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Construction of a Highly Diverse mRNA Library for *in vitro* Selection of Monobodies

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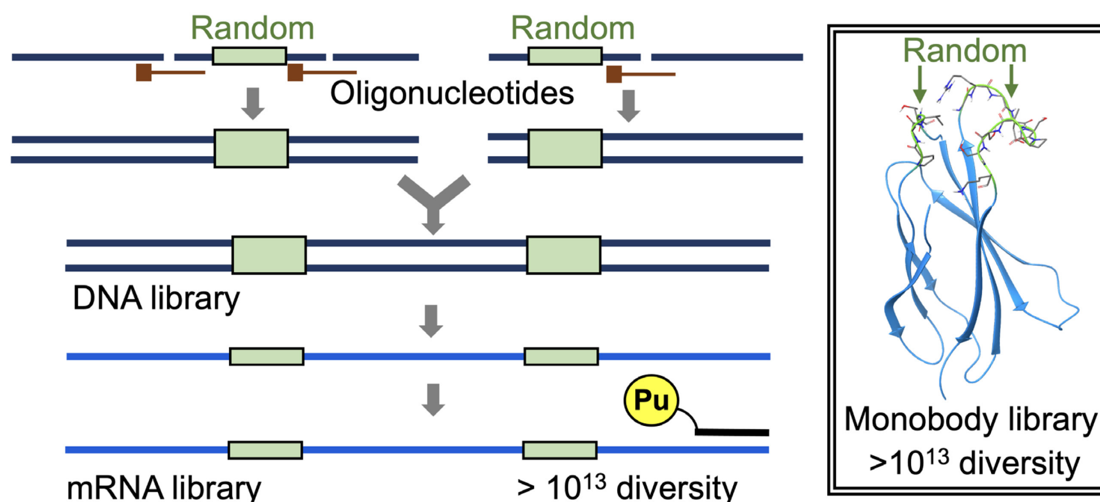
[Abstract] Recently, we developed transcription/translation coupled with the association of puromycin linker (TRAP) display as a quick *in vitro* selection method to obtain antibody-like proteins. For the *in vitro* selection, it is important to prepare mRNA libraries among which the diversity is high. Here, we describe a method for the preparation of monobody mRNA libraries with greater than 10^{13} theoretical diversity. First, we synthesized two long single-stranded DNAs that corresponded to fragments of monobody DNA, with random codons in the BC and FG loops. These oligonucleotides were ligated by T4 DNA ligase with the support of guide oligonucleotides containing 3′ ends that were protected by a modification. After amplifying the product DNAs by PCR, one end of each DNA fragment was digested with the type II restriction enzyme *Bsa*I, and the resulting DNA fragments were ligated using T4 DNA ligase. After amplification of the DNA product, mRNAs were synthesized by T7 RNA polymerase. This method is simple and could be used for the preparation of mRNA libraries for various antibody-like proteins.

Graphic abstract:



Construction of a highly diverse mRNA library.

Keywords: *In vitro* selection, TRAP display, mRNA library, Antibody-like protein, Monobody

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[Background] Various antibody-like proteins (ALPs) have been obtained using an *in vitro* selection approach and employed in the biological and medical fields (Schumacher *et al.*, 2018; Simeon and Chen 2018). During this *in vitro* selection, initial library construction is an important step in obtaining high-affinity binders. For example, to develop a nanobody, the VHH domains of camel antibodies obtained after immunization were used for the construction of an initial library (Arbabi Ghahroudi *et al.*, 1997). Alternatively, the VHH domains from naïve genes or a semi-synthetic or synthetic library could be used as the initial library (Goldman *et al.*, 2006; Monegal *et al.*, 2009; Yan *et al.*, 2014).

For other non-immunoglobulin scaffolds, synthetic libraries were used exclusively as the initial library (Nord *et al.*, 1997; Koide *et al.*, 1998; Beste *et al.*, 1999; Binz *et al.*, 2003; Grabulovski *et al.*, 2007; Olson *et al.*, 2008). Oligonucleotides containing random codons were assembled by extension PCR and ligated to other DNA fragments for synthesis of the DNA library. As randomized codons, NNK codons (with N representing A, C, G, or T; and K representing T or G) were generally used because they cover all 20 amino acids and can reduce the redundancy of codons and the frequency of stop codons. Alternatively, direct codon synthesis using trinucleotide phosphoramidites (Virnekäs *et al.,* 1994) was used to mimic the amino acid frequency in the CDR3 sequences of antibodies (Zemlin *et al.*, 2003; Wojcik *et al.,* 2010; Koide *et al.,* 2012).

The construction of medium-sized libraries (10^{10} diversity) is relatively simple, whereas that of large-sized libraries (10^{13} to 10^{14} diversity) requires special care to maintain the diversity (Olson and Roberts 2007). For example, when a 50 nM DNA product is obtained after 10 cycles of PCR, the concentration of the original template can be calculated as 50 pM. If we use only 100 µl reaction mixture, the maximum diversity that can be achieved using the conditions described above is limited to 3×10^9.

Recently, by modifying the traditional mRNA display (Nemoto *et al.,* 1997; Roberts and Szostak 1997) and the original version of the TRAP display (Ishizawa *et al.*, 2013; Kawakami *et al.*, 2013), we developed an improved TRAP display for the quick *in vitro* selection of ALPs from a large library (>10^{13} theoretical diversity), from which we selected high-affinity monobodies (sub-nM K_D) against the SARS-CoV-2 spike protein (Kondo *et al.*, 2020). In the present article, we describe the preparation of a monobody mRNA library with >10^{13} diversity (Figures 1 and 2). We first divided the monobody gene into two fragments, one containing 8 or 10 random codons in the BC loop sequence (A-fragment) and the other containing 10 or 12 random codons in the FG loop sequence (B-fragment). As a random codon, we used a codon mix with the following ratios: 20% Tyr, 10% Ser, 15% Gly, 10% Trp, and 3% each of all remaining amino acids, with the exception of Cys, which was similar to the original cocktail (30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp, and 2.5% each of all remaining amino acids, with the exception of Cys) (Wojcik *et al.*, 2010; Koide *et al.*, 2012). To prepare a template DNA for each of the two fragments, we ligated 2 to 3 oligonucleotides assembled by guide DNAs using the T4 DNA ligase. We added a modification at the 3′ end of the guide DNAs to prevent them from becoming a primer in the following amplification step. After amplifying the product DNAs by large-scale PCR with minimum amplification cycles (15 ml, 7 cycles), these DNA fragments were digested with *Bsa*I and ligated using T4 DNA ligase. After amplification of the DNA product by large-scale PCR (60 ml, 4 cycles), mRNAs were synthesized by T7 RNA polymerase. This procedure would be useful for the preparation of high-diversity mRNA

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libraries for various ALPs in the future.

A

ATACTAATACGACTCACTATAGGATTAAGGAGGTGATATTTATGCAAGCCAATTCTGGTTCTCTGGAAGTTG
TGGAAGCCAGCCCGACGAGCATTCAGATTTCTTGGGACGCT
-(codon)$_{8,10}$-
GTTCGCTACTATCGCATTACCTATGGCGAAACCGGCGGTAACAGTCCGGTCCAGGAATTTACGGTGCCGGGT
TCAAAATCGACCGCGACGATTTCCGGCCTGAAACCGGGTGTTGATTATACCATCACGGTGTACGCAGTTACC
-(codon)$_{10,12}$-
CCGATTTCTATCAACTACCGCACGGGTGGAGGAGGAGGTAGCTAGGGACGGCGGGCGCGAGGCGGG

B

MQANSGSLEVVEASPTSIQISWDA
-(X)$_{8,10}$-
VRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVT
-(X)$_{10,12}$-
PISINYRTGGGGGS-GRGAGGG

Figure 1. Monobody library sequences. A. DNA sequence of the monobody library [T7 promoter, green; codon mix (20% Tyr, 10% Ser, 15% Gly, 10% Trp, and 3% each of all remaining amino acids, with the exception of Cys), yellow; an21 sequence (the 21-mer sequence for annealing to HEX-PuL), cyan]. **B.** Protein sequence of the monobody library (randomized residues X, yellow).

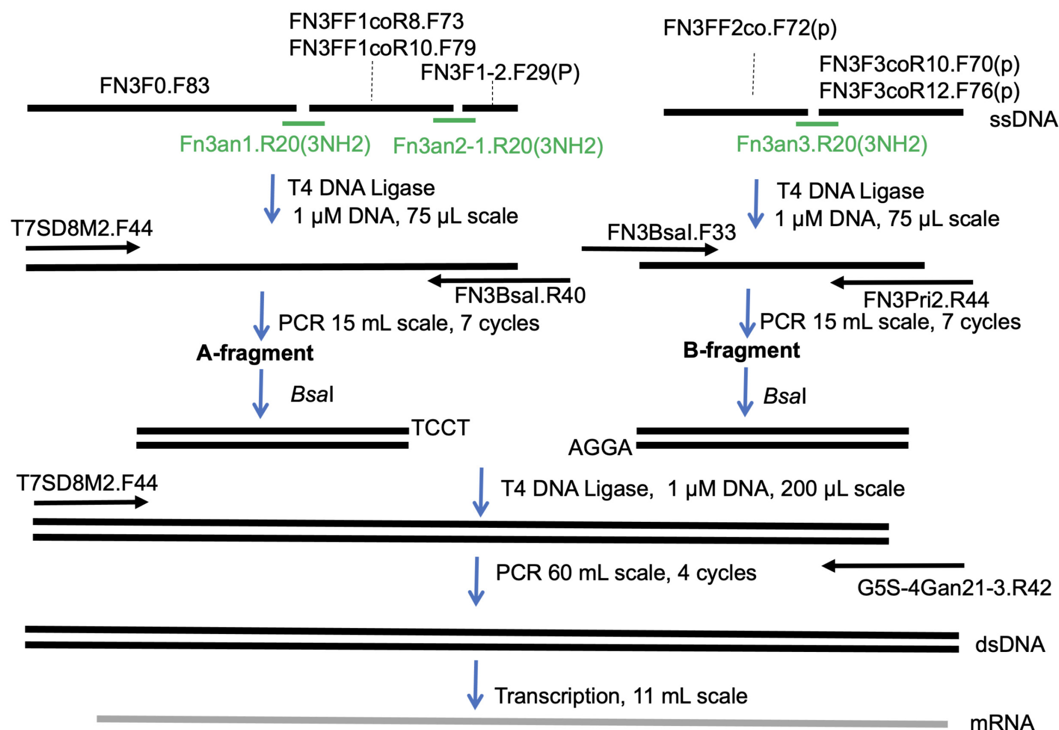

Figure 2. Scheme used for preparation of the monobody mRNA library.

Materials and Reagents

1. Oligonucleotides (sequences and suppliers are listed in Table 1)

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2. T4 DNA ligase (New England Biolabs, catalog number: M0202L)
3. *Bsa* I-HF® (New England Biolabs, catalog number: R3535L)
   Note: This has been replaced with a new version (Bsa I-HFv2®, catalog number: R3733L).
4. *Pfu-S* DNA polymerase prepared in-house (Wang *et al.*, 2004; Kondo *et al.*, 2020)
5. T7 RNA polymerase prepared in-house (Abramochkin and Shrader, 1995; Kondo *et al.*, 2020)
6. ExcelBand 100-bp DNA Ladder (Smobio Technology, catalog number: DM2100)
7. 0.5 mol/L-EDTA Solution (pH 8.0) (Nacalai Tesque, catalog number: 14347-21)
8. Formamide (Nacalai Tesque, catalog number: 16229-95)
9. Glycerol (Nacalai Tesque, catalog number: 17018-25)
10. 40 (w/v) %-Acrylamide/Bis Mixed Solution (37.5:1) (Nacalai Tesque, catalog number: 06121-95)
11. Trimethylolaminomethane (Tris) (Nacalai Tesque, catalog number: 35406-75)
12. Polyethylene Glycol 6000 (PEG 6000) (FUJIFILM Wako Pure Chemicals, catalog number: 169-22945)
13. 1,4-Dithiothreitol (DTT) (Nacalai Tesque, catalog number: 14128-62)
14. Magnesium Chloride Hexahydrate ($MgCl_2$) (Nacalai Tesque, catalog number: 20908-65)
15. Potassium Chloride (KCl) (Nacalai Tesque, catalog number: 28514-75
16. Sodium Chloride (NaCl) (Nacalai Tesque, catalog number: 31320-05)
17. Polyethylene Glycol Mono-*p*-isooctylphenyl Ether (Triton X-100) (Nacalai Tesque, catalog number: 12967-45)
18. Dimethyl Sulfoxide (DMSO) (Nacalai Tesque, catalog number: 13407-03)
19. Magnesium Sulfate Heptahydrate (Nacalai Tesque, catalog number: 06296-25)
20. Spermidine Trihydrochloride (Nacalai Tesque, catalog number: 32110-54)
21. 100 mM dNTP Set (NIPPON GENE CO., LTD., Custom order)
22. CTP, GTP, UTP (JENA BIOSCIENCE, Custom order)
23. ATP (FUJIFILM Wako Pure Chemicals, catalog number: 019-09672)
24. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Mixed, pH 6.7 (Nacalai Tesque, catalog number: 25967-74)
25. Chloroform (Nacalai Tesque, catalog number: 08402-55)
26. 2-Propanol (Nacalai Tesque, catalog number: 29113-95)
27. Ethanol (EtOH) (Nacalai Tesque, catalog number: 14713-95)
28. 2× Ligation solution (see Recipes)
29. 1× PCR solution (see Recipes)
30. 1× RNA Pol solution (see Recipes)
31. DNA loading buffer (see Recipes)

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Table 1. Oligonucleotide sequences and suppliers. NNK codons were prepared via trinucleotide phosphoramidite synthesis (20% Tyr, 10% Ser, 15% Gly, 10% Trp, and 3% each of all the remaining amino acids, with the exception of Cys). Abbreviations: SPC18, spacer 18; Pu, puromycin; HEX, hexachloro-fluorescein.

Names	Sequences (5′ to 3′)	Modification	Supplier
FN3FF1coR8.F73	CGAGCATTCAGATTTCTTGGGACGCTNNKNNKNNKNNKNNKNNKNNKNNKGTTCGCTACTATCGCATTACCTA	5′-Phospholilation	Nippon Bio Service (Japan)
FN3FF1coR10.F79	CGAGCATTCAGATTTCTTGGGACGCTNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKGTTCGCTACTATCGCATTACCTA	5′-Phospholilation	Nippon Bio Service (Japan)
FN3FF2co.F72(P)	AGGAATTTACGGTGCCGGGTTCAAAATCGACCGCGACGATTTCCGGCCTGAAACCGGGTGTTGATTATACCA	5′-Phospholilation	Nippon Bio Service (Japan)
FN3F3coR10.F70(P)	TCACGGTGTACGCAGTTACCNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKCCGATTTCTATCAACTACCG	5′-Phospholilation	Nippon Bio Service (Japan)
FN3F3coR12.F76(P)	TCACGGTGTACGCAGTTACCNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKCCGATTTCTATCAACTACCG	5′-Phospholilation	Nippon Bio Service (Japan)
FN3F1-2.F29(P)	TGGCGAAACCGGCGGTAACAGTCCGGTCC		Fasmac Co., Ltd. (Japan)
FN3BsaI.F33	ACGTTCGGTCTCAAGGAATTTACGGTGCCGGGT		Fasmac Co., Ltd. (Japan)
FN3BsaI.R40	GATCGTGGTCTCTTCCTGGACCGGACTGTTACCGCCGGTT		Fasmac Co., Ltd. (Japan)
FN3Pri2.R44	TAGCTACCTCCTCCTCCACCCGTGCGGTAGTTGATAGAAATCGG		Fasmac Co., Ltd. (Japan)
Fn3an1.R20(3NH2)	TGAATGCTCGTCGGGCTGGC	3′-Amino(C12) modification	Fasmac Co., Ltd. (Japan)
Fn3an2-1.R20(3NH2)	GGTTTCGCCATAGGTAATGC	3′-Amino(C12) modification	Fasmac Co., Ltd. (Japan)
Fn3an3.R20(3NH2)	TACACCGTGATGGTATAATC	3′-Amino(C12) modification	Fasmac Co., Ltd. (Japan)
T7SD8M2.F44	ATACTAATACGACTCACTATAGGATTAAGGAGGTGATATTTATG		Fasmac Co., Ltd. (Japan)
G5S-4Gan21-3.R42	CCCGCCTCGCGCCCGCCGTCCCTAGCTACCTCCTCCTCCACC		Fasmac Co., Ltd. (Japan)
FN3F0.F83	TAATACGACTCACTATAGGATTAAGGAGGTGATATTTATGCAAGCCAATTCTGGTTCTCTGGAAGTTGTGGAAGCCAGCCCGA		Fasmac Co., Ltd. (Japan)
HEX-PuL	<u>CCCGCCTCGCGCCCGCCGTCC</u> $(SPC18)_5$ CC (Pu)	5'-HEX Underline: 2'-OMe-modification	Nippon Bio Service (Japan)

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Equipment

1. T100 thermal cycler (Bio-Rad, catalog number: 1861096J1)
2. Gel Doc EZ (Bio-Rad, catalog number: 1708270)
3. Mini-PROTEAN System (Bio-Rad, catalog number: 1658000FC)
4. Cooling slab electrophoresis device (BIO CRAFT, catalog number: BE130G)

Procedure

A. Preparation of A-fragment DNA of the monobody library

1. Prepare the following solution (35 µl), heat at 95°C for 2 min, and place at room temperature for 5 min:
   FN3F0.F83 (2 µM final concentration), FN3F1-2.F29(P) (2 µM final concentration), FN3FF1coR8.F73 (1 µM final concentration), FN3FF1coR10.F79 (1 µM final concentration), Fn3an1.R20(3NH2) (4 µM final concentration), and Fn3an2-1.R20(3NH2) (4 µM final concentration) in 10 mM Tris-HCl pH 7.5.
2. Add 2× ligation solution (35 µl) to the solution described in (1) and incubate at 25°C for 1 h (see Figure 3A for analysis of the ligation reaction).
3. Add the mixture to 1× PCR solution (15 ml) and dispense 77 µl resulting solution into each well of two 96-well PCR plates. Amplify the DNA using T7SD8M2.F44 and FN3BsaI.R40 under the following conditions: initial activation step at 94°C for 1 min, followed by seven cycles of heat denaturation at 94°C for 20 s, primer annealing at 55°C for 20 s, and primer extension at 72°C for 45 s (see Figure 3B for optimization of the number of thermal cycles).
4. Add 3 M NaCl (1.5 ml).
5. Mix 3 ml phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
6. Mix 6 ml chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
7. Mix 16 ml 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*.
8. After removing the supernatant, add 1 ml 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
9. After removing the supernatant, dry the pellet and dissolve in 0.5 ml 10 mM Tris-acetic acid pH 7.8.
10. Determine the DNA concentration by 12% polyacrylamide gel electrophoresis using the ExcelBand 100-bp DNA Ladder.

B. Preparation of B-fragment DNA of the monobody library

1. Prepare the following solution (35 µl), heat at 95°C for 2 min, and place at room temperature for

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5 min:
FN3FF2co.F72(p) (2 µM final concentration), FN3F3coR10.F70(p) (1 µM final concentration), FN3F3coR12.F76(p) (1 µM final concentration), and Fn3an3.R20(3NH2) (4 µM final concentration) in 10 mM Tris-HCl pH 7.5.

2. Add 2× ligation solution (35 µl) to the solution described in (1) and incubate at 25°C for 1 h (see Figure 3A for analysis of the ligation reaction).
3. Add the mixture to 1× PCR solution (15 ml) and dispense 77 µl resulting solution into each well of two 96-well PCR plates. Amplify the DNA using FN3BsaI.F33 and FN3Pri2.R44 under the following conditions: initial activation step at 94°C for 1 min, followed by seven cycles of heat denaturation at 94°C for 20 s, primer annealing at 55°C for 20 s, and primer extension at 72°C for 45 s (see Figure 3B for optimization of the number of thermal cycles).
4. Add 3 M NaCl (1.5 ml).
5. Mix 3 ml phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
6. Mix 6 ml chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
7. Mix 16 ml 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*.
8. After removing the supernatant, add 1 ml 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
9. After removing the supernatant, dry the pellet and dissolve in 0.5 ml 10 mM Tris-acetic acid pH 7.8.
10. Determine the DNA concentration by 12% polyacrylamide gel electrophoresis using the ExcelBand 100-bp DNA Ladder.

C. Digestion of the A-fragment and B-fragment DNA with *Bsa* I

1. Incubate the reaction mixture (1× CutSmart® buffer, 2 µM A-fragment or B-fragment, 200 units/ml *Bsa* I-HF®; in a total of 400 µl) at 37°C for 4 d.
2. Add 3 M NaCl (40 µl).
3. Mix 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
4. Mix 200 µl chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
5. Mix 400 µl 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*.
6. After removing the supernatant, add 100 µl 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
7. After removing the supernatant, dry the pellet and dissolve in 10 µl 10 mM Tris-acetic acid pH 7.8.

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8. Mix 10 µl resulting solution with 10 µl DNA loading buffer.
9. Subject the solution to 12% polyacrylamide gel electrophoresis (10 cm × 7.3 cm × 0.75 mm, 300 V, 20 min).
10. After cutting the band out of the gel, elute the DNA using 0.3 M NaCl(1.2 ml).
11. Mix 1.2 ml 2-propanol with the solution and centrifuge at room temperature for 15 minutes at 16,000 × *g*.
12. After removing the supernatant, add 100 µl 70% EtOH and centrifuge at room temperature for 5 minutes at 16,000 × *g*.
13. After removing the supernatant, dry the pellet and dissolve in 100 µl 10 mM Tris-acetic acid pH 7.8.
14. Determine the DNA concentration by 12% polyacrylamide gel electrophoresis using the ExcelBand 100 bp DNA Ladder.

D. Ligation of the digested A-fragment and B-fragment DNA

1. Mix the solution containing 2 µM each A-fragment and B-fragment DNA (100 µl) with the 2× ligation solution (100 µl).
2. Incubate the resulting mixture at 25°C for 1 h (see Figure 3C for analysis of the ligation reaction).

E. PCR amplification of the DNA fragment

1. Add the mixture to 1× PCR solution (60 ml) and dispense 77 µl resulting solution into each well of eight 96-well PCR plates.
2. Amplify the DNA fragment using T7SD8M2.F44 and G5S-4Gan21-3.R42 under the following conditions: initial activation step at 94°C for 1 min, followed by four cycles of heat denaturation at 94°C for 20 s, primer annealing at 55°C for 20 s, and primer extension at 72°C for 45 s (see Figure 3D for optimization of the number of thermal cycles).
3. Add 3 M NaCl (6 ml).
4. Mix 8 ml phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
5. Mix 16 ml chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
6. Mix 66 ml 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*.
7. After removing the supernatant, add 1 ml 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
8. After removing the supernatant, dry the pellet and dissolve in 6 ml 10 mM Tris-acetic acid pH 7.8.
9. Determine the DNA concentration by 12% polyacrylamide gel electrophoresis using the ExcelBand 100-bp DNA Ladder.

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F. Preparation of the mRNA library

1. Incubate a mixture of 1× RNA Pol and 50 nM amplified DNA (in a total of 11 ml) at 37°C for 6 h.
2. Add 0.55 ml 0.5 M EDTA pH 8.0 and 1.2 ml 3 M NaCl.
3. Mix 2 ml phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
4. Mix 4 ml chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
5. Mix 9 ml 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*.
6. After removing the supernatant, add 1 ml 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
7. After removing the supernatant, dry the pellet and dissolve in 0.5 ml ultrapure water.
8. Mix 20 μl resulting solution with 150 μl formamide.
9. Subject the solution to 6 M urea 5% polyacrylamide gel electrophoresis (13.8 cm × 12 cm × 2 mm, 40 W, 40 min).
10. After cutting the band out of the gel, elute the DNA using 0.3 M NaCl 50% formamide solution (18 ml).
11. Mix 4 ml phenol:chloroform:isoamyl alcohol (25:24:1) with the resulting solution and centrifuge at room temperature for 5 min at 16,000 × *g*.
12. Mix 8 ml chloroform with the water layer and centrifuge at room temperature for 5 min at 16,000 × *g*.
13. Mix 18 ml 2-propanol with the water layer and centrifuge at room temperature for 15 min at 16,000 × *g*
14. After removing the supernatant, add 1 ml 70% EtOH and centrifuge at room temperature for 5 min at 16,000 × *g*.
15. After removing the supernatant, dry the pellet and dissolve in 20 μl ultrapure water.
16. Determine the mRNA concentration by measuring the OD at 260 nm. Store at -80°C.

G. Preparation of the mRNA/HEX-PuL complex

1. Before the first round of selection, heat the solution (25 mM HEPES-K pH 7.8, 200 mM potassium acetate, 4-8 μM RNA, and 4 μM HEX-PuL, in a total of 80 μl) at 95°C for 2 min, then cool to 25°C. Use the resulting complex for the first round of selection.

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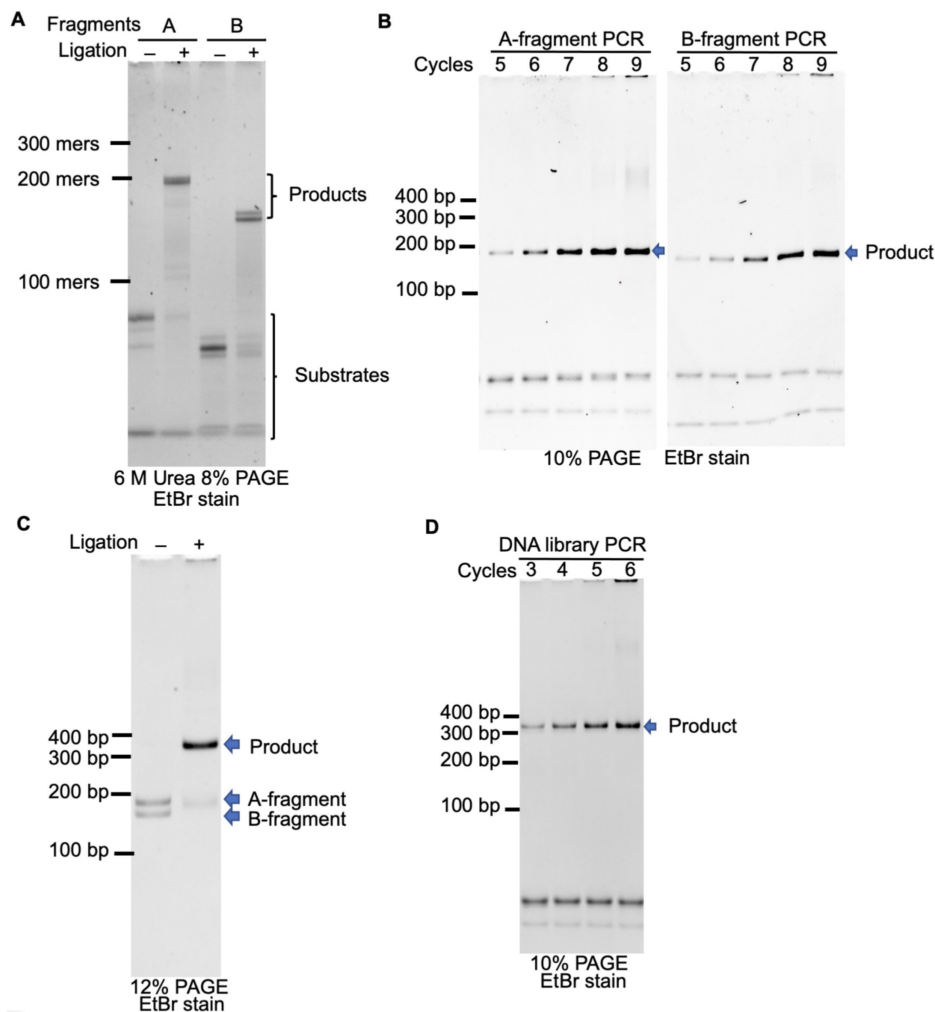

Figure 3. PAGE analysis of the reaction in each procedure. A. Ligation of oligonucleotides during Procedures A and B. **B.** Optimization of the number of thermal cycles in Procedures A and B. The reaction was performed in 10 μl solution. **C.** Ligation of the *Bsa*I-treated A- and B-DNA fragments in Procedure D. **D.** Optimization of the number of thermal cycles in Procedure E. The reaction was performed in 10 μl solution.

Data analysis

The 44 clones in the DNA library were cloned, and the sequences were analyzed by Sanger sequencing. Fourteen single-base deletions and 19 single-base insertions were observed in the backbone sequence; they were likely by-products of the oligonucleotides used in the construction of the DNA template. As a result, the reading frame was maintained in 19 out of 44 clones. The number of codons in the random sequences is summarized in Table 2. The observed frequency showed good agreement with the theoretical frequency.

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Table 2. Analysis of the codons in the BC and FG loop sequences of 44 clones.

Amino acids	Number of observed codons	Observed frequency (%)	Theoretical frequency (%)
Ala	25	3.3	3
Cys	0	0.0	0
Asp	26	3.4	3
Glu	31	4.1	3
Phe	22	2.9	3
Gly	98	12.8	15
His	39	5.1	3
Ile	22	2.9	3
Lys	17	2.2	3
Leu	16	2.1	3
Met	28	3.7	3
Asn	17	2.2	3
Pro	18	2.4	3
Gln	26	3.4	3
Arg	18	2.4	3
Ser	52	6.8	10
Thr	30	3.9	3
Val	52	6.8	3
Trp	65	8.5	10
Tyr	162	21.2	20
Stop	0	0.0	0

Notes

The theoretical diversity of the ssDNA, dsDNA, and mRNA libraries was calculated as follows:

A-fragment in Procedure A: 1 μM oligonucleotides (75 μl scale); 75 pmol, 4.5×10^{13} molecules

B-fragment in Procedure B: 1 μM oligonucleotides (75 μl scale); 75 pmol, 4.5×10^{13} molecules

A-fragment in Procedure A: 43 nM dsDNA product after 7 cycles (15 ml scale); 0.64 nmol, 3.9×10^{14} molecules, 0.6×10^{13} diversity with 64 copies

B-fragment in Procedure B: 38 nM dsDNA product after 7 cycles (15 ml scale); 0.57 nmol, 3.4×10^{14} molecules, 0.5×10^{13} diversity with 64 copies (only the complementary DNA was synthesized in the 1st cycle)

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dsDNA product in Procedure D: 1 μM dsDNA (200 μl scale); 200 pmol, 1.2 × 10^{14} molecules

dsDNA product in Procedure E: 33 nM dsDNA product after 4 cycles (60 ml scale), 2.0 nmol, 1.2 × 10^{15} molecules, 7.5 × 10^{13} diversity with 16 copies

mRNA product in Procedure F: 50 nM DNA template (11 ml scale); 0.55 nmol, 3.3 × 10^{14} molecules, the diversity was limited by Procedure E.

Recipes

1. 2× Ligation solution
   100 mM Tris-HCl pH 7.5
   12 mM $MgCl_2$
   1 mM ATP
   16% PEG 6000
   20 mM DTT
   4,000 U/ml T4 DNA ligase
2. 1× PCR solution
   10 mM Tris-HCl pH 8.4
   100 mM KCl
   0.1% (v/v) Triton X-100
   2% (v/v) DMSO
   2 mM $MgSO_4$
   0.2 mM each dNTP
   0.375 μM each primer
   2 nM *Pfu-S* DNA polymerase
3. 1× RNA Pol solution
   40 mM Tris-HCl pH 8.0
   1 mM spermidine
   0.01% (v/v) Triton X-100
   10 mM DTT
   25 mM $MgCl_2$
   5 mM each NTP
   1 μM T7 RNA polymerase
4. DNA loading buffer
   80 mM Tris-acetate pH 8.0
   2 mM EDTA
   20% (v/v) glycerol

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Competing interests

The authors declare no competing interests.

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