Ph.D.™ Peptide Display Cloning System

M13KE is a simple M13 derivative in which cloning sites have been introduced at the 5′ end of gene III for display of short peptide sequences as N-terminal pIII fusions. Because this is a phage, rather than a phagemid vector, all 5 copies of pIII on the surface of each virion will be fused to the cloned peptide. Since displayed proteins longer than 20–30 amino acids have a deleterious effect on the infectivity function of pIII, this vector is suitable only for the display of short peptides. Additionally, the vector does not carry a plasmid replicon or antibiotic resistance, so it is necessary to propagate the vector as phage, rather than a plasmid (i.e., titer for plaques, not colonies). This simplifies the intermediate amplification steps during biopanning considerably, as it is not necessary to express antibiotic genes before plating, or to use helper phage during amplification. The steps necessary to clone a peptide library into M13KE are outlined below. To clone a single peptide sequence, reactions can be scaled down.

Materials

M13KE Extension Primer (16 µg)

M13KE gIII Cloning Vector (20 µg)

Design and Cloning of Synthetic Oligonucleotide Inserts

The following procedure is specific for the M13 cloning vector M13KE, but could easily be adapted for other phage (but NOT phagemid) vectors.

1. Design a library oligonucleotide following the convention in Figure 1. Bear in mind that the sequence VPFYSHS preceding the leader peptidase cleavage site is part of the pIII signal sequence and should not be altered. The first residue of the displayed peptide will immediately follow this sequence. For randomized positions, relative representations of each amino acid can be improved by limiting the third position of each codon to G or T (= A or C on the synthetic library oligonucleotide). We recommend including a short spacer sequence between the randomized segment and the first native pIII residue to improve target accessibility to the displayed peptide, e.g. the spacer Gly-Gly-Gly between the random peptide and the Ser-Ala-Glu (SAE) shown in Figure 1. This sequence can also include a protease cleavage site to allow elution of bound phage by protease digestion (50, 51). Pentavalency of the displayed peptide does not prevent protease release of bound phage (unpublished data). The oligonucleotide should be synthesized on a minimum of 0.2 µmol scale, gel-purified (48), and accurately quantitated by measuring the OD_{260} in a spectrophotometer (1 absorbance unit at 260 nm = 20 µg/ml of single stranded DNA).

2. Anneal 5 µg of the library oligonucleotide with 3 molar equivalents of the universal extension primer 5′-^{HO}CATGCCCGGGTACCTTTCTATTCTC-3′ (approximately 4 µg for a 90-nucleotide library oligonucleotide) in a total volume of 50 µl TE containing 100 mM NaCl. Heat to 95°C and cool slowly (15–30 minutes) to less than 37°C in a thermal cycler or water bath.

3. Extend the annealed duplex as follows (mix in the given order)

H_2O	119 µl
10X NEBuffer 2	20
annealed duplex	50
10 mM dNTPs	8
Klenow fragment (NEB #M0210) (5 Units/µl)	3
	200 µl

   Incubate at 37°C for 10 minutes, then 65°C for 15 minutes. Save 4 µl for later analysis (Step 5).

4. Digest the extended duplex as follows:

Extension reaction	196 µl
H_2O	154
10X NEBuffer 3	40
EagI (NEB #R0505) (10 Units/µl)	5
Acc65I (NEB #R0599) (10 Units/µl)	5
	400 µl

   Incubate at 37°C for 3–5 hours. Purify the DNA by phenol/chloroform extraction, chloroform extraction and ethanol precipitation. KpnI (NEB #R0142) may be substituted for Acc65I but, digestion should be carried out sequentially in NEBuffers 1 and 3, respectively.

5. Gel-purify the digested duplex on an 8% nondenaturing polyacrylamide gel (48), including as molecular weight markers, 4 µl of the undigested duplex as well as Low Molecular Weight DNA Ladder (NEB #N3233). Visualize by ethidium bromide staining, and excise the digested duplex from the gel, minimizing UV exposure time. Mince the excised band and elute the DNA by shaking overnight in several volumes of 100 mM sodium acetate, pH 4.5, 1 mM EDTA, 0.1% SDS at 37°C.

6. Briefly microfuge to separate the gel fragments from the elution buffer, and transfer the supernatant to a clean tube. Repeat wash to improve yield, if desired. Purify the DNA duplex from the supernatant by phenol/chloroform extraction, chloroform extraction and ethanol precipitation (48). Resuspend the pellet in 50 µl of TE and quantitate a small amount by PAGE or spectrophotometrically. One µg of purified insert is more than sufficient for a library of complexity 10^9.

7. For a high-complexity library, digest 10–20 µg of M13KE vector with 10 units/µg of EagI and Acc65I in a volume of 40 µl of 1X NEBuffer 3 per µg of DNA (total volume = 400–800 µl). Use KpnI in place of Acc65I if you used KpnI for insert digestion. Gel purify using standard methods (β-Agarase, QIAGEN®, etc). Quantitate a small amount of purified cut vector on an agarose gel or spectrophotometrically.

8. Optimize the ligation conditions. Suggested starting parameters per 20 µl ligation: 40 and 100 ng of cut vector; 3:1, 5:1 and 10:1 molar excess of cut duplex; 2 µl of 10X ligase buffer; and 200 units (= 3 Weiss units) of T4 DNA Ligase (NEB #M0202). Incubate overnight at 16°C.

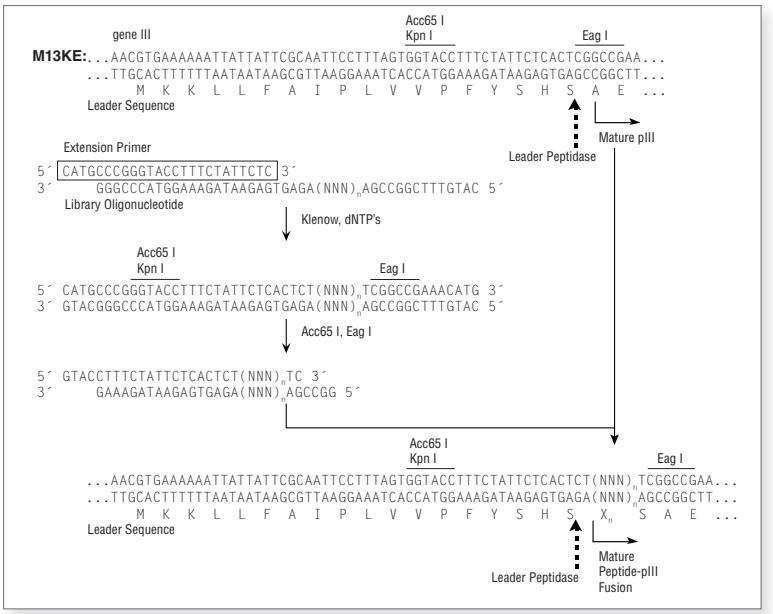
9. Heat-kill the test ligations at 65°C for 15 minutes, then electroporate 1 µl of each into 100 µl of electrocompetent ER2738 or other F^+ strain (for suggested electroporation parameters see step 8 in the previous section). Outgrowths are carried out in 1 ml of SOC medium for 30–45 minutes at 37°C with shaking.

10. Prepare 10, 100, and 1000-fold dilutions of the outgrowth in LB. Transfer 10 µl of each dilution to a test tube containing 3 ml of top agar + 200 µl of a mid-log culture of ER2738, equilibrated at 45°C. Vortex briefly and spread on LB/IPTG/Xgal plates. Incubate overnight at 37°C and count blue plaques the next day.

11. Scale up the protocol using the highest plaque/µg ratio to desired library complexity. For example, a library with a compexity of 1 x 10^9 clones would require a 5 µg ligation if the test ligations yield a ratio of 2 x 10^8 plaques/µg of vector. Use no more than 500 µl per individual ligation reaction; use multiple tubes if necessary.

FIGURE 1: **Construction of a peptide library in M13KE**

Schematic shows the sequence of the peptide cloning site as well as the strategy for designing and cloning a peptide library into M13KE. The sequence of the extension primer is outlined. N = A, G, C or T; X = any user defined or randomized amino acid.



SOC

Dissolve 20 g tryptone, 5 g yeast extract, 0.5 g NaCl in 950 ml deionized H_2O

Add 10 ml of 250 mM KCl and adjust pH to 7.0 with NaOH

Bring the solution to 1 liter and sterilize by autoclaving

Just before use, add $MgCl_2$ to 10 mM and glucose to 20 mM, from sterile stock solutions

PEG/NaCl

20% (w/v) polyethylene glycol-8000, 2.5 M NaCl

Autoclave, store at room temperature

TBS

50 mM Tris-HCl, pH 7.5, 150 mM NaCl

12. Purify the large-scale ligation by phenol/chloroform extraction, chloroform extraction and ethanol precipitation. Wash with 70% ethanol to desalt. Resuspend the DNA in low salt buffer and electroporate as described above. To reduce the likelihood of cells picking up more than one DNA sequence, the ligation should be divided and electroporated using as many cuvettes as convenient. For a 10–20 µg scale ligation we typically carry out 100 electroporations, using 3 µl of resuspended ligated DNA per 100 µl of electrocompetent cells.

13. Add 1 ml of SOC to each cuvette immediately after electroporation. For high-complexity libraries it may be convenient to pool the SOC outgrowths in groups of 5. Each outgrowth (or pool of 5) should be incubated for 30–45 minutes (no longer) before amplification. Titer several outgrowths or pools (as in Step 10) prior to amplification in order to obtain library complexity.

14. Amplify the electroporated cells by adding 20 ml of pooled SOC outgrowths to 1 liter of early-log cells (OD_{600} 0.01–0.05) in LB medium. Incubate with vigorous aeration (250 rpm) at 37°C for 4.5 to 5 hours. Centrifuge at 5000 g for 20 minutes at 4°C. Transfer the supernatant to a clean bottle and discard the cells.

15. Recover the phage from the supernatant by adding 1/6 volume of 20% PEG/2.5 M NaCl and incubating overnight at 4°C. Pellet the phage by centrifugation at 5000 g for 20 minutes at 4°C. Discard the supernatant.

16. Thoroughly resuspend the phage pellet in 100 ml of TBS by gently rocking over ~1–3 hour or overnight at 4°C. Remove residual cells by centrifugation at 5000 g for 10 minutes at 4°C.

17. Transfer the supernatant to a new tube and discard the pellet. Reprecipitate the phage by adding 1/6 volume of 20% PEG/2.5 M NaCl and incubating for 1 hour at 4°C. Centrifuge at 5000 g for 20 minutes and discard the supernatant.

18. Resuspend the final library in 10–40 ml of TBS by gentle rocking for 24–48 hours at 4°C. For long-term storage, add an equal volume of sterile glycerol, mix thoroughly and store at –20°C. The titer of the library should remain constant for several years at this temperature. Further amplification of the library is not recommended, as sequence biases may occur upon reamplification.

Ordering Information

PRODUCT	NEB #	SIZE
Ph.D. Peptide Display Cloning System	E8101S	20 µg
COMPANION PRODUCTS		
M13KE Phage	N0316S	0.04 ml
Ph.D.-12 Phage Display Peptide Library Kit	E8110S	10 panning experiments
Ph.D.-12 Phage Display Peptide Library Kit	E8100S	10 panning experiments
Ph.D.-12 Phage Display Peptide Library Kit	E8120S	10 panning experiments

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