Chapter 21

In Vitro Selection of Unnatural Cyclic Peptide Libraries via mRNA Display

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Abstract

The ribosomal synthesis of drug-like peptides containing unnatural amino acids is possible due to the broad substrate specificity of the ribosome. In this protocol, a reconstituted *Escherichia coli* ribosomal translation system (PURE) is adapted to incorporate unnatural amino acids into mRNA-displayed peptide libraries, which are used in in vitro selection.

Key words: Unnatural amino acids, Peptide library, mRNA display, In vitro selection, mRNA-peptide fusion, Ligand discovery, PURE system, Ribosomal translation

1. Introduction

Vast peptide libraries can now be reliably created and subjected to in vitro selection in order to find novel ligands for a wide variety of biomolecules and materials. The techniques for the creation of the most diverse peptide libraries involve the biochemical machinery of translation. As a result, until recently, the peptide libraries created using these methods contained linear peptides composed of natural amino acids. These peptides often suffer from poor biostability. The recent development of translation systems reconstituted entirely from purified components (1, 2) as well as an ever-expanding list of unnatural aminoacyl-tRNAs (3–5) have enabled the ribosomal synthesis of cyclic peptides (6–10) composed primarily of unnatural amino acids (11, 12). Cyclization and incorporation of unnatural building blocks are two common methods to make peptides more drug-like (13).

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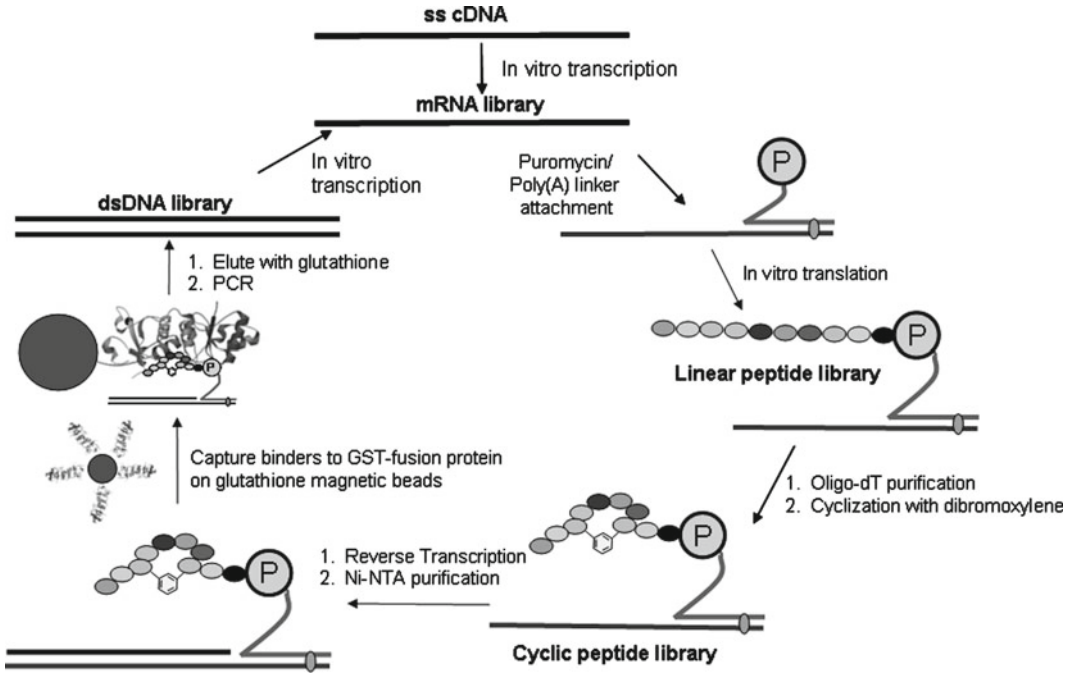

Fig. 1. In vitro selection cycle. Starting with a single-stranded cDNA, an mRNA library is prepared by in vitro transcription (Subheading 3.6). This mRNA is then photo-crosslinked to a puromycin-containing linker (Subheading 3.7). In vitro translation leads to a linear peptide (Subheading 3.8) which is purified on Oligo(dT)-Cellulose (Subheading 3.9). Cyclization is performed on the oligo(dT) column (Subheading 3.9). After reverse transcription and Ni-NTA purification (Subheading 3.10) the library of mRNA peptide fusions is incubated with the desired immobilized target (Subheading 3.10). Binders are eluted and the resulting mRNA-peptide fusions are amplified by PCR (Subheading 3.11).

The following section describes a protocol for the creation of cyclic, unnatural peptide libraries using mRNA display (14, 15). The protocol is summarized in Fig. 1. Although more sophisticated methods for attachment of unnatural amino acids onto tRNA have been developed, this protocol focuses on unnatural amino acids that have already been described as efficient substrates for in vitro translation (12). In a typical mRNA display selection, the initial troubleshooting may take 1–2 months. Once established, each round of selection will take ~3 days. In vitro selections typically converge on a small number of sequences within ten rounds. Thus the entire selection process may take 3–5 months of focused work. This may seem daunting at first, but when one considers that during this process over 10^{13} different unnatural, cyclic peptides have been created and sorted, the overall efficiency is quite remarkable.

Compared to a standard mRNA display experiment which utilizes cell extracts (16), this protocol requires the use of the PURE translation system. This system is commercially available from a number of sources and is suitable for mRNA display for peptides containing all natural amino acids. It is recommended that the user who is unfamiliar with mRNA display begins with this system

before investing the time in developing the customizable versions of the system which are required for unnatural amino acid mRNA display.

2. Materials

2.1. Urea PAGE Purification of Library DNA

1. Owl P10DS Dual Gel System (Thermo Scientific).
2. Elutrap Electroelution System (includes Elutrap chamber and BT1 and BT2 membranes) (Whatman). Store the membranes at 4°C (see Note 1).
3. 20 × 20 cm glass-backed TLC plate with fluorescent dye excitable at 254 nm.
4. UVP Compact Lamp, 254 nm, 4 W (UVP).
5. EC Apparatus electrophoresis power supply EC 600 or other power supply with constant watt capabilities.
6. Urea PAGE loading buffer: 8 M Urea, 2 mM Tris–HCl, pH 7.5, 20 mM EDTA, 0.25% bromophenol blue (w/v), 0.25% xylene cyanol.
7. SequaGel sequencing system (National Diagnostics).
8. Ammonium persulfate (APS): prepare a 10% solution (w/v) in ddH_2O freshly (see Note 2).
9. *N′N′N′N′*-tetramethylethlyenediamine (TEMED). Store at 4°C.
10. 5× TBE buffer: 445 mM Tris base, 445 mM boric acid, 10 mM EDTA (see Note 3).
11. 3 M NaOAc, pH 5.2. Store at –20°C.
12. 100% Ethanol and 70% ethanol. Store at –20°C.

2.2. PCR and TOPO Cloning

1. Taq DNA polymerase (various suppliers). Store at –20°C.
2. 10× ThermoPol PCR buffer (New England Biolabs) (see Note 4). Store at –20°C.
3. dNTP solution (various suppliers). Store at –20°C.
4. Agarose (various suppliers). Choose molecular biology grade.
5. 5× Agarose gel loading buffer with Orange G as the dye (0.25% Orange G (w/v), 30% glycerol, 6 mM EDTA) (see Note 5). Store at room temperature.
6. 100 bp DNA ladder (various supplies). Store at –20°C.
7. Ethidium bromide solution (10 mg/mL). Store at room temperature. Ethidium bromide is a mutagen. Wear gloves and avoid exposure. Dispose of ethidium bromide accordingly.

8. QIAquick Gel Extraction Kit (Qiagen). Store at room temperature.
9. TOPO TA Cloning Kit (Invitrogen). Store the components according to manufacturer's instructions.
10. LB-Agar plates with 50 μg/mL ampicillin. Store at 4°C.

2.3. Transcription and Purification of mRNA

1. The equipment necessary for Urea PAGE purification of nucleic acids (see Subheading 2.1).
2. T7 RNA polymerase (various suppliers). Store at −20°C.
3. 10× Transcription buffer (400 mM Tris–HCl, pH 7.8, 0.1% Triton X-100). Store at 4°C.
4. NTP solutions: 100 mM each NTP (Sigma-Aldrich), pH adjusted to 7–8 with NaOH (check pH with a pH paper). Sterile filter through 0.22 μm syringe filter and aliquot. Store at −80°C.
5. 300 mM spermidine. Store at −20°C.
6. 1 M $MgCl_2$. Store at room temperature.
7. 1 M DTT, freshly made (see Note 6).
8. 0.1 mg/mL Inorganic pyrophosphatase from *Escherichia coli* (Sigma-Aldrich) (see Note 7). Store at −20°C.
9. RNase inhibitor (various suppliers). Usually supplied as 40 U/μL. store at −20°C.
10. Solid urea.
11. 0.5 M EDTA, pH 8.0. Store at room temperature.
12. Turbo DNase (Applied Biosystems/Ambion). Store at −20°C.

2.4. Psoralen Photo-Crosslinking

1. 365 nm handheld UV lamp (UVP).
2. 96-Well round bottom crosslinking plate (Costar).
3. XL-PSO oligonucleotide: 5′-PsoC6-$(uagccggug)_2'_{\text{-OMe}}$-15xA-2xSpacer 9-ACC-Puro-3′ (see Note 8). Dissolve in ddH_2O to a final concentration of 125 μM. Aliquot and store at −20°C.
4. 1 M HEPES-KOH, pH 7.6. Store at room temperature.
5. 1 M KCl. Store at room temperature.
6. 25 mM spermidine. Store at −20°C.
7. 3 M KOAc, pH 5.5. Store at −4°C.

2.5. Translation

1. Econo-Pac column (Bio-Rad).
2. 50 mL Nalgene Oakridge centrifuge tubes (Thermo Fisher Scientific).
3. B-PER reagent (in phosphate buffer) (Thermo Scientific/Pierce). Store at room temperature.
4. Ni-NTA Agarose (Qiagen). Store at 4°C.

5. 100 mg/mL ampicillin and 25 mg/mL kanamycin stocks. Store at –20°C.
6. 1 M IPTG. Store at –20°C.
7. Slide-A-Lyzer dialysis cassettes (Thermo Scientific/Pierce) (see Note 9).
8. His compatible protease inhibitor set VII (Merck/EMD) (see Note 10). Store at –20°C.
9. Ni-NTA wash buffer (I): 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole. Add 5 mM 2-mercaptoethanol before use. Store at 4°C.
10. Ni-NTA elution buffer (I): 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole. Add 5 mM fresh 2-mercaptoethanol before use. Store at 4°C.
11. Enzyme dialysis buffer with glycerol: 50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 30% glycerol. Add 2-mercaptoethanol to 7 mM before use. Store at 4°C.
12. BeadBeater with a medium blender chamber and glass beads (BioSpec Products).
13. Ribosome buffer A: 10 mM Tris–HCl pH 7.5, 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 0.25 mM EDTA. Add 2-mercaptoethanol to 7 mM before use. Store at 4°C.
14. Ribosome buffer B: 10 mM Tris–HCl pH 7.5, 10 mM $Mg(OAc)_2$, 500 mM NH_4Cl. Add 2-mercaptoethanol to 7 mM before use. Store at 4°C.
15. Ribosome buffer B with 30% sucrose: 10 mM Tris–HCl pH 7.5, 10 mM $Mg(OAc)_2$, 500 mM NH_4Cl, 30% sucrose (w/v). Add 2-mercaptoethanol to 7 mM before use. Store at 4°C.
16. Ribosome buffer C: 10 mM Tris–HCl pH 7.5, 10 mM $Mg(OAc)_2$, 60 mM NH_4Cl, 0.5 mM EDTA. Add 2-mercaptoethanol to 3 mM before use. Store at 4°C.
17. Ultracentrifuge with Type Ti70.1 rotor (Beckman Coulter).
18. Quick-Seal ultracentrifuge tubes and tube rack (Beckman Coulter).
19. Standard 3× polymix buffer: in a 15-mL Falcon tube, add 4 mL ddH_2O and add final concentrations of 8 mM putrescine, 1 mM spermidine, 5 mM K_2HPO_4, 95 mM KCl, 5 mM NH_4Cl. Adjust pH to 7.70–7.80 with 600 mM HCl. Add $Mg(OAc)_2$ toa final concentration of 5 mM. In a second 15-mL Falcon tube, prepare 3 mL solution of 0.5 mM $CaCl_2$. Mix the two solutions well by pouring one tube of solution into another. Repeat this at least ten times. Add ddH_2O to a total volume of 9.75 mL. Filter the solution through 0.22 μm syringe filter and place at room temperature until use.

20. Creatine kinase: dissolve lyophilized creatine kinase (Roche Applied Science) in 10 mM Tris–HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl and adjust OD_{280} to 10. This solution should be stored at 4°C for not more than 1 week.
21. Creatine phosphate: dissolve creatine phosphate potassium salt (Merck/EMD) in ddH_2O to make 0.5 M solution. Store at −80°C.
22. Nucleoside 5′-diphosphate kinase from bovine liver (Sigma-Aldrich). Store at 4°C.
23. Myokinase from rabbit muscle (Sigma-Aldrich). Store at 4°C.
24. Putrescine dihydrochloride (Sigma-Aldrich) 2.4 M. Store at −20°C.
25. (6R,S)-5,10-formyl-5,6,7,8-tetrahydrofolic acid (Schircks Laboratory): dissolve at 2 mg/mL in 20 mM DTT and adjust pH to 7 with KOH. Store at −80°C.
26. 25 mM ATP and 25 mM GTP potassium salt solution: because sodium inhibits translation, the sodium salt form of ATP and GTP should be exchanged to potassium. Mix thoroughly 200 μL of 100 mM ATP or GTP from Subheading 2.3 with 20 μL 3.81 M KCl and 600 μL ethanol from −20°C. Centrifuge immediately at 16,000 × *g* for 5 min at 4°C. Wash the pellets with 70% ethanol twice and once with 100% ethanol. Aspirate all the remaining liquid and air-dry the pellets. Resuspend the pellets in 200 μL ddH_2O. Dilute the solutions to 1:5,000 and measure the OD_{260}. Calculate the concentration of ATP or GTP by using 13,700 OD_{260} of GTP = 1 M and 15,400 OD_{260} of ATP = 1 M. Make up a solution containing both 25 mM ATP and 25 mM GTP based on the calculations. Store the 100 μL aliquots at −80°C.
27. Deacylated *E. coli* total tRNA (Roche Applied Science): dissolve tRNA at 100 mg/mL in 1 M Tris–HCl (pH 9). Incubate at 37°C for 2 h. Dialyze overnight against 50 mM Tris–HCl (pH 9). Precipitate with 0.1 volume of KOAc, pH 5.5 and 3 volumes of ethanol. Wash the pellet with 70% ethanol. Air-dry at room temperature. Resuspend the tRNA in ddH_2O and adjust the concentration to 100 mg/mL. Store at −80°C in small aliquots (see Note 11).
28. 2.85 M KCl/1 M $MgCl_2$. Store at room temperature.
29. All amino acids and analogs are dissolved in ddH_2O at 1, 10, or 50 mM, and the pH was adjusted to 7.0–7.5 with 1 M KOH. Filter through 0.22 μm syringe filter and aliquot. Store at −20°C.
30. Isotopically labeled ^{35}S-Met [specific activity: >1,000 Ci (37.0 TBq)/mmol] (Perkin-Elmer). Upon receiving, thaw and store in 10 μL aliquots at −80°C.

2.6. Oligo(dT) Purification and Cyclization

1. Millipore UltraFree-MC centrifugal filter devices with Durapore membrane (Millipore).
2. Beckman Coulter scintillation counter.
3. Oligo(dT)-Cellulose Type 7 (GE Healthcare). Store at –20°C.
4. 5 mg/mL glycogen (Applied Biosystems/Ambion). Store at –20°C.
5. Oligo(dT) binding buffer: 20 mM Tris–HCl, pH 7.8, 10 mM EDTA, 1 M NaCl, 0.2% Triton X-100. Add 0.5 mM fresh TCEP before use (see Note 12). Store at 4°C.
6. Oligo(dT) wash buffer: 20 mM Tris–HCl, pH 7.8, 0.3 M NaCl, 0.1% Triton X-100. Add fresh TCEP to 0.5 mM before use. Store at 4°C.
7. Cyclization buffer: 20 mM Tris–HCl, pH 7.8, 0.66 M NaCl, 3 mM α,α′-dibromo-*m*-xylene (Sigma-Aldrich/Fluka), 33% acetonitrile (v/v), 0.5 mM TCEP (fresh). To make the cyclization buffer: prepare a stock solution of 30 mM Tris–HCl, pH 7.8 and 1 M NaCl. Store at room temperature. Before use, prepare a 10 mM solution of dibromo-*m*-xylene in acetonitrile. Mix 2.64 mL of 30 mM Tris–HCl, pH 7.8 and 1 M NaCl with 1.32 mL of 10 mM dibromo-*m*-xylene in acetonitrile. Discard any unused dibromo-*m*-xylene.

2.7. Reverse Transcription and Ni-NTA Purification

1. Superscript III First Strand Synthesis Kit (Invitrogen). Store at –20°C.
2. Denaturing Ni-NTA binding buffer: 100 mM NaH_2PO_4, 10 mM Tris–HCl, 6 M guanidinium hydrochloride, 0.2% Triton X-100. Adjust pH to 8.0. Add 2-mercaptoethanol to 5 mM before use. Store at 4°C.
3. Ni-NTA wash buffer (II): 100 mM NaH_2PO_4, 300 mM NaCl, 0.2% Triton X-100. Adjust pH to 8.0. Add 2-mercaptoethanol to 5 mM right before use. Store at 4°C.
4. Ni-NTA elution buffer (II): 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.2% Triton X-100. Adjust pH to 8.0. Add 2-mercaptoethanol to 5 mM before use. Store at 4°C.

2.8. Selection

1. Pierce magnetic glutathione beads (Thermo Fisher Scientific).
2. Magnetic separation stand (Invitrogen).
3. Fixed speed Labquake tube rotators (Thermo Fisher Scientific).
4. GST beads wash buffer: 125 mM Tris–HCl, pH 8.0, 150 mM NaCl. Store at 4°C.
5. Selection buffer: 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 4 mM $MgCl_2$, 0.25% Triton X-100. Store at 4°C.

6. GSH elution buffer: 250 mM Tris–HCl, pH 9.0, 500 mM NaCl, 100 mM reduced glutathione, 1% Triton X-100. Prepare fresh and filter through 0.22 μm syringe filter.
7. Highly purified protein selection target (see Note 13).
8. 10 mg/mL BSA (New England Biolabs). Store at –20°C.
9. Dialysis buffer: 0.1% Triton X-100 in ddH_2O. Pre-cool at 4°C.

2.9. PCR Amplification of Selected Fusions

1. PCR primers for mRNA-peptide fusions. The 5′ primer sequence is: TAATACGACTCACTATAGGGTTAACTTT AGTAAGGAGG. The 3′ primer sequence is: CTAGCTACC TATAGCCGGTGGTGATG.
2. Phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich). Store at 4°C.

3. Methods

Prior to beginning an in vitro selection there are several factors that need to be considered.

3.1. Target

For the entire selection ~1 mg of a particular protein target is required. We typically overexpress proteins in bacteria as GST fusions.

3.2. Unnatural Building Blocks

A random peptide library composed of all natural amino acids has been successfully used in mRNA display and has been described in a previous protocol (16). However, there are a wide variety of unnatural amino acid analogs that are efficient substrates for translation (12). Many of these can simply be substituted for their natural amino acid counterparts. For this example, we have replaced six of the natural amino acids with unnatural counterparts (Fig. 2), but we have in other cases replaced as many as 12 natural amino

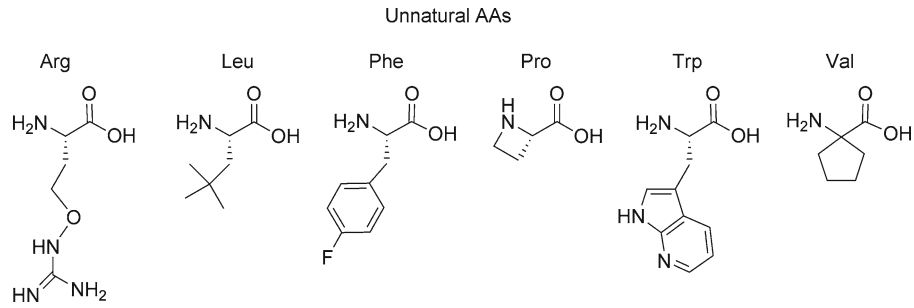

Fig. 2. Unnatural amino acids used in this peptide library. Written above each unnatural amino acid is the natural amino acid it is replacing in the library.

acids. One consideration is that once the in vitro selection has been completed, it is typically necessary to synthesize the unnatural peptides on the solid phase, so it is wise to consider the availability of the corresponding Fmoc unnatural amino acids.

3.3. Library Creation

The flexibility of mRNA display allows the synthesis of linear or cyclized peptides. This library includes fixed cysteines at both termini for cyclization. However, it is possible to encode a mixture of linear and cyclic peptides by omitting the second cysteine. In this case, the cyclization size is not fixed and the peptides can be linear (see Note 14) when no cysteine is present in the library region. One can encode for an enhanced percentage of cysteine codons in the random region by using the codon NNB (B = T, G, or C) (see Note 15).

The DNA sequence of the random library is: TAATACGACTCACTATAGGGTTAACTTTAGTAAGGAGG ACAGCTAAATGTGCNNSNNSNNSNNSNNSNNSNN SNNSNNSNNSNNSNNSTGCGGCTCCGGTAGCTTAGG CCACCATCACCATCACCACCGGCTATAGGTAGCTAG.

The detailed sequence composition of the library is as follows:

1. TAATACGACTCACTATA: T7 promoter, followed by GGG with the first "G" as the transcription start.
2. TTAACTTTAG: Epsilon enhancer (1).
3. TAAGGAGG: Shine Dalgarno sequence, also known as the ribosome binding site.
4. ACAGCTAA: the Spacer between ribosome binding site and the start codon, with "AA" at end (17).
5. ATGTGC$(NNS)_{12}$TGC: translation start codon for methionine (ATG) and the codon for the fixed cysteine (TGC), followed by 12 random NNS codons. N denotes A, T, C, or G, and S denotes G, or C. The translated peptides have the sequence of MCX_{12}CGSGSLGHis_6, where X can be one of the natural or unnatural amino acids.
6. GGCTCCGGTAGCTTAGGC: codons for GlySerGlySer-LeuGly, the flexible linker with two out-of-frame stop codons.
7. CACCATCACCATCAC: codons for His5 tag. The sixth His of His6 tag is in the following sequence.
8. CACCGGCTAT: hybridization region for the Psoralen crosslinker (XL-PSO oligonucleotide, see below). The CAC encodes the sixth His of the intact His6 tag.
9. AGGTAGCTAG: 3′-UTR to allow those non-crosslinked peptides to release at the in-frame TAG stop codons.

To construct the mRNA library to be used in the PURE system, DNA oligonucleotides with the antisense sequence of the above library DNA are synthesized and purified by Urea PAGE. Two

hundred-nanomole scale synthesis with good quality is usually enough, although we prefer 1 μmol scale.

The antisense sequence of the library DNA is named as CX12REV: CTAGCTACCTATAGCCGGTGGTGATGGTGAT GGTGGCCTAAGCTACCGGAGCCGCASNNSNNSNNS NNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNN SNNGCACATTTAGCTGTCCTCCTTACTAAAGTTAACCC TATAGTGAGTCGTATTA.

A DNA oligonucleotide with the T7 promoter (TAATAC GACTCACTATA) is annealed to the 3′ region of the antisense library DNA. The formation of the DNA duplex in the T7 promoter is necessary to initiate the transcription reaction and library mRNA is then transcribed.

The sequence of the XL-PSO oligonucleotide (18) is:
5′-PsoC6-$(uagccggug)_{2'\text{-}OMe}$-15xA-2xSpacer9-ACC-Puro-3′.

The 5′ end of the XL-PSO oligonucleotide is a psoralen moiety which is attached through a C6 alkyl chain to the 5′-phosphate of the linker. The linker hybridization sequence is prepared from 2′-*O*-methyl-RNA phosphoramidites to enhance the pairing stability between mRNA and the XL-PSO oligonucleotide. Flexible spacers are used to tether 5′-dAdCdC-puromycin to the 3′-end of a stretch of Poly(A). The Poly(A) is designed for purification of the cross-linked mRNA by oligo(dT)-cellulose.

3.4. Urea PAGE Purification of Library DNA

3.4.1. Urea PAGE

1. Set up a large 8% SequaGel (20 cm × 20 cm) on an Owl P10DS Dual Gel System. Use the prep comb to make a single large well. Use 1× TBE in both chambers.
2. Pre-run the gel for 20–30 min by using constant power at 25 W supplied by EC Apparatus electrophoresis power supply EC 600.
3. Flush out the urea from well before loading samples.
4. Dissolve the synthesized DNA library in ddH_2O. Take out one-fifth of the DNA solution and mix well with an equal volume of Urea PAGE loading buffer. Load the DNA sample slowly into the well and assemble the lid.
5. Run the gel at constant power of 25 W until the bromophenol blue reaches the bottom of the gel. This may take 90 min.
6. Turn off the power supply and disassemble the gel system. Transfer the gel from the glass plates to Saran wrap on both sides. Visualize the DNA band by UV shadowing and cut it out with a fresh razor blade.

3.4.2. Electroelution

1. Assemble the Whatman Elutrap electroelution System.
2. Put the gel slice into the chamber, and mush it into small pieces with a pipette tip. Fill the chamber with 0.5× TBE buffer until the gel is covered.

3. Run the electroelution for 2 h at 300 V on a Bio-Rad PowerPac basic power supply.
4. At the end of running, switch the electrodes and run it backwards for 1 min at 300 V.
5. Remove the solution from the small chamber between the two membranes with a plastic dropper, and expel into a 1.6 mL tube.
6. Measure the volume of eluent and add 0.1 volume of 3 M NaOAc and 3 volumes of 100% ethanol. Mix well and freeze the tube at –20°C for 30 min.
7. Spin the tube at 16,000 × *g* for 20 min at 4°C.
8. Discard the supernatant and add 500 μL of 70% ethanol.
9. Spin again at 16,000 × *g* for 1 min.
10. Discard the supernatant and air-dry the pellet for 5–10 min at room temperature.
11. Dissolve the DNA in ddH_2O and measure the absorbance at 260_{nm} on a spectrophotometer. Calculate the concentration by using the online software Oligonucleotide Properties Calculator (http://www.unc.edu/~cail/biotool/oligo/index.htmL). Adjust the concentration to 50 μM and store the DNA at –20°C.

3.5. PCR and TOPO Cloning

3.5.1. PCR Amplification of Library DNA

1. Obtain a 5′ and 3′ primer for amplification of the library DNA. The 5′ primer sequence is: TAATACGACTCACTATAGG. The 3′ primer sequence is: CTAGCTACCTATAGCCGGTGG.
2. Resuspend the primers in ddH_2O and adjust the concentration to 10 μM.
3. Prepare a series of tenfold diluted stock of the library DNA from Subheading 3.4. The concentrations of the diluted stock DNA are 5, 0.5, and 0.05 μM.
4. Prepare a series of PCR reaction mixtures in 0.2 mL PCR tubes containing 5, 0.5, and 0.05 pmol of the library DNA (1 μL of the diluted stock DNA), 50 pmol of each primer (5 μL of 10 μM primer), 2 μL of 10 mM dNTP mix, 10 μL of 10× ThermoPol buffer, and add ddH_2O to 100 μL.
5. Place the tubes on a PCR machine and denature the template at 94°C for 2 min. Take the tubes out and sit on ice for 2 min. Add 1 μL Taq DNA polymerase (5 U/μL) to each tube. Program the PCR machine to run 30 cycles of PCR of 94°C for 15 s, 60°C for 30 s, and 72°C for 45 s and start the PCR.
6. While the reactions are cycling, prepare a 2% Agarose gel with 1× TBE. Add 2 μL ethidium bromide solution (10 mg/mL) per 50 mL gel.

7. Monitor the PCR cycling. When the cycles have reached number 10, pause the PCR cycling at 2 s before the end of the elongation step (72°C, 45 s total). Take 4 μL out of each PCR reaction and pipette into 4 μL 5× Agarose gel loading buffer with Orange G as the dye which was previously aliquoted into a 96-well plate on ice. Do this for cycles 12, 14, 16, etc., until the end of PCR.
8. Run the samples on the Agarose gel together with 5 μL 100 bp DNA ladder in an adjacent lane.
9. Observe the PCR product on a UV transilluminator.
10. Based upon the results of the gel, determine when the PCR product has reached a plateau in concentration. Note the number of cycles.
11. Repeat the PCR with the condition (dilution of library DNA and cycles of PCR) from above and proceed to TOPO TA cloning.

3.5.2. TOPO Cloning and Sequencing

1. Follow the instructions of the TOPO TA Cloning Kit from Invitrogen and ligate the PCR product directly with the TOPO TA cloning vector.
2. Transform the ligation mixture into Top 10 competent cells and plate onto LB plates with 50 μg/mL ampicillin.
3. Incubate the plate overnight at 37°C in an incubator chamber.
4. Sequence at least 20 clones to ensure that the library was synthesized as designed and there is no strong bias in the nucleotide composition. If there is a bias, consider resynthesizing the library DNA.

3.6. Transcription and Purification of mRNA

1. Set up the transcription reaction by combining the following reagents: 100 μL of 10× Transcription buffer, 8.33 μL 300 mM spermidine, 25 μL 1 M $MgCl_2$, 10 μL 1 M DTT, 50 μL each of the 100 mM NTPs, 40 μL extra 100 mM GTP, 5 μL RNase inhibitor (40 U/μL), 10 μL Inorganic pyrophosphatase, 50 μL T7 RNA polymerase (20 U/μL), and library DNA (add volume sufficient for 5–50 nM final concentration). Add ddH_2O to 1 mL and mix well.
2. Incubate overnight at 37°C in an incubator chamber.
3. DNase treatment: remove the transcription reaction from the incubator and add 50 μL Turbo DNase. Incubate for 15 min at 37°C. After incubation, add 435 mg solid urea so that the final concentration of urea is 8 M. Add 100 μL 0.5 M EDTA and 20 μL Urea PAGE loading buffer and mix well.
4. Heat the mixture at 90°C for 5 min and place on ice until loading.

5. Purify the mRNA as described in Subheading 3.6. Use 3 M KOAc, pH 5.5 and 100% ethanol to precipitate the mRNA after electroelution (see Note 16). Wash the pellet twice with 70% ethanol. Air-dry the pellet. Dissolve the mRNA in ddH_2O at a final concentration of 50 μM and store at −20°C.

3.7. Psoralen Photo-Crosslinking

1. In a 1.5 mL tube, add the following reagents: 8 μL 1 M HEPES-KOH, pH 7.6, 40 μL 1 M KCl, 16 μL 25 mM spermidine, 0.8 μL 0.5 M EDTA, 24 μL of 50 μM mRNA, 24 μL of 125 μM XL-PSO oligonucleotide, and 287 μL ddH_2O. Mix well and spin briefly to collect the liquid. Aliquot into four 0.2 mL PCR tubes with 100 μL in each tube.
2. Place the PCR tubes in a PCR machine, heat to 70°C for 5 min, then cool to 25°C over 5 min (0.1°C/s).
3. After this, transfer the mixtures to a crosslink plate, 100 μL per well.
4. Place the plate on a stable surface in a cold room. Put a 365-nm handheld UV lamp on top of the plate, making sure the window of the UV lamp is directly above the wells with crosslinking mixtures.
5. Turn on the UV lamp and irradiate the plate for 20 min at 4°C (see Note 17).
6. Transfer the mixture into a 2-mL tube and precipitate the mRNA with 3 volumes of ethanol and 0.1 volume of 3 M KOAc. Wash the pellet twice with 70% ethanol. Air-dry the pellet at room temperature.

3.8. Translation

3.8.1. Expression and Purification of AARS

1. Inoculate a 30-mL solution of LB media with appropriate antibiotics, grow overnight at 37°C, shaking at 200–250 rpm in a 250-mL flask.
2. Inoculate 500 mL of LB media with antibiotics with 25 mL of the starter culture from above (5% inoculation volume), and shake at 250 rpm at 37°C in a 2 L flask.
3. Grow until the OD_{600} of 0.6 is reached (60–120 min).
4. Add 50 μL 1 M IPTG to a final concentration of 0.1 mM.
5. Grow for another 4–5 h at 37°C.
6. Harvest the cells by centrifugation at 4,000 × *g* for 25 min 4°C. The cell pellets can be stored at −20°C.
7. Take frozen cells from the freezer and resuspend in 25 mL B-PER phosphate buffer along with 100 μL His compatible protease inhibitor cocktail (Merck/EMD).
8. Transfer to a 50 mL Nalgene Oakridge centrifuge tube.
9. Shake gently for 15 min at room temperature on a rocking platform shaker.

10. Centrifuge the lysate at 16,000 × *g* for 30 min at 4°C to pellet debris. Transfer the supernatant to a 50 mL Falcon tube.
11. Add 3 mL Ni-NTA Agarose slurry (mix well before using) to the lysate and shake in a rocking platform shaker at 4°C for 60 min.
12. Load all the solutions onto a Bio-Rad Econo-Pac column. Remove bottom cap and allow to drain by gravity. Wash the Falcon tube with the flow through to ensure all the Ni-NTA Agarose is transferred into the column.
13. Wash with 2 × 15 mL Ni-NTA wash buffer (I).
14. Elute with 6 mL Ni-NTA elution buffer (I).
15. Dialyze the eluents in a 3–12 mL volume Slide-A-Lyzer dialysis cassette overnight at 4°C against 500 mL enzyme dialysis buffer with glycerol. Change the dialysis buffer next morning and continue the dialysis for another 4–5 h.
16. Take out the protein solutions from the Slide-A-Lyzer with a needle and transfer into a 15-mL Falcon tube. Use the dialysis buffer as the blank and measure the OD_{280} on a spectrophotometer. Calculate the protein concentration according to each protein's calculated extinction coefficient. Aliquot into small amounts and flash freeze at −80°C (see Note 18).

3.8.2. Purification of Escherichia coli Ribosomes

Ribosomes are prepared at 4°C from *E. coli* strain A19 (19, 20).

1. Prepare a 150-mL LB media in a 500-mL flask and 4 × 2,000 mL of LB media in 4 × 8 L flasks. Adjust the pH to 7 if necessary. Autoclave for 20 min at 121°C. Cool down to room temperature.
2. Inoculate the 150-mL LB from A19 glycerol stock for overnight starter culture.
3. Inoculate 4 × 2,000 mL LB with 30 mL starter culture and shake at 250 rpm and 37°C.
4. Check the OD_{600} and stop the growth when OD_{600} = 0.6–0.8, about 2.5 h.
5. Pour the culture into 4 × 2.25 L centrifuge bottles and cool down on ice.
6. Pellet the cells at 4,000 × *g* for 30 min at 4°C.
7. Resuspend and combine the cells with a total of 600 mL ribosome buffer A in a 1 L bottle and spin at 4,000 × *g* for 30 min at 4°C.
8. Freeze the bottle with bacterial pellet at −20°C.
9. Resuspend the pellet in 14 mL of ribosome buffer A and transfer to the medium blender chamber of the BeadBeater. Rinse the centrifuge bottle with 3 mL ribosome buffer A and transfer to the chamber. Repeat the rinse once.

10. Add pre-cooled glass beads to fill the blender chamber. Assemble the BeadBeater according to manufacturer's instructions.
11. Induce cell lysis with 6 × 20 s pulses. Allow the chamber to cool down for 40 s between pulses.
12. Transfer the glass beads-lysate mixture into a centrifuge tube. Use 5 mL ribosome buffer A to rinse the blender chamber and transfer to the centrifuge tube. Repeat the rinse once.
13. Centrifuge at 16,000 × *g* for 15 min at 4°C.
14. Carefully transfer the supernatant into a clean 50 mL Falcon tube and keep on ice. Centrifuge again if necessary.
15. Distribute 15 mL of cold ribosome buffer B with 30% sucrose into each of four ultracentrifuge tubes on ice.
16. Carefully layer 7.5 mL of the cleared lysate onto the sucrose in the four tubes.
17. Seal all the tubes by using the Quick-Seal Tube Rack.
18. Spin at 300,000 × *g* for 2.5 h in Type Ti70.1 rotor at 4°C.
19. Cut off the top part of the tubes and discard the supernatant. Wash the pellet with ribosome buffer A, taking care to remove the loosely packed brown flocculent material on top of the clear ribosome pellet.
20. Add 1 mL ribosome buffer B and a magnetic stir bar to each tube. Place the tubes in beakers containing half-full ice water. Resuspend the ribosome pellet with gentle stirring. Make sure no water gets into the tubes.
21. Combine the ribosomes and repeat the ultracentrifugation once.
22. Repeat steps 19–20 (see Note 19), use 2 mL buffer C to resuspend the ribosome pellet.
23. Determine the concentration based on $OD_{260} = 1{,}000$ being equal to 23 nmol of ribosomes. Aliquot the ribosomes in 20 μL portions on ice and flash freeze in liquid nitrogen (see Note 20). Store at −80°C.

3.8.3. Translation

1. Prepare a standard 3× polymix buffer.
2. Place a 15-mL falcon tube on ice. Add 3.33 mL of 3× polymix buffer supplemented with 1 mM DTT, 2 mM ATP, 2 mM GTP, 10 mM Creatine phosphate, and 30 μM (6R,S)-5,10-formyl-5,6,7,8-tetrahydrofolic acid. Add final concentrations of 4 μg/mL creatine kinase, 3 μg/mL myokinase, 1.1 μg/mL nucleotide diphosphate kinase, 1 μg/mL inorganic pyrophosphatase, 0.2 μM MTF, 1.0 μM IF1, 0.3 μM IF2, 0.7 μM IF3, 3.2 μM EF-Tu, 0.6 μM EF-Ts, 0.5 μM EF-G, 0.3 μM RF1, 0.4 μM RF3, 0.1 μM RRF, 0.5 μM ribosomes, and 0.05 OD_{260} U/L total tRNA. In addition, the reaction contains

natural amino acids (200 μM each), unnatural amino acids (400 μM to 6.6 mM) (see Note 21), and AARSs (0.1 μM MetRS, 0.3 μM LeuRS, 0.6 μM GluRS, 0.2 μM ProRS, 1.0 μM GlnRS, 1.0 μM HisRS, 0.25 μM PheRS A294G, 1.5 μM TrpRS, 0.2 μM SerRS, 0.2 μM IleRS, 0.4 μM ThrRS, 0.6 μM AsnRS, 0.6 μM AspRS, 0.5 μM TyrRS, 0.5 μM LysRS, 0.4 μM ArgRS, 0.2 μM ValRS, 0.2 μM AlaRS, 0.5 μM CysRS, and 0.06 μM GlyRS). 0.2 μM of ^{35}S-Met is added to isotopically label the peptides.

3. Start translation by addition of photo-crosslinked mRNA (see Subheading 3.7) to 1.0 μM followed by incubation for 1 h at 37°C.
4. At the end of incubation, add KCl and $MgCl_2$ to a final concentration of 550 and 50 mM, respectively (see Note 22). Mix by inverting the tube several times.
5. Incubate the tube at room temperature for 15 min.
6. Transfer the tube to a −20°C freezer and incubate overnight.

3.9. Oligo(dT) Purification and Cyclization

1. Take six 20 mL Bio-Rad Econo-Pac columns and rinse with ddH_2O.
2. Weigh 1 g of Oligo(dT)-cellulose in a 50 mL Falcon tube. Add 30 mL ddH_2O to swell the cellulose.
3. Transfer 5 mL Oligo(dT)-cellulose into each of six Econo-Pac column.
4. Allow the water flow through.
5. Add 10 mL of oligo(dT) binding buffer to the columns and drain. Repeat once.
6. Cap the bottom of the columns with a yellow end cap.
7. Take the translation reaction out of the −20°C freezer and vortex to resuspend any precipitates that might have formed. Remove 5 μL for scintillation counting to determine the total radioactivity of ^{35}S-Met added to the translation reaction.
8. Equally transfer the rest of the translation reaction to the columns, 2.6 mL for each column.
9. Rinse the translation reaction tube three times with 5 mL oligo(dT) Binding buffer and transfer to one of the columns.
10. Add more binding buffer to the columns so that each has a final volume of 20 mL. Cap the columns tightly on both ends.
11. Place the columns on a rocking platform shaker in 4°C cold room and shake for 30 min.
12. Place the columns on a column rack and carefully remove the bottom end caps. Drain the binding buffer from the columns into a disposable tube. Discard the liquid radioactive waste accordingly.

13. Add 25 mL oligo(dT) wash buffer and allow to drain. Repeat this once.
14. While the columns are draining, prepare the cyclization buffer.
15. Replace bottom end caps tightly to the columns. Add 6 mL of cyclization buffer to each column.
16. Shake the columns at room temperature for 30 min (see Note 23).
17. Drain the cyclization buffer, and wash the columns twice with 20 mL wash buffer. The first wash should contain 5 mM 2-mercaptoethanol and second wash should have 0.5 mM TCEP as the reducing agent (see Note 24).
18. After the columns have completely drained, elute the columns eight times with 1 mL ddH_2O with 0.5 mM TCEP.
19. Take 1 μL from each eluent and count in a scintillation counter.
20. Combine fractions with significant radioactivity and transfer into 6×50 mL Nalgene Oakridge centrifuge tubes and add 0.8 mL 3 M KOAc, pH 5.5, 32 mL 100% ethanol and 0.8 mL 5 mg/mL glycogen.
21. Freeze the tube in the −20°C freezer for 30 min.
22. Centrifuge at 16,000×*g* for 20 min at 4°C.
23. Discard the supernatant (see Note 25).
24. Resuspend the pellet in each tube with 500 μL 70% ice cold ethanol and transfer to a new microcentrifuge tube. Repeat twice. Combine all the washes in the microcentrifuge tube.
25. Centrifuge for 2 min at maximum speed on a bench-top centrifuge.
26. Discard the supernatant accordingly (see Note 25).
27. Wash the pellet with 500 μL 70% ethanol and centrifuge. Discard the supernatant accordingly. Spin again and completely remove residual liquid with a pipette and air-dry for 10 min at room temperature.
28. Calculate the pmol amount of the mRNA-peptide fusion based on the scintillation counts. Re-dissolve the pellet in ddH_2O so that the final concentration of the fusion is 100 nM (0.1 pmol/μL) (see Note 26).
29. If necessary, use centrifugal filter device to filter out residual Oligo(dT)-cellulose in the fusion solution.

3.10. Reverse Transcription and Ni-NTA Purification

1. In a 2-mL microcentrifuge tube, add the mRNA-peptide fusion from Subheading 3.9 and 0.5 mM dNTPs, 0.5 μM RT-primer TTTTTTTTTTTTTTTGTGATGGTGATGGTGGCC TAAGC. Put the tube in a heating block and incubate for 5 min at 65°C. Immediately place the tube on ice and incubate for at least 1 min (see Note 27).

2. Add reagents to a final concentration of 5 mM $MgCl_2$, 1 mM DTT, 2 U/μL RNaseOUT, 5 U/μL Superscript III in a total volume of 2 mL (see Note 28). Mix well.
3. Incubate for 30 min at 55°C for elongation, then 15 min at 70°C to inactivate the Superscript III (see Note 29).
4. Transfer 1 mL Ni-NTA Agarose to an Econo-Pac column (see Note 30). Add 4 mL Ni-NTA denaturing binding buffer and drain the column. Cap the bottom end of the column tightly.
5. Transfer the RT reaction to the column. Rinse the tube with 1 mL Ni-NTA denaturing binding buffer and transfer to the column. Repeat the rinse two more times.
6. Add a total of 10 mL Ni-NTA denaturing binding buffer to the column.
7. Shake the column on a rocking platform shaker for 1 h at 4°C.
8. Drain the column. Use a 15-mL Falcon tube to collect the flow through.
9. Wash the column with 10 mL Ni-NTA wash buffer (II). Repeat the wash twice. Each time collect the wash in a new 15 mL falcon tube.
10. Elute six times with portions of 0.5 mL Ni-NTA elution buffer (II). Collect eluents in 1.5 mL microcentrifuge tubes.
11. For each elution, let the elution buffer sit on the beads for 5 min by capping the bottom end of the column.
12. Take 100 μL from the flow through and three washes, and 5 μL of each eluent and count in the scintillation counter.
13. Combine all eluents with high counts, and dialyze against 1 L pre-cooled selection buffer in a cold room overnight.
14. Precipitate the fusion with ethanol and NaOAc (see Note 31). Redissolve in 1 mL selection buffer. Place on ice before proceeding to selection.

3.11. Selection

1. Take two 1.5 mL tubes and add 200 μL of Pierce Magnetic Glutathione beads to each tube. Label one tube "GST" for negative selection and the other "GST fusion" for positive selection.
2. Add 1 mL GST Beads Wash buffer to each tube and mix the beads by tapping the tube gently. Place the tubes on the magnetic stand. Use a pipette to remove the supernatant while holding the magnetic stand. Be careful not to disturb the beads (see Note 32).
3. Repeat the wash two more times.
4. Add 1 mL GST beads wash buffer and 10 μM purified GST to the tube labeled "GST". Add 1 mL GST beads wash buffer

and 10 μM purified GST fusion of target protein to the tube labeled "GST fusion".

5. Incubate at 4°C for 1 h with rotation on a tube rotator.
6. At the end of incubation, briefly spin the tubes to collect the beads and liquid. Place the tubes on the magnetic stand and remove the supernatant.
7. Wash the beads twice with 1 mL GST beads wash buffer and once with 1 mL selection buffer.
8. Add dialyzed or precipitated mRNA-peptide fusions from Subheading 3.10 to the tube labeled "GST". Add BSA to a final concentration of 0.1 mg/mL. Place the tube labeled "GST fusion" on ice until use.
9. Incubate the tube labeled "GST" at 4°C for 1 h with rotation.
10. Separate the supernatant from the beads in the tube labeled "GST" using the magnetic stand and transfer to the tube labeled "GST fusion".
11. Incubate at 4°C for 1 h with rotation.
12. Remove the supernatant from the beads using the magnetic stand and save it in a new tube. The supernatant is the flow through.
13. Wash the beads three times with 400 μL selection buffer. Save the wash in a new tube each time.
14. Elute six times with portions of 100 μL GSH elution buffer.
15. At each elution step, let the elution buffer sit on beads for 5 min before separation on the magnetic stand.
16. Take 10 μL from the flow through and three washes, and 1 μL of each eluent, and count in the scintillation counter. Count all the magnetic beads in two labeled tubes.
17. Combine all eluents with high counts, and dialyze against 1 L pre-cooled dialysis buffer in the cold room overnight.

3.12. PCR Amplification of Selected Fusions

1. Set up a series of pilot PCR reactions using 10, 20 and 30 μL dialyzed eluent from Subheading 3.11 in 100 μL PCR reactions. Run the cycles and check PCR products on a 2% Agarose gel as described in Subheading 3.5. Analyze the intensity of DNA bands to find out the optimum conditions for PCR.
2. Set up a large-scale PCR using multiple tubes or PCR strips and use all the eluents from Subheading 3.11 to amplify the selected fusions.
3. After PCR, combine all the reaction mixtures and add equal volume phenol:chloroform:isoamyl alcohol (25:24:1). Vortex the tube vigorously and spin at maximum speed for 2 min to separate the organic and aqueous phases.

4. Transfer the upper phase to a new tube and precipitate with ethanol and NaOAc.
5. Redissolve the DNA in 500 μL ddH_2O. This is the template for the next round of selection (see Note 33).

3.13. Additional Rounds of Selection

Once the first round of selection is completed in Subheading 3.12, repeat the selection for additional 6–10 rounds. Use a 1–2 mL of translation reaction. Scale down all the subsequent steps accordingly (see Note 34). The progress of the selection can be monitored by measuring the percentage of ^{35}S peptides that are eluted from the GSH column for that round. Once this value plateaus, proceed to sequencing.

3.14. Analysis of Selected Sequences

1. Clone and sequence the library as described in Subheading 3.5. Usually one 96-well plate of clones is enough to find several families of unnatural peptide selection winners.
2. Once the individual sequences are obtained, they can be aligned manually or using web-based alignment programs. For sequence alignments, Clustal W2 (Jalview V. 2) is used (21). Unnatural amino acids were assigned based on the tRNA/AARS pairs responsible for the incorporation into peptides.
3. Select a few sequences from each family for further study.
4. Synthesize the peptide sequences using a solid phase peptide synthesizer.

4. Notes

1. Other gel elution methods such as crush and soak can be used. Electroelution with the Elutrap is relatively fast and efficient in nucleic acid recovery from PAGE gels.
2. One can also make a 10% APS stock solution and store at 4°C for up to a month. We prefer freshly made APS.
3. It is not necessary to filter the TBE buffer as the EDTA in the buffer is sufficient to inhibit DNase and RNase activity.
4. Standard PCR buffer can also be used. We prefer ThermoPol buffer for its optimized components for use with Taq polymerase and the inclusion of 0.1% Triton X-100 to reduce nonspecific binding.
5. Standard agarose gel loading buffer can also be used but the bromophenol blue in the standard loading buffer might interfere with the visualization of the PCR product. Orange G is smaller than bromophenol blue and would not block the visibility of smaller DNA molecules.

6. A stock solution of DTT can be made and aliquots can be stored at –20°C.
7. Inorganic pyrophosphatase can be stored as 1 mg/mL in enzyme dialysis buffer (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 50% glycerol). Dilute the 1 mg/mL stock with the enzyme dialysis buffer to 0.1 mg/mL for routine use.
8. Purchase the XL-PSO oligonucleotide from a good resource such as Integrated DNA Technologies (IDT) or the Keck facilities at Yale. Dissolve the oligonucleotide in ddH_2O. Desalt on a NAP-25 column (GE Healthcare). Measure the OD_{260} on a spectrophotometer and adjust the concentration to 125 μM. Aliquot and cover with aluminum foil. Store at –20°C.
9. Other dialysis units or cassettes can be used, such as dialysis tubing or D-Tube dialyzer from Merck/EMD Chemicals. The molecular weight cut-offs should be smaller than the molecules that need to be dialyzed.
10. The protease inhibitor should not contain any EDTA or EGTA as these are chelating agents and will strip the nickel from Ni-NTA Agarose.
11. Some of the total tRNAs prepared from *E. coli* extracts are intrinsically acylated with their cognate natural amino acids. They need to be deacylated before use in PURE translation system. After deacylation and dialysis, the natural amino acids are removed from tRNAs, and the tNRAs can then be acylated with unnatural amino acids in PURE system.
12. Make a fresh stock of 0.5 M TCEP and dilute 1- to 1,000-fold into solution before use.
13. We chose GST fusion for its broad use in successful expression of soluble proteins in *E. coli*. We also use GST alone to perform the negative selection. In the negative selection, the peptides that bind to GST and the support matrix (agarose or other matrix) are removed from the library. The rest of the peptides are then selected on a GST fusion target. Other proteins with different tags can be used, but the negative selection needs to be adjusted accordingly.
14. Although we have not performed an exhaustive search it appears that peptides with a single cysteine form linear peptides containing a bis-thioether adduct between the cysteine and 2-mercaptoethanol.
15. One negative consequence of using the codon NNB is that it leads to more exaggerated biases between other amino acids. For example, in an NNB library, the ratio of serine to tryptophan is 5:1, vs. only 3:1 in an NNS or NNK library.

16. For mRNA that will be used in translation, use KOAc instead of NaOAc to precipitate. Residual Na^+ in the mRNA preparation will interfere with translation.
17. Longer exposure is not necessary. The efficiency of photocrosslinking is usually around 50%.
18. It is important to aliquot the purified proteins in small volumes. Do not freeze and thaw the proteins more than twice.
19. If the ribosomes are to be used to study translation factors or their mutants, the trace factor activity that remains after second wash can be further reduced by washing the ribosomes a third or even fourth time.
20. It is important to aliquot the purified ribosomes in small amount. Do not freeze and thaw the ribosomes more than once. Discard the remaining ribosomes after thawing.
21. The concentrations of the amino acids need to be optimized for each new combination. The analogs are initially utilized at 400 μM and the concentration is increased if misincorporations are observed in translation reactions with FLAG or His-tagged templates. The process is outlined in ref. 12.
22. It is convenient to make a stock solution of KCl/$MgCl_2$. Mix 2.85 M KCl and 1 M $MgCl_2$ in a ratio of 11.9:3.1. Add 0.32 volume of this mixture to the translation reaction. The final concentrations of KCl and $MgCl_2$ in the reaction are approximately 500 and 50 mM, respectively.
23. It is necessary to release the pressure at least once during cyclization by opening the bottom cap while holding the column upside down, since the acetonitrile evaporates and pressure can be built inside the column. Use a paper towel to cover the cap when opening. Use caution to avoid any radioactive contamination.
24. The 2-mercaptoethanol in the first wash is used to neutralize the residual dibromo-*m*-xylene in the cyclization buffer.
25. Be sure to discard the supernatant properly since it contains residual ^{35}S.
26. The amount of mRNA-peptide fusion obtained after oligo(dT) purification varies from 1 to 100 pmol.
27. In the later rounds of selection, the volume needed for reverse transcription is usually less than 400 μL. Multiple PCR tubes and PCR machine can be used.
28. DTT is always added ahead of RNaseOUT.
29. After reverse transcription, do not denature the duplex.
30. In the later rounds of selection, the volume of Ni-NTA Agarose required is usually less than 400 μL. Centrifugal filter devices can be used to simplify the purification of mRNA-peptide fusion through Ni-NTA agarose.

31. In the later rounds of selection, the volume of mRNA-peptide fusion is small and no precipitation is required. The solution after dialysis can be directly used in selection.
32. The magnetic beads cannot withstand high speed centrifugation. Spin briefly if necessary.
33. Save half of the purified PCR template from the previous round in case anything goes wrong.
34. In the later rounds of selection, sometimes PCR amplification of selected mRNA templates can be difficult. Conditions for pilot PCR need to be explored and optimized. More eluent volume or cycles might be necessary for PCR. If PCR is successful, repeat the previous round of selection without the negative selection or use a larger volume of translation reaction.

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References

1. Forster, A. C., Weissbach, H., and Blacklow, S. C. (2001) A simplified reconstitution of mRNA-directed peptide synthesis: activity of the epsilon enhancer and an unnatural amino acid, *Anal. Biochem.* 297, 60–70.
2. Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001) Cell-free translation reconstituted with purified components, *Nat. Biotechnol.* 19, 751–755.
3. Hartman, M. C. T., Josephson, K., and Szostak, J. W. (2006) Enzymatic aminoacylation of tRNA with unnatural amino acids, *Proc. Natl. Acad. Sci. U. S. A.* 103, 4356–4361.
4. Murakami, H., Ohta, M., Ashigai, H., and Suga, H. (2006) A highly flexible tRNA acylation method for non-natural polypeptide synthesis, *Nat. Methods* 3, 357–359.
5. Merryman, C., and Green, R. (2004) Transformation of aminoacyl tRNAs for the *in vitro* selection of "drug-like" molecules, *Chem. Biol.* 11, 575–582.
6. Goto, Y., Ohta, A., Sako, Y., Yamagishi, Y., Murakami, H., and Suga, H. (2008) Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides, *ACS Chem. Biol.* 3, 120–129.
7. Kawakami, T., Ohta, A., Ohuchi, M., Ashigai, H., Murakami, H., and Suga, H. (2009) Diverse backbone-cyclized peptides via codon reprogramming, *Nat. Chem. Biol.* 5, 888–890.
8. Sako, Y., Morimoto, J., Murakami, H., and Suga, H. (2008) Ribosomal synthesis of bicyclic peptides via two orthogonal inter-side-chain reactions, *J. Am. Chem. Soc.* 130, 7232–7234.
9. Yamagishi, Y., Ashigai, H., Goto, Y., Murakami, H., and Suga, H. (2009) Ribosomal synthesis of cyclic peptides with a fluorogenic oxidative coupling reaction, *Chembiochem* 10, 1469–1472.
10. Millward, S. W., Takahashi, T. T., and Roberts, R. W. (2005) A general route for post-translational cyclization of mRNA-displayed libraries, *J. Am. Chem. Soc.* 127, 14142–14143.
11. Josephson, K., Hartman, M. C. T., and Szostak, J. W. (2005) Ribosomal synthesis of unnatural peptides, *J. Am. Chem. Soc.* 127, 11727–11735.
12. Hartman, M. C. T., Josephson, K., Lin, C.-W., and Szostak, J. W. (2007) An expanded set of amino acid analogs for the ribosomal translation of unnatural peptides, *PLoS ONE* 2, e972.

13. Adessi, C., and Soto, C. (2002) Converting a peptide into a drug: strategies to improve stability and bioavailability, *Curr. Med. Chem.* 9, 963–978.
14. Roberts, R. W., and Szostak, J. W. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins, *Proc. Natl. Acad. Sci. U. S. A.* 94, 12297–12302.
15. Nemoto, N., Miyamoto-Sato, E., Husimi, Y., and Yanagawa, H. (1997) In vitro virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro, *FEBS Lett.* 414, 405–408.
16. Takahashi, T. T., and Roberts, R. W. (2009) In vitro selection of protein and peptide libraries using mRNA display, *Methods Mol. Biol.* 535, 293–314.
17. http://www.ambion.com/techlib/append/rbs_requirements.html. (Accessed 1 August 2010).
18. Kurz, M., Gu, K., and Lohse, P. A. (2000) Psoralen photo-crosslinked mRNA-puromycin conjugates: a novel template for the rapid and facile preparation of mRNA-protein fusions, *Nucleic Acids Res.* 28, E83.
19. http://www.daimi.au.dk/~g960801/Protocols/Riboso.pdf. (Accessed 1 August 2010).
20. Spedding, G. (1990) *Ribosomes and protein synthesis: a practical approach*, Oxford University Press, London.
21. Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., and Barton, G. J. (2009) Jalview Version 2–a multiple sequence alignment editor and analysis workbench, *Bioinformatics (Oxford, England)* 25, 1189–1191.