preselection of target specific clones. They used the FliTrx Peptide Library for epitope mapping of antibodies. In this case antibodies for detection of positive clones already exist. If the peptide library is applied to a target without specific detection reagents provided, it is recommended to fuse or conjugate the target molecule to a peptide-tag or protein which has

![Fig. 16.4. Selection procedure of the “FLITRX™ Random Peptide Display Library”.](image)
the following properties: (i) it must not be a protein or homolog already existing in *E. coli*, and (ii) a specific antibody against it should be available. Possible peptide tags are, e.g., His- or FLAG-tags (37) and possible proteins are, e.g., GFP (green fluorescent protein) and GST (glutathione-S-transferase). For individual characterization western blots or sequencing of single clones are suitable.

1. If the selection procedure is carried out against molecules with small molecular weight, they should be conjugated to a carrier protein (like BSA, OVA, KLH) (see Note 28).

2. Proliferation of the FliTrx™ peptide library (see Note 29):
   a. Inoculate 50 ml IMC-Medium with tenfold of the peptide library (at least 1.8 × 10⁹ clones).
   b. Incubate with shaking overnight at 25°C until saturation is reached.

3. Immobilization of the target molecule (see Note 29):
   a. Add 300 μl 20 μg/ml target molecule (in sterile water or buffer) (see Note 30) in each of 32 microtiter wells (see Note 31)
   b. Incubate overnight at 4°C.

4. Tryptophan induction:
   a. Inoculate 50 ml IMC-Medium (containing 100 μg/ml L-tryptophan) with 100-fold of the peptide library (detection by OD measurement).
   b. Incubate for 6 h at 25°C.

5. In the meanwhile, pour off the coating solution of the microtiter wells, rinse them with sterile water and block them with 1% (w/v) non-fat dry milk in 150 mM NaCl (300 μl/well for 1 h at room temperature and 50 rpm agitation) (see Note 32).

6. Panning:
   a. After 6 h induction, mix tryptophan culture (see Notes 33 and 34) with free-carrier protein (2% (w/v); final concentration) (see Note 35), 1% (w/v) non-fat dry milk and 300 μl 5 M NaCl (150 mM), add IMC-Medium to 10 ml final volume (see Note 32).
   b. Add 300 μl tryptophan culture mix per well, agitate them gently at 50 rpm (see Note 36) for 1 min and leave them bench-top for 1 h at room temperature.
   c. Remove the solution containing non-binding bacteria carefully (e.g., by pipetting).
   d. Add 300 μl IMC-medium to each well, incubate for 5 min at room temperature and gently agitate (50 rpm). Remove medium and repeat washing step another four times (see Note 37).
e. Decant the washing solution after the last step, add 300 μl IMC-medium to each well and vortex the whole plate for 30 s (see Note 38).

f. Inoculate 10 ml IMC-medium with the total eluate volume and cultivate it overnight at 25°C/180 rpm for the next selection round or centrifuge eluted cells for 2 min (2,000 × g), decant supernatant, resuspend the cells in 1/3 initial volume of IMC medium and spread them on RMG-Amp plates (overnight 25°C).

3.5. Purification of Peptide Aptamers

Once a protein or a peptide is identified from a library screen, it needs to be characterized in more detail and in complex with the target protein. The characterization might include measurements by ELISA, surface plasmon resonance (SPR), fluorescence or circular dichroism, depending on the nature of the selected interaction pair. For this purpose, the enriched binder needs to be present in purified form and sufficient amount. This can be achieved by both synthesizing the peptide and purifying it via reversed phase HPLC or by recombinant overexpression in E. coli and subsequent purification. For the latter, the binder is most often fused to a protein that can be purified by affinity chromatography, such as GST, chloramphenicol acetyl transferase (CAT) or maltose binding protein (MBP). Another possibility is to add a short peptide tag, e.g., six histidine residues, to the identified protein. By simultaneously inserting a proteolytic cleavage site, e.g., factor Xa (FXa, recognition site: Ile-Glu/Asp-Gly-Arg, cleavage after Arg), the enriched protein or peptide can be cleaved off from the fusion protein or tag.

The advantage of a fusion protein lies in a lower susceptibility to proteolytic degradation during expression in E. coli and easy purification. In addition, the tendency to form aggregates resulting in inclusion bodies is also reduced. However, it should be noted that each selected protein or peptide has to be treated individually with a defined expression and purification protocol.

Possibly the most common used fusion protein for the purification of recombinant proteins is GST. The native substrate of GST is glutathione (GSH), which binds to GST with high affinity. Consequently, during affinity chromatography, where GSH is covalently coupled to a carrier matrix (column or beads), the recombinant GST fusion protein binds to the GSH matrix. Elution of the GST fusion protein from the matrix is performed by adding soluble GSH, which competes with the immobilized GSH. The selected protein or peptide is subsequently isolated from the fusion protein by proteolytic degradation and purification via reversed-phase HPLC.

In the following protocol, the GST affinity purification of a helical peptide, followed by FXa cleavage and a reversed phase
high-performance liquid chromatography (RP-HPLC) is described. A schematic representation of the individual steps is described in Fig. 16.5.

1. Harvest *E. coli* cells from an expression culture and freeze them for 20 min at –80°C.

2. Resuspend the cells in GST binding buffer on ice (see Note 39) and transfer the lysate into a 50 ml polypropylene (PP) tube.

3. Lyse the cells either by sonication for 10 min on ice (with repeating 1 min interruptions to cool down the solution) or by french press.

4. Transfer the cell lysate into precooled SS34 centrifuge tubes and centrifuge for at least 30 min at 19,000 rpm (Sorval SS34 rotor; 41,000 *g*) at 4°C to spin down cell debris.

5. Transfer the supernatant into a pre-cooled 50 ml PP tube and sonicate again for 1 min on ice (see Note 40).
6. Load the supernatant via a superloop on the GST column (see Note 41) connected to a peristaltic pump or HPLC device that allows monitoring the absorbance of the loaded proteins by UV detection at 280 nm (see Note 42).

7. Wash the immobilized fusion protein with at least 60 column volumes of GST binding buffer to increase purity of the recombinant protein.

8. Elute the GST fusion protein from the column with elution buffer, containing 10 mM GSH. The protein should elute in the first 2–10 ml.

9. Transfer the elution fractions into dialysis tubes and exchange the buffer by extensive dialysis into FXa buffer at 4°C on a magnetic mixer (see Note 43).

10. Cleave the selected peptide or protein from GST by adding 1U of FXa enzyme per 10 μg of purified protein to the solution and incubate at 4°C or room temperature for an appropriate time (see Note 44).

11. Add 1 mM PMSF (phenylmethylsulfonyl fluoride) to stop the reaction. If appropriate, the cleavage efficiency can be checked on an SDS gel, where a clear shift from the upper GST fusion protein to the now remaining GST protein should be visible.

12. Load the FXa digest to a reversed phase column connected to an HPLC system that allows to monitor UV absorbance and to run gradients (see Note 45).

13. Separate the selected peptide or protein from GST using an appropriate gradient of ddH2O 0.1% TFA to ACN 0.1% TFA (e.g., 1%/min) and collect the fractions containing the peptide/protein (see Note 46).

14. Concentrate the samples collected from the RP-HPLC run under vacuum (e.g., lyophilizer or speed-vac) and resuspend them in an appropriate buffer for determination of peptide/protein concentration.

4. Notes

1. *E. coli* XL1-blue cells can also be used, but it should be made sure that the chosen cell line for amplification of the phage-library contains the F-episome, which is needed for F-pili formation. The F-episome contains a tetracycline resistance, which is why tetracycline is added to liquid cultures and plates.

3. Non-fat milk powder (approx. 2–5% (w/v)) can be used alternatively for blocking and/or washing. Due to different protein sizes in the milk, it might be more efficient – and cheaper – than BSA. Only in cases of streptavidin–biotin interactions, the biotin in the milk powder will interfere.

4. For easy preparation of M9 medium prepare a stock of 5 × M9 salts: 64 g Na₂HPO₄ · 7 H₂O (or 33.9 g Na₂HPO₄ anhydrous), 15 g KH₂PO₄, 2.5 g NaCl, 5.0 g NH₄Cl, add ddH₂O to 1 l and autoclave in aliquots of 100 or 200 ml. For 1 l minimal medium mix 200 ml sterile M9 salts, 2 ml sterile 1 M MgSO₄, 20 ml sterile 20% glucose, 1 ml sterile 0.1 M CaCl₂, ddH₂O ad 1 l. It is very important to use ultra-clean water and bottles to avoid any contamination with nutrients which would have an adverse effect on the mDHFR selection. All solutions should be cooled to at least 60°C before combining. To avoid precipitation, it is best to autoclave the water and CaCl₂ together first (for example, for 1 l of media, first autoclave approximately 750 ml UHP water and 1 ml 0.1 M CaCl₂), then add the rest of the components, with gentle stirring or swirling. The final volume can be adjusted with sterile ddH₂O.

5. It is important to decrease the temperature slowly so that the oligonucleotides can anneal properly.

6. For information on phage display vectors, also see the web page: http://www.biosci.missouri.edu/SmithGP.

7. Libraries can be transformed chemically or by electroporation. Usually electroporation yields higher transformation rates than chemical transformation. In either case, make sure to plate an amount of cells, which can easily be counted to determine the transformation rate and the library size (see Section 2.1).

8. It is important to work very accurate as phages are highly infectious and will infect any other *E. coli* culture susceptible for phage infection. Furthermore, if more than one person in the lab is working with phages, make sure to use completely different equipment and if possible have a spatial separation between respective lab members to avoid cross-contamination. Filter tip pipettes should be used for all phage-related work.

9. Shearing forces can cause damage to the pili of a considerable amount of cells, reducing the amount of infection events. Thus, e.g., Erlenmeyer flasks without baffles are recommended.

10. Directly shaking the culture upon infection of the phages might cause damage to a considerable amount of pili, due to immediate shearing forces.

11. Temperature and duration of the incubation can be varied, if the yield of phages is too low. For example let the cultures grow at 28°C and longer than 6 h if necessary.
12. To determine the phage concentration using a Photo-Spectrometer use the following equation (38):

\[
\frac{\text{Pfu}}{\text{ml}} = \frac{\text{DF} \times 6 \times 10^{16} \times (A_{260} - A_{320})}{\text{Size of genome in nt}}
\]

with Pfu = phage forming units and DF = dilution factor.

13. Always check the plates for white plaques, as this means contamination with wild type phages, which are ubiquitous and contaminate easily.

14. Usually the washing steps become more stringent the more panning rounds are performed. That is, in panning round 1 wash the tube 10 x with TBS-T 0.05%, in panning round 2 wash 20 x with TBS-T 0.05%, and so on. Other parameters can be varied, like incubation time of washing steps (e.g., add washing buffer and incubate the immunotube for 5 min under gentle agitation).

15. This elution procedure is a very general method and can be applied to most libraries. Before starting, pre-titration of the amount of neutralization buffer to neutralize 1 ml of elution buffer (to pH 7.0) is recommended. There are many other possibilities to elute phages, depending on the target. Elution can be performed by adding high-ligand concentrations that compete with the phages, by adding trypsin, or high-salt concentrations (25, 39–43).

16. The number of panning rounds can vary, depending on the target. It is useful to check the pools for enriched sequences to decide whether more panning rounds need to be performed or not. To make sure that no phages are enriched that bind to plastic or BSA, which is often used as blocking reagent, phages can first be incubated on immunotubes immobilized with BSA (subtractive panning). After ~30 min incubation, phages can be transferred to the immunotube containing the target. BSA binders will remain in the tube containing BSA.

17. Overnight growth is also possible for double-stranded plasmid preparation. However, in general it is not recommended to grow phage infected cells for prolonged times due to possible genetic instabilities.

18. If the signals in the phage ELISA are too high, less phages can be used (down to 1 x 10^{10} phages per well).

19. In the PCA, the murine mDHFR (UniProtKB/SwissProt entry P00375) is split into mDHFR fragment 1 (amino acids 1-105; also named DHFR1, F[1, 2] or DHFR[1, 2] in some publications) and mDHFR fragment 2 (amino acids 106–186; also named DHFR2, F[3] or DHFR[3]).
20. We typically clone the proteins of interest X and Y via NheI and Ascl restriction sites into a pQE16 derivative (Qiagen) containing a G/S linker tagged to mDHFR fragment 1 (pAR230d-X-mDHFR1; ampicillin resistance) or fragment 2 (pAR300d-Y-mDHFR2; chloramphenicol resistance \((8, 13, 44)\).

21. For the CANDI procedure, a competing peptide is cloned via NheI and Ascl in a third plasmid (pAR210d) from the same series as mentioned in Note 20, but lacking the mDHFR fragments and with tetracycline resistance \((13)\).

22. For stable propagation of expression constructs encoding toxic or hydrophobic proteins, the cells should express the lacI gene product that represses protein expression prior to IPTG induction. If lacI is not encoded in one of the vectors, it can be obtained by cotransforming the repressor plasmid pREP4 (KanR; Qiagen).

23. Cells should not be able to grow without IPTG in the presence of the lacI gene product. Therefore, they should also be plated on M9 minimal medium (+ Amp, Cm, Kan, TMP) without IPTG to confirm that there is no contamination and mDHFR activity is restricted to the screened interaction.

24. For weak interactions or toxic proteins, the use of many antibiotics might compromise growth. Consequently, Amp and Cm can be omitted as cell growth in the presence of TMP requires mDHFR complementation and thus selects for the presence of both mDHFR plasmids. Kan is required to maintain the lacI expressing pREP4, and Tet is essential in the case of CANDI for maintaining the plasmid with the competitor.

25. During later selection rounds, individual clones can be analyzed.

26. This may take up to 48 h for the first selection round. With each round, the bacterial growth rate will increase and the final OD\(_{600}\) will be reached faster.

27. The final OD\(_{600}\) should not be higher than 0.5 or the selection pressure will diminish, because a high-cell density results in the release of complex metabolites into the medium.

28. Conjugation to the carrier protein facilitates the adsorption to the plastic surface of microtiter plates by hydrophobic interactions and enables binding of library peptides with minimized steric inhibition. An additional tool to avoid selection of carrier protein specific peptides would be to change between two different carrier proteins.

29. **Steps 2 and 3** should be done in parallel.

30. The coating concentration of the target molecule can be reduced during several selection rounds to increase the selection pressure on single bacterial clones.
31. Lu et al. (16) performed panning in 60 mm dishes. 96-well microtiter plates provide a better surface to volume ratio and therefore an increased coating area.

32. Fimbriae at the *E. coli* surface are able to interact with oligosaccharides. This is important if the target molecules are glycosylated (like antibodies). In this case α-methyl mannoside (1%) (w/v) (16) should be also added.

33. Recommended: OD-measurement to detect bacterial concentration in tryptophan culture. Lu et al. (36) recommend $\text{OD}_{550} = 0.8–1.2$ and 10 ml induced cell culture for panning. A defined cell number helps to evaluate the enrichment of clones over the selection rounds. Due to the enrichment, the amount of cells can be reduced in later selection rounds. To be absolutely sure about the number of colony forming units (cfu) it is also possible to plate a small aliquot of tryptophan culture on RMG-Amp plates and incubate at 30°C overnight.

34. For characterization of different selection rounds, prepare glycerol stocks of overnight cultures before tryptophan induction.

35. The free carrier protein should be the same as used for coating, especially if the target molecule is conjugated to different carriers.

36. Flagella are sensitive to mechanical shearing. Every step in the panning procedure should be performed carefully.

37. Selection stringency can be increased by a higher number of washing steps or longer incubation for each washing step.

38. For observing recovery of eluted clones, it is recommended to remove 10 μl of eluate, plate it on RMG-agar plates and incubate overnight at 30°C.

39. Working on ice and fast handling as well as adding 1 mM PMSF or 1 mM EDTA decreases proteolysis and results in a higher yield of purified protein. Presence of lysozyme (0.2 mg/ml) enhances the lysis process.

40. Due to the shearing forces of the ultrasound, remaining DNA in the supernatant will be disrupted. Addition of DNase also reduces the DNA amount in the cell lysate.

41. GSH sepharose beads can also be used for batch purification in a small reaction tube.

42. If no UV detector is available, the collected fractions during the elution can also be tested for enzymatic activity of GST.

43. The amount of dialysis volume depends on the volume of the eluted protein, but should at least be around six liters for 2 ml of eluted protein. Alternatively, cleavage on the column can be performed so that the proteins are not eluted from the
column by GSH. Instead, the column is equilibrated in the FXa buffer and the enzyme is injected manually with a syringe to the column. After incubation, the peptide or protein is eluted by washing the column.

44. The cleavage conditions need to be optimized separately for each protein and depend on the pH of the FXa buffer (optimum between pH 6.5 and pH 8) as well as on the incubation temperature and incubation time.

45. The target protein can be recovered from the FXa digest and separated from the GST fusion partner using either size exclusion chromatography, an ion exchange column or a reversed phase column, depending on the nature of the selected peptide or protein.

46. The elution volume of the identified peptide or protein depends on its hydrophobicity. This elution volume should not overlay with the elution of the GST protein from the RP column. If this is the case, either the gradient needs to be changed or a FXa cleavage on the GST column needs to be performed.

References


