pSKAN Phagemid Display System

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Contents

The pSKAN Phagemid Display System was developed by Prof. Dr. J. Collins and Dr. P. Röttgen at the GBF Braunschweig, Germany. The system is patented.

A license is required for the commercial use of any products developed with pSKAN.

The pSKAN Phagemid Display System

The pSKAN Phagemid Display System is an alternative to antibodies or immunoglobulin domain presenting phagemids. Since the pSKAN phagemid displays a small and constrained hypervariable amino acid loop, more than 3×10^7 variants can be tested for the specific binding to a target molecule of interest. The variants with the optimal (in general the highest) affinity can be selected and propagated without having to use animals or tissue cultures. The sequence of the binding domain can be determined easily by DNA sequencing. In comparison to antibodies, the pSKAN System is able to target very small epitopes, since it searches for binding sites with "only a fingertip" instead of "two whole hands" like antibodies. Within a period of only three weeks, the scientist is able to receive the target molecule of interest.

Applications:

- Development of new affinity matrices for a protein of choice
- Screening for novel protease inhibitors with potential clinical applications
- Tools for the detection of proteins in, e.g., food technology
- High-throughput screening of protein-protein interactions
- Selection of proteins with specific binding properties (high affinity binders)
- Ligand screening
- Development of pharmaceuticals and diagnostics
- Detection of allergens
- Of special interest: 3-D structure-based protein design
- and many more!

Advantages:

- Can select very high-affinity binders
- Low dissociation due to low flexibility and rigid structure of the hypervariable loop
- Easy and reliable modelling because of the highly constrained epitope
- Stability of the libraries due to the strong repression of the λ-promoter in *E. coli* WK6λmutS
- Production of a soluble protein possible
- Helpful for subsequent protein structure modelling

Advantages over immunoglobulins:

- Ease of handling and rapidity (three weeks!)
- Low costs
- Animals or tissue cultures become superfluous

Advantages over immunoglobulin phagemids:

- Targets smaller, recessed epitopes of limited accessibility
- Monovalent display gives high selectivity

1. Background

The pSKAN Phagemid Display System can be used to select for and produce novel proteins which bind to target molecules of interest. Examples are the selection of new protease inhibitors (see chapter 6) or the creation of new peptide sequences which can be used like a monovalent antibody for affinity purification.

The pSKAN Phagemid Display System produces molecular repertoires containing millions of site-specific mutant proteins in a form which enables an adsorption-based selection of both, gene and gene product. This property allows for repeated cycles of selection and amplification which results in the enrichment of novel proteins with the desired binding affinity for particular target molecules of interest.

The technology simultaneously selects variant genes and gene products. This is achieved by using the single-stranded bacteriophage M13 propagation system. The pSKAN phagemid containing a phage origin of replication is packed into a phage protein-coated particle on superinfection by a helper phage (M13K07 or derivatives). The phagemid encodes a hybrid protein consisting of a fusion between one of the phage coat proteins (pIII) and the human pancreatic secretory trypsin inhibitor (hPSTI) which presents a hypermutated amino acid loop (figures 1 and 2). This hPSTI-pIII$_{M13}$ fusion protein replaces generally one (or two) of the five wild type pIII proteins of the phage coat, leading to a monovalent display of the hypervariable moiety (genetic details see chapter 3).

Originally, the hPSTI protein presents a loop of 7 amino acids stretched between two disulfide bridges on the protein surface. MoBiTec supplies three variations of this pSKAN library: due to the introduction of different oligonucleotides, the loop consists of 6, 7 or 8 hypervariable amino acids. The three libraries are referred to as HyC, HyB, and HyA, respectively (Hy for hypervariable; HyC has a 7 amino acid loop of which only 6 are hypervariable). By randomly mutagenizing the hPSTI gene sequence for these amino acid loops, three different libraries of phagemids are constructed containing tens of millions of ligand variants. Some of these gene product variants will have the desired binding affinities for the particular target molecule of interest. The subset of phagemids with the desired binding properties is selected by, for example, binding on a surface coated with the target molecule; the poorly binding majority of phagemids being washed away. The strongly binding population is eluted and amplified by reinfection and cultivation in *E. coli*. About three successive rounds of such binding and amplification lead to the enrichment of one (or very few) variant ligands which exhibit the desired binding characteristics for the target molecule. The three libraries are normally treated separately during the selection process, but a combination of the libraries is possible as well.

After selecting the peptide sequence of interest, the production of a soluble hPSTI protein is possible as well. For this purpose, the DNA sequence of the selected hypervariable loop can be cloned into a vector like pMAMPF (reference #4). pMAMPF vectors produce a soluble variant of the hPSTI protein. By exchanging the DNA sequence of the hPSTI loop in pMAMPF with the DNA sequence of the selected pSKAN phagemid, a small soluble protein of 6.1 kDa can be produced in *E. coli*.

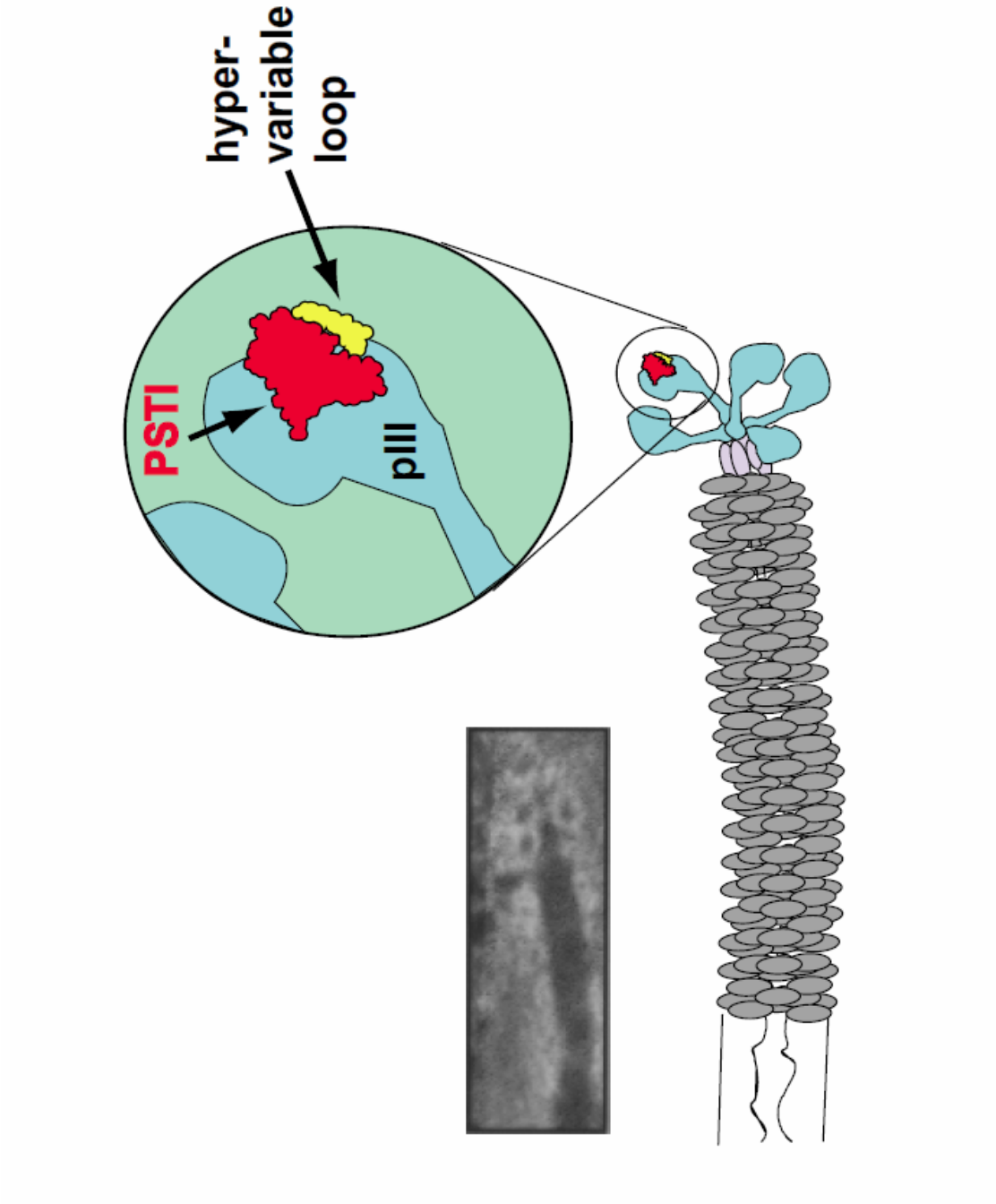


Fig. 1: The infectious phagemid is displayed as an electron-microscopy picture (insert) and as a schematic drawing. The hybrid protein is shown enlarged as a drawing. Size of the phage: 60 x 8800 Å (0.006 x 0.88 µm); size of the hPSTI protein: 6.1 kDa (production of a soluble hPSTI protein: see page 7).

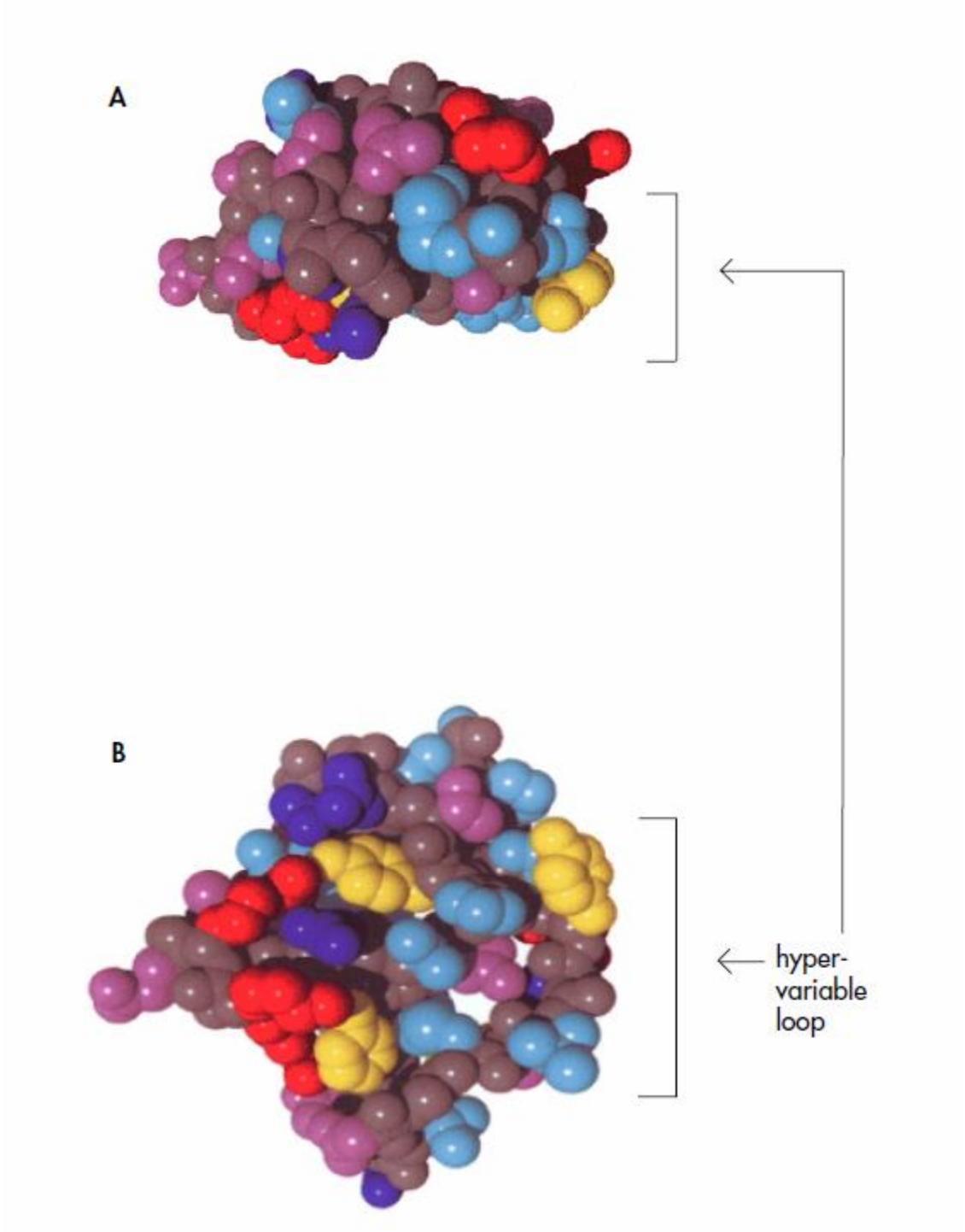


Fig. 2: The protein crystal structure of hPSTI in the van-der-Waals presentation
A: top view B: side v

2. Advantage of This System Over Other Systems (Immunoglobulins, etc.)

The pSKAN Phagemid Display System represents an alternative to the peptide or peptide analogue libraries and the RNA evolutive technology in the search for novel ligands. Different from these latter systems, the pSKAN phagemid presents the variable epitope under defined conformational restraints on the protein surface between two disulfide bridges. Over a linear cross-section of 30-40 Å a binding site of 5-7 amino acids is displayed without problems of steric hindrance from neighboring molecules, allowing targeting of specific regions. The crystal and solution (NMR data) structures of hPSTI and a number of variants are known so that the structure of new variants, which differ only in the loop, can be accurately estimated and modelled. The selected variants can serve as excellent "lead molecules" for the development of synthetic mimotopes.

Advantage over other phage display systems

As mentioned in chapter 1. the pSKAN phagemid presents only one or two hybrid hPSTI-$pIII_{M13}$ molecules. Such essentially monovalent phagemids exhibit a strong selective advantage for variant molecules with high avidity. In phage display systems, where the pIII gene is replaced on the DNA level by a recombinant gene producing the hybrid protein, the fusion protein replaces all five pIII molecules. In such a system, phages which display molecules with less affinity will also bind (via five variable moieties) to the target molecule of interest resulting in the selection of a less specific variant molecule.

Advantage over immunoglobulins

Compared to immunoglobulins, the pSKAN phagemid has the following advantages: The development and production of polyclonal and monoclonal antibodies is time-consuming. Furthermore, animals (mostly mice) have to be injected and finally are normally killed. For polyclonal antibodies this process has to be repeated for every new production. For monoclonal antibodies, the selected cell lines have to be kept, grown and harvested for production, which is a rather expensive process. In addition, antibodies are large molecules consisting of four peptide chains. Thus, once parts of the molecules unfold, refolding is rather improbable. The large antibodies try to find a marked epitope of a molecule with two binding sites ("with two hands") while the pSKAN phagemid searches for an epitope with a small amino acid loop ("with a fingertip"). Antibodies have problems targeting small epitopes due to steric hindrance by their surface peptide loops. The hPSTI protein on the contrary is small and consists of only one peptide chain. Compared to antibodies, the pSKAN Phagemid Display System is inexpensive, easy to handle, does not require involved laboratory equipment and surveillance and results in a variant protein within a very short period of time.

Advantage over immunoglobulin phagemids

The pSKAN Phagemid Display System also has advantages over immunoglobulin domain-presenting phagemids for "screening" small epitopes. In comparison to F_{ab} phagemids (hypervariable CDR3 region), the varied epitope of pSKAN is small and constrained as an extended peptide. The immunoglobulin phagemids only display up to 10^5 variations, a small number compared to the $3x10^7$ variants displayed by the pSKAN system.

Clinical potential

hPSTI is based on a small human acute response protein (human pancreatic secretory trypsin inhibitor) which is present in high levels in serum. This might be of advantage at a later stage if a direct clinical application is envisaged for the variant protein (e.g., development of new protease inhibitors with therapeutic potential; see chapter 6.2.).

3. Genetic Information on the System

The pSKAN phagemid, which combines genetic elements from phage and plasmid, uses the ColE1 plasmid origin for replication in *E. coli*. Upon superinfection with the helper phage M13K07, single stranded DNA is produced from the phage fd origin. The phagemid DNA is then packaged into the M13K07 phage coat, creating a new phagemid generation with pSKAN-DNA. A hybrid protein consisting of a fusion between one of the M13 phage coat proteins (pIII) and the human pancreatic secretory trypsin inhibitor (hPSTI) is expressed from the phagemid DNA. This fusion protein is regulated by the λpL promotor. Thus, it is repressed in the λ-lysogenic *E. coli* WK6λmutS which produces the λ-repressor cI. hPSTI-pIII$_{M13}$ is only expressed after superinfection with the helper phage. The induction mechanism, which is initiated by the superinfection, is not fully understood. hPSTI-pIII$_{M13}$ can replace one or more of the five wild type pIII$_{M13}$ coat proteins during phage coat assembly. Statistically, only one hybrid protein is integrated in every second phagemid. Thus, a population of phages is produced, which monovalently displays the hybrid protein with the hypervariable loop (see figures 1 and 2) and which contains pSKAN phagemid DNA. When the new phagemid generation infects *E. coli* again, the phagemid DNA remains in form of a plasmid in the cell and no further phages are produced. For phagemid production a new infection with helper phage is essential (see figure 3). Since the helper phage is an M13 derivative, the cells are not lysed; the phages are excreted. The host strain needs to carry the F-episome encoding the sex pili which serve as phage receptors during the infection.

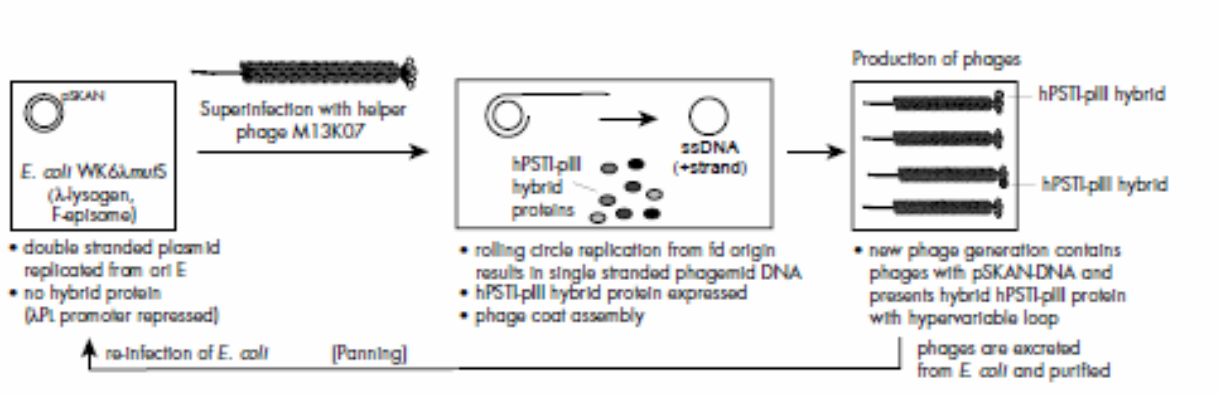


Fig. 3: pSKAN Phagemid life cycle in *E. coli* (squares)

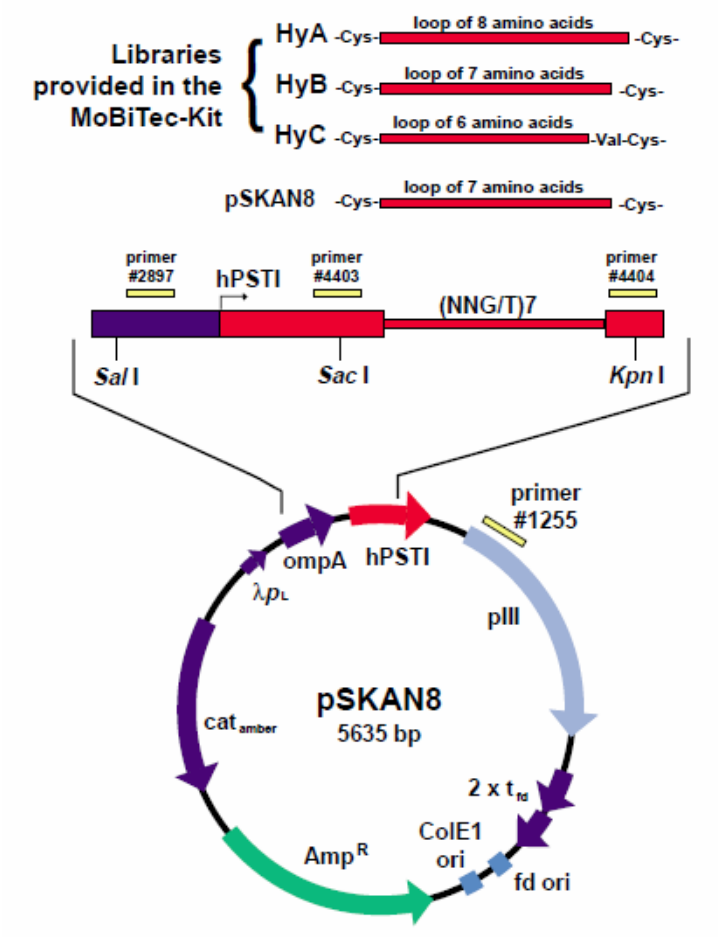
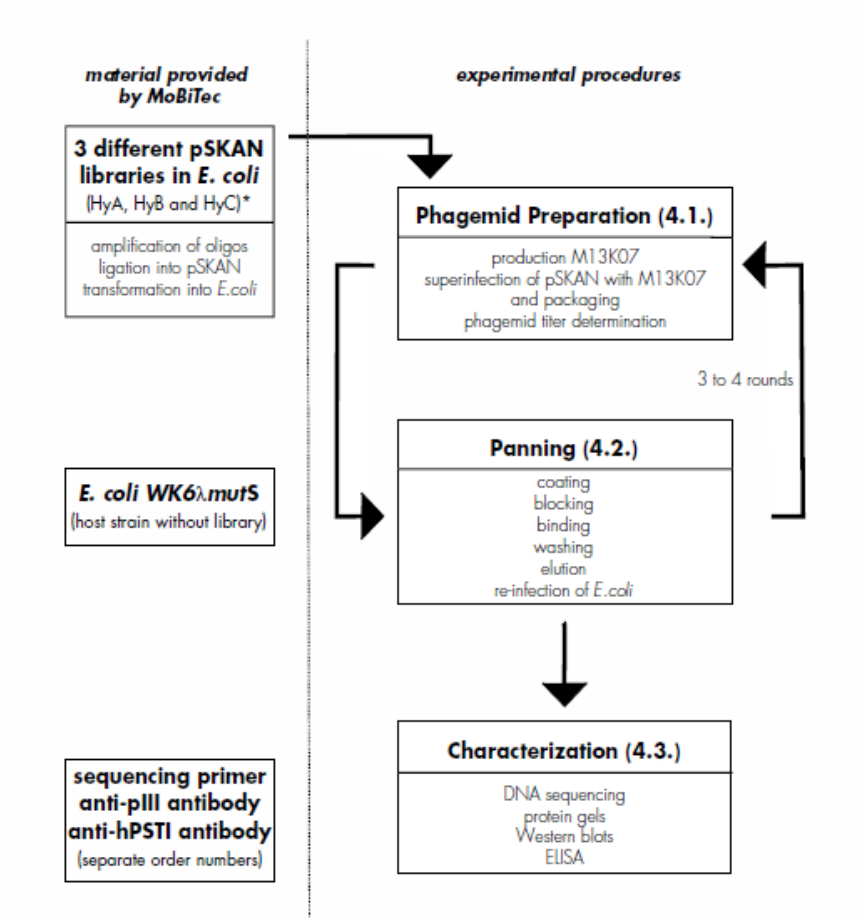


Fig. 4: Phagemid vector construction. The phagemid contains the genetic elements: selection marker Amp^R (ampicillin resistance); ColE1 ori: plasmid origin of replication; fd ori: origin of replication for the fd phage; λpL: λ-promoter left; ompA: signal sequence for secretion; hPSTI: gene of the human pancreatic secretory trypsin inhibitor; the hPSTI gene contains the inserted randomized sequence (NNG/T)n with n being 6 (HyC), 7 (HyB), or 8 (HyA), Hy for hypervariable; pIII: gene for the phage coat protein III; 2 x t_{fd}: two phage fd transcription terminator sequences; cat_{amber}: chloramphenicol resistance is inactive due to an amber mutation.

4. Protocols

Note: MoBiTec conveniently provides the pSKAN libraries HyA, HyB, and HyC ready-to-use in three 1-ml aliquots of *E. coli* WK6λmutS. The host strain (without phagemid) for re-infection is supplied with the libraries. Therefore, after the preparation of the phagemids, the customer is immediately able to start the selection of ligands, the so called "panning" procedure.
If there is an interest in preparing your own custom-made pSKAN DNA library: the original pSKAN8 vector which was also used to prepare the above mentioned libraries is available at MoBiTec as well (see chapter 7).



*HyA (n=8), HyB (n=7), HyC (n=6); n indicating the number of hypervariable amino acids in the loop

Buffer compositions, media, and antibiotics are listed in chapter 4.5.
An example suitable as positive control is described in chapter 6.1.
We highly recommend the performance of this positive control!

General knowledge: Users of this kit should be familiar with molecular biology and microbiological techniques!

4.1. Preparation and quantification of phagemid particles

Note: each amplification of a library causes the loss of peptide variants, therefore we strongly recommend working with the original library stocks provided by MoBiTec which has 10^7 variants per library. The host strain WK6λmutS without phagemid (for re-infection) should be kept on minimal medium during cell-keeping in order to select for the F-episome, which carries proAB for the complementation of the proline synthesis deficiency.

4.1.1. Helper phage: M13K07 phage stocks

The preparation of M13K07 helper phages should be started from a single fresh phage plaque. Therefore, inoculate 20 ml of LB medium with a single colony of *E. coli* WK6 cells and incubate over night at 180 rpm and 37 °C. Use 200 µl of this culture to inoculate 20 ml LB medium and incubate at the same conditions until the culture reaches the logarithmic growth phase (2-3 hours; OD_{600} = 0.5). Mix 1 µl of a M13K07 phage stock solution and 0.5 ml of logarithmic growing WK6 cells with 3 ml of molten LB top agar (about 40 °C) and pour the mixture onto a LB agar plate. Incubate over night at 37 °C. The next day, use a disposable Pasteur pipette to pick a single, well separated phage plaque and inoculate 20 ml of LB (2x)/Km medium (100 ml Erlenmeyer flask). Incubate over day at 37 °C on a shaker at 180 rpm. Inoculate 2 x 500 ml LB (2x)/Km medium with 10 ml preculture and incubate overnight (37 °C, 180 rpm).

The next day, centrifuge four 250 ml aliquots for 15 minutes at 8,000 rpm and 4 °C (e.g., GS3 rotor, Sorvall RC5C). Transfer the supernatant to centrifuge bottles and centrifuge again. Transfer the supernatant again, add 0.15 volumes of PEG/ NaCl solution, mix, and incubate on ice for at least 2 hours.

Centrifuge for 40 minutes at 8,000 rpm (GS3 rotor), decant the supernatant, repeat the centrifugation for 1 minute at 4,000 rpm, and remove last traces of supernatant using a pipette. Resuspend each PEG pellet in 2.5 ml PBS solution and collect the resuspended phages in one SS34 centrifuge bottle. To clear the suspension, centrifuge again for 10 minutes at 12,000 rpm (SS34 rotor). Recover the supernatant (pipette), add NaN_3 to a final concentration of 0.02%, and store the phages at 4 °C.
Note: about three to four successive rounds of binding and amplification lead to the enrichment of one, or very few, variant ligands which exhibit the desired binding characteristics for the target molecule. Therefore, the following protocols have to be repeated at least three times.

4.1.2. Packaging of pSKAN phagemids

Inoculate 20 ml LB medium containing 250 µg/ml Amp and 20 µg/ml Tc in a 200 ml Erlenmeyer flask with 200 µl from the frozen *E. coli* WK6λmutS stocks containing the pSKAN phagemids, as provided in the MoBiTec kit or from the previous panning step. Incubate overnight at 37 °C.

Note: an inoculation with 200 µl provides more than sufficient cells than possible peptide variants (10^7 per library). The concentration (cells/ml) for each lot is indicated on the data sheet provided with the product.

For packaging the phagemids, inoculate 100 ml of LB/Amp/Tc medium (1L Erlenmeyer flask) with 1 ml of the overnight culture. Incubate at 37 °C and 180 rpm until OD_{600} = 0.5 (~ 2.5 hours). Add 500 µl of the helper phage M13K07 stock solution (10^{11}-10^{12} cfu/ml; see 4.1.1.), incubate at 37 °C without shaking for 15 minutes, and then at 37 °C and 180 rpm overnight.
Note: some scientists have observed higher phagemid yields when Kanamycin (end concentration 50 µg/ml) was added 0.5 to 1 hour after infection with the helper phage, leaving sufficient time for the expression of the helper phage's Km resistance gene.

The next day, centrifuge 10 minutes at 8,000 rpm (GSA rotor), decant the supernatant into a fresh bottle, and repeat the centrifugation step. Add 0.15 vol. of PEG/NaCl solution to the supernatant and incubate on ice for at least two hours. Centrifuge for 30 minutes at 10,000 rpm (GSA rotor), decant the supernatant, and repeat the centrifugation. Remove the supernatant completely, dissolve the pellet in 1 ml of PBS buffer, and transfer the solution to an Eppendorf reaction tube. Centrifuge for 10 minutes at 13,000 rpm (batch centrifuge) recover the cleared solution, and add NaN_3 (final concentration of 0.02%). Store at 4 °C.

4.1.3. Determination of phagemid titers (colony forming units [CFU] assay)

Inoculate 20 ml of LB/Tc (20 µg/ml) medium with 200 µl of an *E. coli* WK6λmutS overnight culture (37 °C; LB/Tc) and incubate at 37 °C and 180 rpm for 2 to 3 hours (OD_{600} = 0.5).

For each phagemid probe fill seven to eight wells of a sterile 96-well culture dish with 90 µl of autoclaved water and prepare dilution series by transferring 10 µl aliquots (dilutions 10^{-1} to 10^{-7} or 10^{-3} to 10^{-10}).

Add 100 µl of logarithmic growing cells, mix, and incubate for 30 min at 37 °C. Spot 20 µl portions of each well onto LB/Amp/Tc (phagemid titer) and LB/Km/Tc (helper phage titer) agar plates, and incubate overnight at 37 °C. As a control use 20 µl of non-infected log-phase cells spotted onto LB/Amp/Tc, LB/Km/Tc, and LB/Tc agar plates.

4.2. Panning: selection of ligands

Note: several panning procedures can be applied at this stage. The protocols below describe the standard procedure with microtiter plates for the coating step which is used at the GBF Braunschweig, Germany. Alternative coating protocols involve tubes, magnetic beads, or the concentration of the ligands. Variations are also possible - and advisable - at the elution step. An example for phagemid titers resulting from a panning procedure is given in chapter 6.1. It is recommended to perform this example as positive control.

4.2.1. Panning procedure

4.2.1.1. Coating of microtiter plates

Fill 100 µl of ligand solution (100 µg/ml PBS) into the wells of a 96-well microtiter plate (e.g., Nunc, Maxisorb; has a hydrophilic surface for the binding of ligands). Incubate overnight at 4 °C or at least 2 hours at room temperature.

Note: one well is sufficient per ligand and per reaction condition (e.g., different elution buffers; see figure 6). One well should be left without ligand as a negative control. If phagemids are enriched in this negative control after 3-5 rounds, they might have an affinity to the blocking reagent (skim milk). In such a case, it is advisable to start a new panning procedure.

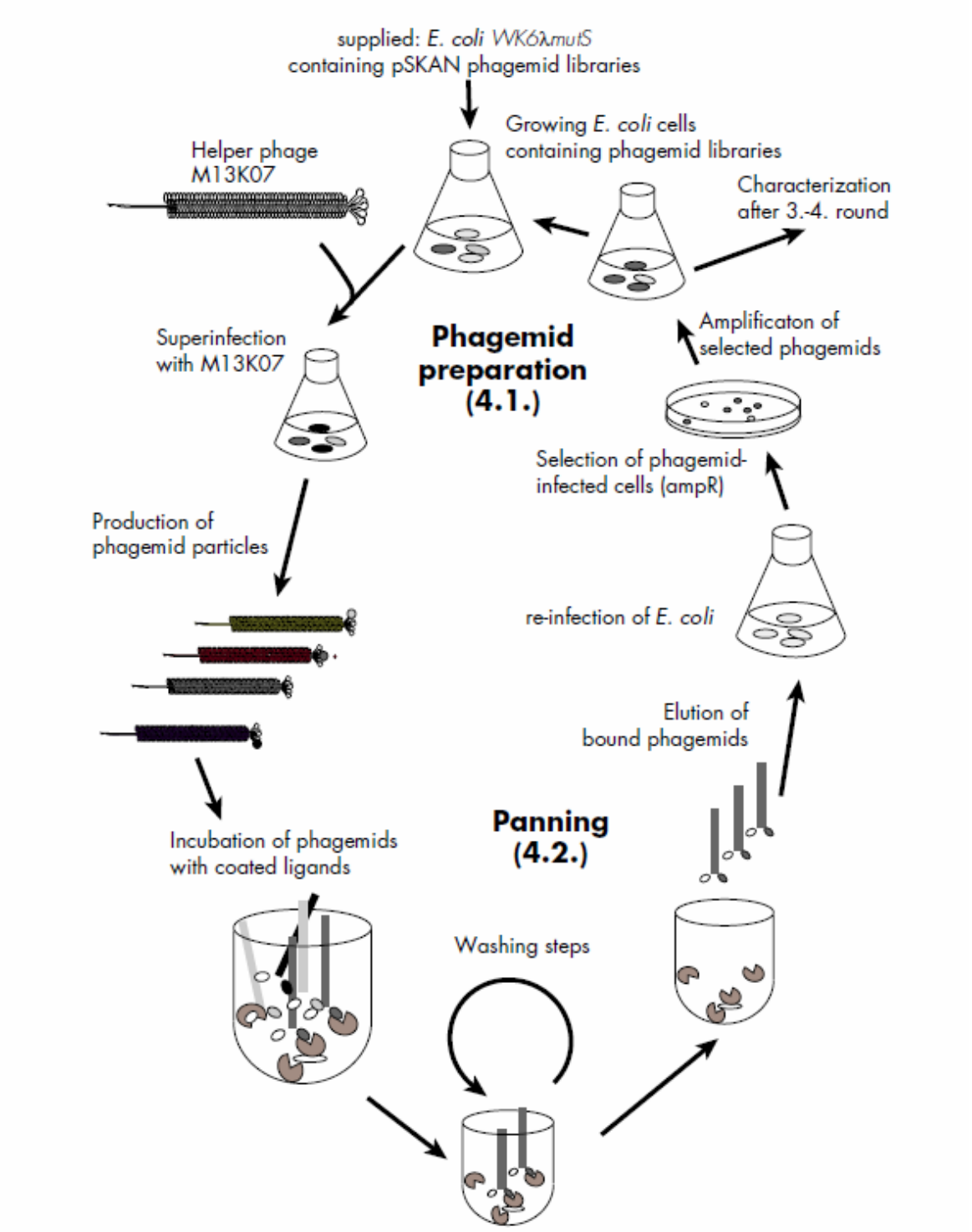


Fig. 5: General application protocol

Shake out the wells, slap the plate onto a paper towel, and wash the wells once with T-PBS solution (ELISA plate washer).

4.2.1.2. Blocking

In order to block any free positions, fill the wells with 400 µl of blocking solution and incubate at room temperature for ~ 1 hour. Shake out the wells, slap the plate onto a paper towel, and wash the wells once with T-PBS.

4.2.1.3. Binding

Fill the coated and, as a negative control, one uncoated well with 100 µl of phagemid preparations diluted 1:1 with blocking solution or PBS (~ 10^{10} – 10^{11} phages/well) and incubate at room temperature for at least 1 hour.

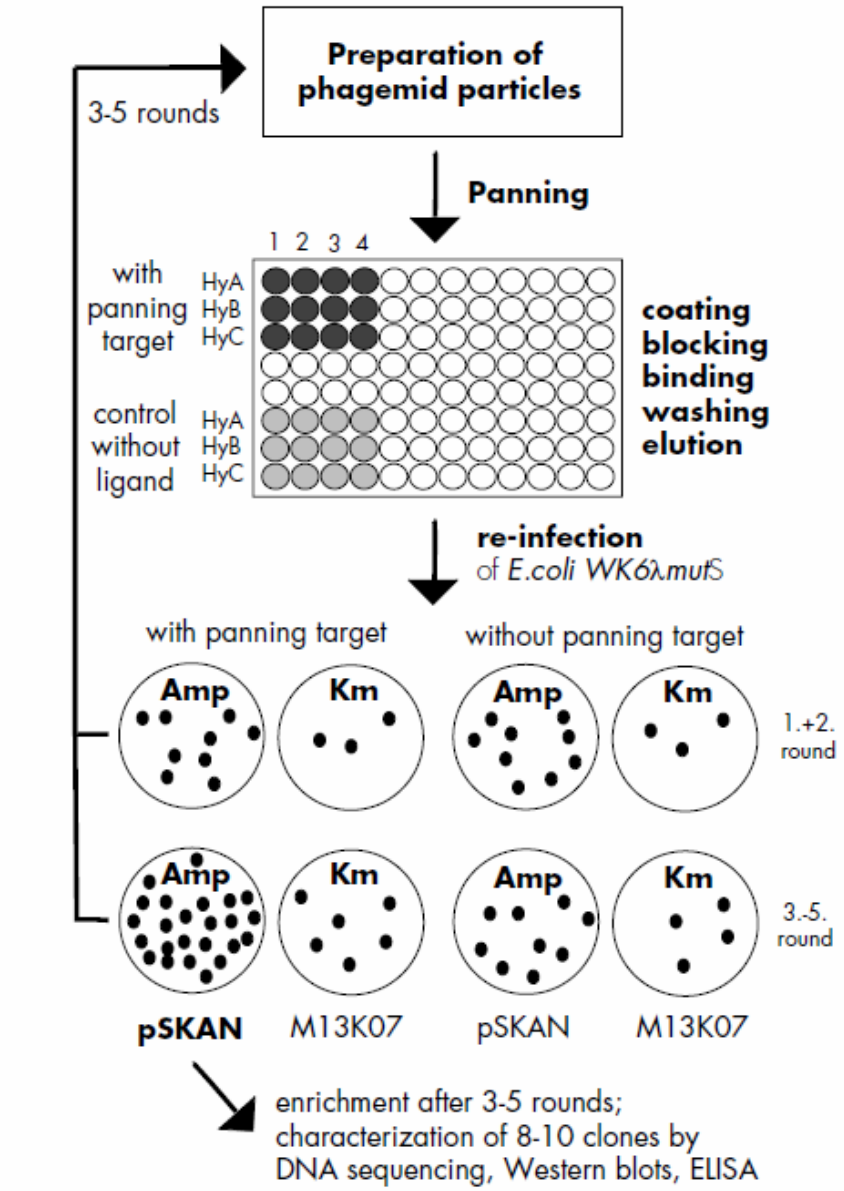
4.2.1.4. Washing

Shake out the wells and slap the plate onto a paper towel. In the first round of panning wash the wells once with T-PBS, incubate for 10 minutes with 400 µl blocking solution, wash again with T-PBS, and finally two times with water. During all further rounds repeat the T-PBS washing steps three times. All washing steps may be carried out by using an ELISA plate washer.

4.2.1.5. Elution

Slap out the plate and fill the wells with 100 µl of elution-buffer. Incubate at room temperature for 15 minutes and transfer the solution to an Eppendorf reaction tube containing 6 µl Tris (2 M).

Determine the titer of eluted phages as described under 4.1.3.



*1-4 indicating different reaction conditions (e.g., variation in elution buffer)

Fig. 6: Panning with microtiter plates. Per reaction condition (#1-4) one well per library (HyA, HyB, HyC) is coated with the ligand of interest. After 3-5 rounds an increase in the pSKAN phage titer is observed. The control without panning target shows the background of unspecifically binding phages. The helper phage titer (M13K07) serves as additional control.
Km, kanamycin; Amp, ampicillin.

4.2.2. Reinfection of *E. coli* cells

Mix the eluted phages and 10 ml of *E. coli* WK6λmutS log-phase cells (37 °C; LB/Tc-culture) and incubate for 30 min at 37 °C. Collect the cells by centrifugation (5 minutes, 8,000 rpm, SS34 rotor) and resuspend the pellet in 400 µl of LB/Amp (250 µg/ml)/Tc (20 µg/ml) medium. Plate 200 µl aliquots onto LB/Amp/Tc agar plates and incubate overnight at 37 °C.

4.2.3. Packaging of phagemids from re-infected cells

Resuspend the complete lawn of the re-infected *E. coli* cells in 20 ml of LB/Amp/Tc medium and use 2 ml for inoculation of 50 ml LB/Amp/Tc medium (250 ml Erlenmeyer flask). Incubate at 180 rpm and 37 °C for 1 hour, add 100 µl of M13K07 stock solution (10^{11}-10^{12} cfu/ml; see 4.1.1.), and incubate for 15 minutes at 37 °C without shaking. Continue the incubation at 37 °C and 180 rpm overnight.

The next day, collect the phages as described under 4.1.2.

After each round of panning, the titers of kanamycin-resistant (Km^R; helper phage) and ampicillin-resistant (Amp^R, pSKAN) phagemid particles were determined according to 4.1.3.

Note: an increase in eluted pSKAN phages should be recognizable after three to four rounds (see figure 6). Example titer results are given in chapter 6.1.2.

4.3. Characterization

4.3.1. DNA Sequencing

Note: after three to four rounds of panning, a first characterization of the selected phagemids is advisable. For this purpose, sequencing the single clones is the easiest procedure.

Use standard protocols for DNA sequencing. Suitable primers are available at MoBiTec (see chapter 7). The primer sequences are as follows:

- for single- and double-stranded DNA (binding site at the start of pIII; sequencing towards hPSTI):

primer # 1255 5´ GGGATTTTGCTAAACAAC 3´

- for double-stranded DNA (esp. Alf-sequencing; binding site before hPSTI leader sequence; sequencing towards hPSTI):

primer # 2897 5´ GGAGGTCTAGATAACGAGG 3´

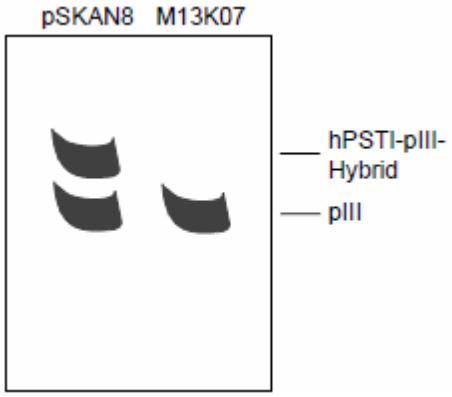
Note: we recommend double-stranded sequencing since primer # 1255 also recognizes a sequence on the helper phage M13K07, which would be added for the production of single-stranded DNA.

4.3.2. Protein gels and Western blots

Note: protein gels and Western blots are used as a control for the integration of hybrid proteins in the phagemid particles. An anti-pIII antibody is available at MoBiTec (see chapter 4.7. and 7). Alternatively, an hPSTI antibody is available (see chapter 4.7.) which can be used to detect hPSTI directly. The antibody is also suitable to detect soluble hPSTI. In a Western blot the hPSTI protein yields one band at 6.1 kDa. Phagemids containing an hPSTI-pIII hybrid protein will yield two bands in Western blots, one resulting from the non-hybrid pIII proteins, the other from the larger fusion protein hPSTI-pIII. Phagemids without hybrid protein yield only one band (for pIII). Such phagemids can occur when a stop codon is present within the selected hypervariable loop. Cells without a fusion protein grow faster and could thus potentially be enriched. This control will exclude a false positive result.

Use standard protocols for polyacrylamide gel electrophoresis (PAGE) of proteins, blotting, and protein detection by specific antibodies.

Fig. 7: Schematic presentation of a Western blot using the pIII antibody. A phagemid displaying a hybrid protein, such as pSKAN8 or a phagemid selected during the panning procedure, shows two bands: one for pIII and one for the larger hybrid protein hPSTI-pIII. A phage which does not present a hybrid protein, like M13K07 or a pSKAN phagemid with a stop codon within the hypervariable region, shows only one band.



4.3.3. Phagemid ELISA

Note: in order to test the binding capacity of the isolated phagemid variants an ELISA can be used. An example of an ELISA result is given in chapter 6.1. (Fig. 8).

To coat a microtiter plate, fill 100 µl solution of the desired ligand, e.g., at 100 µg/ml PBS, into the wells and incubate at 4 °C overnight.

Slap out the wells and wash them once with T-PBS solution. Add 400 µl of blocking solution, incubate for 1 hour at room temperature, and wash again. Incubate with phages (4.2.2.) diluted 1:1 in blocking solution for 3 hours and wash two times with T-PBS. Incubate with a commercially available anti-M13 antibody (for instance, anti-pVIII, peroxidase labeled; diluted 1:5,000 in blocking solution) for 1 hour and wash the wells three times with T-PBS. Incubate with 100 µl of staining solution, stop the reaction after 30 minutes by adding 50 µl of 2 M H_2SO_4, and measure the absorption at 492 nm.

To exclude any false positive results, the following negative controls are recommended for the phagemid ELISA:

a) well without ligand – to exclude unspecific phagemid binding to the plate or the blocking reagent)
b) well without phages – to exclude unspecific binding of the M13 antibody
c) well without M13 antibody – as control for the antibody-specific color reaction

4.4. Antibiotics, media, and buffers

Antibiotics (stock solutions)

Ampicillin (Amp, 50 mg/ml):
Dissolve 1g of ampicillin (sodium salt) in 20 ml water and sterilize by filtration
Tetracycline (Tc, 20 mg/ml):
Dissolve 400 mg of tetracycline in 20 ml of ethanol (50%)
Kanamycin (Km, 50 mg/ml):
Dissolve 1 g of kanamycin in 20 ml of H_2O and sterilize by filtration.

Store all antibiotics at -20 °C

M9 medium (1x)

M9 salt solution (10x)	100 ml
Glucose (40% w/v)	12.5 ml
$CaCl_2$ (1 M)	100 µl
$MgSO_4$ (1 M)	1 ml
$FeCl_3$ (1 mM)	0.5 ml
Thiamine (10 mg/ml)	100 µl
H_2O	ad 1 l

all solutions are autoclaved or sterile filtered (Thiamine) separately

M9 salt solution (10x)

Na_2HPO_4*2 H_2O	74.1 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
H_2O ad	1 l
autoclaved	

LB stock solution (2x)

Tryptone	10 g
Yeast extract	10 g
NaCl	5 g
H_2O ad	500 ml
autoclaved and stored at 4 °C	

Agar stock solution (2x)

Agar	15 g
H_2O ad	500 ml
autoclaved and stored at 4 °C	

LB medium

Tryptone	10 g
Yeast extract	10 g
NaCl	5 g
H_20 ad	1,000 ml
autoclaved	

LB agar plates

For preparation of 40 agar plates melt 500 ml of agar stock solution, add 500 ml of LB stock solution, mix, and pour into sterile petri dishes.

For selective plates add 5 ml of ampicillin (50 mg/ml), 1 ml of tetracycline (20 mg/ml), or 1 ml of kanamycin (50 mg/ml) stock solution, respectively.

LB top agar

LB medium containing 0.6% Agar

PEG/NaCl-solution: (16.7%/3.3 M)

PEG 8,000	100 g
NaCl	116.9 g
H_2O	475 ml

PBS-buffer (1x, pH 6.8-7.0)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4 +2 H_2O	1.43 g
KH_2PO_4	0.2 g
H_2O ad	1,000 ml

For panning and phagemid ELISA:

T-PBS solution:

PBS-buffer containing 0.5% Tween 20

Blocking solution:

PBS-buffer containing 2% skim milk powder

Elution buffer:

glycine (0.1 M; pH 2.2)

Staining solution for phagemid ELISA:

Na_2HPO_4 (0.2 M)	3.1 ml
Citric acid (0.2 M)	2.9 ml
H_2O	6 ml
o-Phenylenediamine	4.8 mg
H_2O_2 (30%)	1.5 µl

4.5. Bacterial strains

- *E. coli* WK6λmutS cells with and without the pSKAN libraries
  (supplied by MoBiTec)

Genotype *E. coli* WK6λmutS:
galE, strA, nalT, (lac-proAB), λ+, mutS::Tn10, F´ [lacIq, lacZ M15, proAB+];

The host strain WK6λmut S without phagemid (for re-infection) should be kept on minimal medium (M9) during cell-keeping in order to select for the F-episome, which carries proAB for the complementation of proline synthesis deficiency. The transposon Tn10 carries the Tc resistance gene. The original strain WK6mutS, which does not contain the λ lysogen, is described in reference 13.

- For the helper phage: *E. coli* WK6 or any *E. coli* F+-strain (not provided)
  M13 and derivatives require the presence of an F-episome since the here encoded sex pilus serves as phage receptor.

4.6. Antibodies

- Anti-pIII (g3p) antibody
  mouse monoclonal IgG, unlabeled; use 1:1000 for Western blots (see chapter 4.3.2. and reference 14).
- Anti-hPSTI antibody
  mouse monoclonal IgG, unlabeled, use 1:1000 for Western blots and ELISA. Recognizes soluble hPSTI as well as the hPSTI in the pIII-hPSTI fusion protein of pSKAN.

5. Literature

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6. Examples, Application

6.1. Positive control: selection and characterization of chymotrypsin-binding hPSTI-variants

The following application example describes the use of one of the supplied phagemid libraries for the selection of protein variants with altered binding specificity, namely novel chymotrypsin inhibitors; see also reference 12. **We highly recommend performing these experiments as positive controls.**

The phagemid pSKAN8, which was used for the construction of the pSKAN phagemid libraries HyA, HyB, and HyC, encodes an hPSTI-pIII fusion protein which is a specific inhibitor of the protease trypsin. pSKAN8 can be purchased separately, see chapter 7.

6.1.1. Methods

Preparation of phagemid particles

Phagemids were packaged from library HyB according to 4.1.2. using $3x10^{11}$ (Km^R cfu/ml) M13K07 helper phages. The final preparation contained $2x10^{12}$ Amp^R cfu/ml and $8x10^{10}$ Km^R cfu/ml (determined according to 4.1.3.).

Panning procedure

Coating (according to 4.2.1.1.)

One well of a 96-well microtiter plate (Nunc, Maxisorb) was filled with 100 µl of α-chymotrypsin (bovine; 100 µg/ml PBS) and incubated overnight at 4 °C. The next day the plate was washed twice with T-PBS.

Blocking

The chymotrypsin-coated well and an additional uncoated well, to be used as a negative control, was blocked according to 4.2.1.2.

Binding (according to 4.2.1.3.)

100 µl of phagemid preparations were pipetted in the coated and uncoated wells, both blocked, and incubated for 3 hours on a rocker at room temperature. For the first cycle the phagemid preparation mentioned above was diluted by a factor of 10 in blocking solution. For all of the successive cycles phages prepared from re-infected cells (see below) were diluted by a factor of 2. For exact titers see Table 2.

Washing

The washing procedure, according to 4.2.1.4., is summarized in Table 1. All washing steps were performed using a Titertek Mikroplate Washer S8/12 (Flow Laboratories GmbH, Meckenheim, Germany), superwash mode, 600 µl washing solution per well.

Table 1: Washing procedure

procedure	**round 1**	**round 2**	**round 3**
washing with T-PBS	2x	3x	5x
incubation with blocking solution	10 min.	10 min.	10 min.
washing with T-PBS	2x	3x	5x
incubation with blocking solution	5 min.	5 min.	5 min.
washing with T-PBS	2x	3x	5x
washing with H_2O	2x	2x	2x

Note: this washing procedure has been proven as very selective. In some cases a less stringent procedure may be necessary.

Elution (according to 4.2.1.5.)

Re-infection and packaging of phagemids from re-infected cells.

The eluted phages were used for re-infection of *E. coli* cells as described in 4.2.2. and plated separately.

Resulting bacterial lawns were resuspended and phagemid particles for the next round of panning were prepared according to 4.2.3.

6.1.2. Results

Enrichment of chymotrypsin-binding variants

The panning experiment was done for a total number of five cycles. After each round of panning the titers of kanamycin-resistant (Km^R; helper phage) and ampicillin-resistant (Amp^R; phagemid containing) phage particles were determined according to 4.1.3. for both input solutions and eluates (Table 2).

Table 2: An example of phage titers during an anti-chymotrypsin panning

panning target					
	round 1	round 2	round 3	round 4	round 5
input					
α-chymotrypsin					
Amp^R	$2x10^{11}$	$1x10^{11}$	$1x10^{11}$	$3x10^{11}$	$1.5x10^{11}$
Km^R	$8x10^9$	$3x10^{10}$	$7.5x10^9$	$4x10^9$	n.d.
none					
Amp^R	$2x10^{11}$	$4x10^{11}$	$3x10^{11}$	$5x10^{11}$	$1x10^{10}$
Km^R	$8x10^9$	$8x10^9$	$1x10^{10}$	$1x10^{10}$	n.d.
eluate					
α-chymotrypsin					
Amp^R	$2x10^4$	$5x10^4$	$4.5x10^6$	$2x10^7$	$2x10^7$
Km^R	n.d.	n.d.	$5.4x10^4$	$1x10^5$	$2x10^6$
none					
Amp^R	$2x10^5$	$5x10^4$	$4.3x10^4$	$7x10^4$	$4x10^4$
Km^R	n.d.	n.d.	$1.5x10^3$	$3x10^3$	$2x10^4$

Titers are given as ampicillin-resistant (Amp^R) or kanamycin-resistant (Km^R) colony forming units (cfu) per ml; n.d.: titer not determined.

Explanation

As can be seen from Table 2 the titer of input phages were roughly the same in all rounds of panning in both cases. In the case of chymotrypsin an increase in eluted phages can be definitively recognized for the first time after the third round ($4.5x10^6$ vs. $4.3x10^4$ Amp^R cfu/ml). This leads to a further, stable enrichment during round four and five ($2x10^7$ Amp^R cfu/ml). The background of unspecifically binding phages (ca. $5x10^4$) is represented by the negative control (without target).

Characterization of enriched variants

DNA Sequencing:
The enrichment of chymotrypsin-binding variants within the eluted phage population was verified by DNA sequencing of the hypervariable region of randomly selected single clones. (Normally, ten single clones were taken from the titration plates after round three to five and sequenced using primer # 2897.) Regarding the chymotrypsin-panning, only three different clones (Chy1, Chy4, and Chy5*) were found among six random clones from round three. An additional clone (Chy8*) appeared in round four. The predominant clone of round three was further enriched to 80% (8/10) and 100% (10/10) of the clones sequenced after round four and five. All of these clones show homology to each other as well as to known chymotrypsin inhibitors. Within the control an enrichment of single clones could not

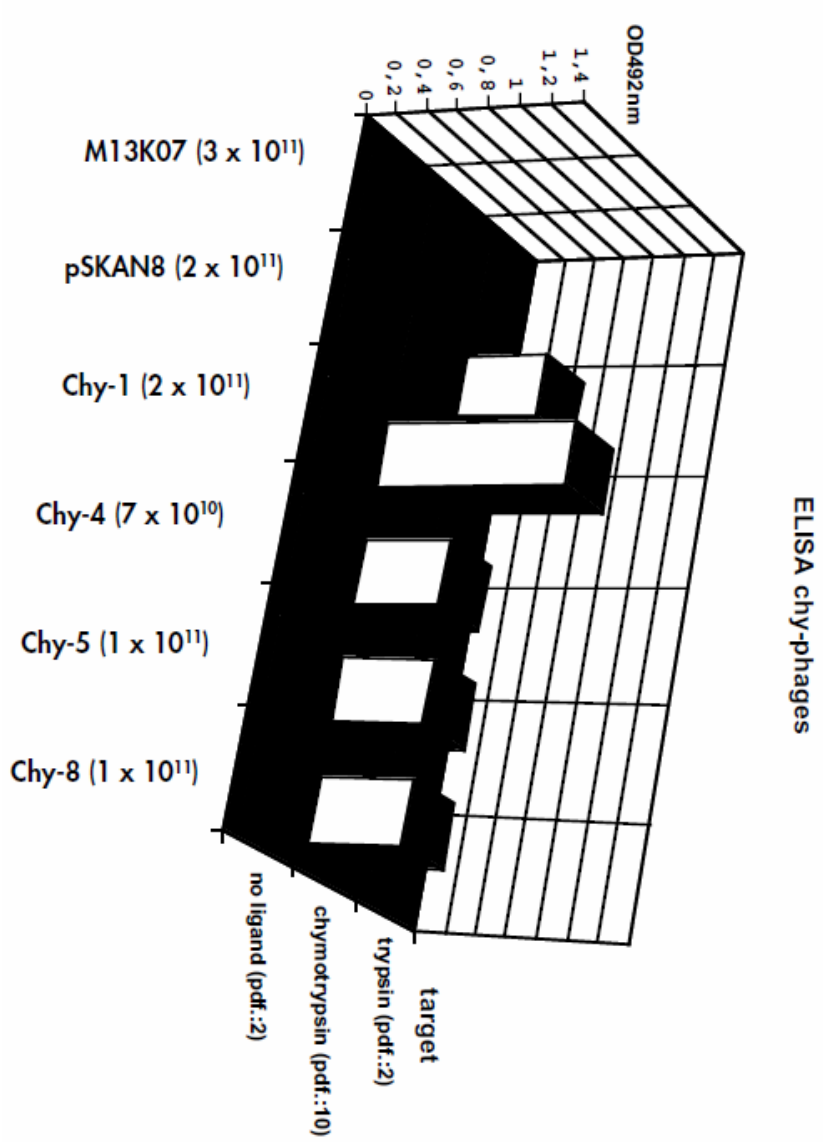
be detected during rounds three to four. All of the clones sequenced were clearly unrelated in sequence, and 30% to 60% of them contained stop codons.

*for sequences see Röttgen and Collins, (reference 12, page 20)

Phagemid ELISA
To verify that the isolated variants are able to bind their target, phagemid particles were used for a phagemid ELISA according to 4.3.3. For results, see Fig. 8, page 24.

Fig. 8 shows that all of the isolated clones indeed are chymotrypsin binders and show no affinity to closely related targets like trypsin or unspecific binding to the coated surface.

Fig. 8: ELISA of chymotrypsin binding phages
Each row was coated with the target proteins noted on the right and each column was incubated with phage particles noted below. M13K07: helper phage particles; pSKAN8: phages presenting the original hPSTI-variant; Chy-1 to Chy-8: phage clones isolated by anti-chymotrypsin panning. The phage titers of the original preparations are noted in brackets, and dilutions of these used per row are given as phage dilution factors (pdf).



Concluding remark

The described experiment was one of a series of panning experiments done at the GBF, demonstrating the usefulness of the pSKAN Phagemid Display System for affinity selection approaches.

The data presented here may serve as a clue for your own panning experiments. Corresponding experiments may also be included as positive controls.

6.2. Highly effective protease inhibitors from variants of human pancreatic secretory trypsin inhibitor (hPSTI): an assessment of 3-D structure-based protein design (reference 11, page 20)

Variants of hPSTI were generated in order to design extremely strong and specific human leukocyte elastase (HLE) inhibitors. HLE is involved in several disorders, like adult respiratory distress syndrome (ARDS) or septic shock. Thus, an HLE inhibitor could have potential therapeutic applications. Szardenings *et al.* (reference 11) were able to develop an excellent inhibitor with a minimum of two amino acid exchanges.

Protein purification:

0.05% Tween-80 and 5.5% perchloric acid were added subsequently to the supernatant of a typical 1L batch fermentation. The precipitate was removed by centrifugation after 30 minutes, and the solution re-adjusted to pH 8.0 by the addition of a saturated solution of Tris. Salts were removed by dialysis against water in a dialysis tubing (3 kDa cut-off). Further purification was carried out by filtration through a DEAE Sephacel ion exchanger. The flow-through and the first fractions obtained by washing with 20 mM Tris pH 8.0 were adjusted to pH 2.7 with citric acid and applied to an S-Sepharose fast flow column. The column was washed with 10 mM citric acid (pH 2.7), and most hPSTI variants could be obtained from a NaCl gradient (0-1 M NaCl) at about 0.6 M NaCl. Depending on batch and particular variant, the hPSTI already represented about 50% of the entire protein content of the peak fractions as judged by SDS PAGE. The protein was either purified further by an additional chromatography on a Mono-S column, or the fractions were dialyzed against water and lyophilized. The final purification was carried out by reverse-phase chromatography on a ProRP column running a gradient from 0 to 60% acetonitrile containing 0.1% TFA.

Note: for such an application, the production of soluble protein is essential, i.e., the protein has to be produced without the pIII-portion. Therefore, the DNA sequence of interest has to be cloned into another vector before protein production (see pMAMPF vectors described in reference 4).

7. Order Information, Shipping, and Storage

Order#	Product	Quantity
PSKAN	pSKAN libraries (3) in E.coli, incl. E.coli strain WK6 λmutS	1 mL each
shipped on dry ice; store at -80 °C		
PS1255	sequencing primer # 1255 (18 mer), lyophilized	500 pm
PS2897	sequencing primer # 2897 (19 mer), lyophilized	500 pm
PSKAN4	pMAMPF-3-PSTI4 vector, lyophilized DNA	5 µg
PSKAN8	pSKAN8 vector,lyophilized DNA	5 µg
shipped at RT; store at 4 °C		
PSKAN2	Anti-hPSTI - antibody (SPINK1), monoclonal mouse	100 µl
PSKAN3	Anti-PIII - antibody, monoclonal mouse	100 µl
shipped on blue ice; store at -20 °C		