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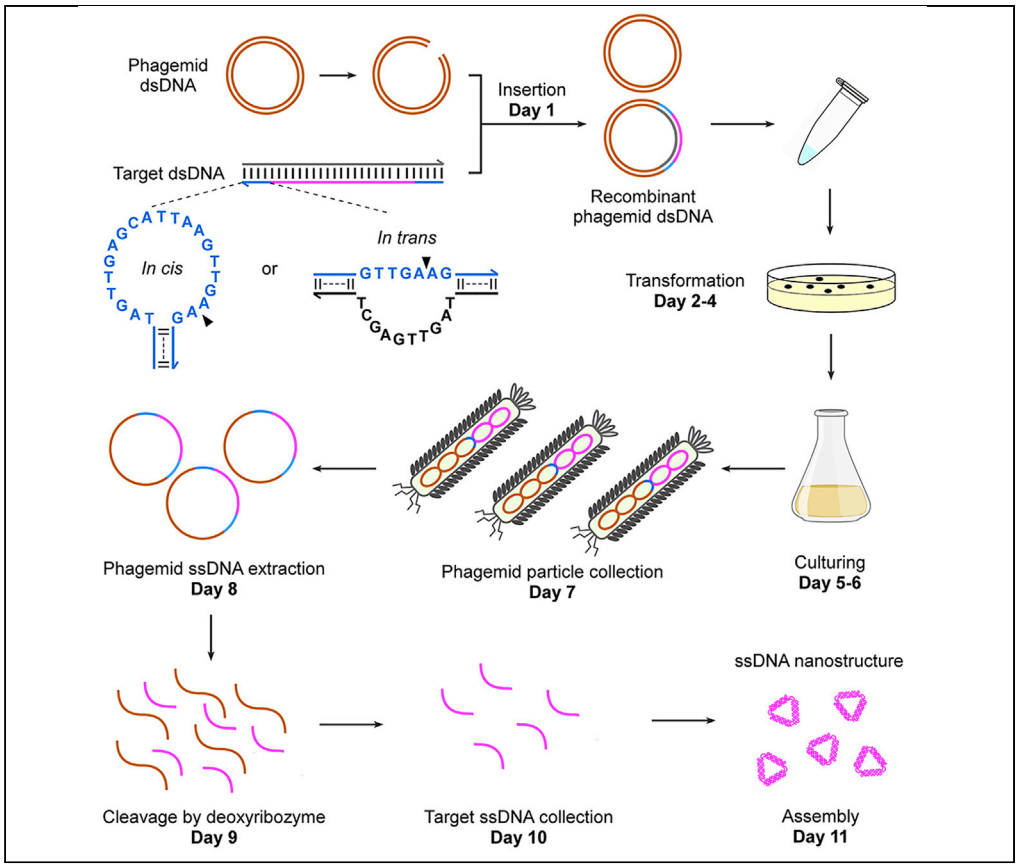
Protocol

Biotechnological production of ssDNA with DNA-hydrolyzing deoxyribozymes



Preparation of long single-stranded (ss)DNA in large quantities with high efficiency and purity remains a synthetic challenge. Here, we present a protocol for using DNA-hydrolyzing DNA enzymes (deoxyribozymes) for efficient biotechnological production of milligrams of ssDNA with a customizable sequence up to a few kilobases. Our protocol provides a convenient yet economical way to store the sequence information of target ssDNA on phages for selective mass production on demand.

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Highlights

Storage of target ssDNA on phages for selective mass production on demand

Controllable release of specific ssDNA targets by *trans*-cleavage of DNA

Preparation of long ssDNA in large quantities with high efficiency and purity

Self-folding of kilo-based long ssDNA into desired nanoshapes

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Biotechnological production of ssDNA with DNA-hydrolyzing deoxyribozymes

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SUMMARY

Preparation of long single-stranded (ss)DNA in large quantities with high efficiency and purity remains a synthetic challenge. Here, we present a protocol for using DNA-hydrolyzing DNA enzymes (deoxyribozymes) for efficient biotechnological production of milligrams of ssDNA with a customizable sequence up to a few kilobases. Our protocol provides a convenient yet economical way to store the sequence information of target ssDNA on phages for selective mass production on demand.

For complete details on the use and execution of this protocol, please refer to Jia et al. (2021).

BEFORE YOU BEGIN

Despite of the mature of DNA synthesis by solid-phase chemistry, the efficiency decreases as the length of ssDNA increases, with the limitations of low yield and high cost for >120-nt (nucleotide) DNAs. In recent years, long ssDNA exhibited great potential in DNA nanotechnology, DNA sequencing, gene knockin, and so on. Therefore, urgently needed is a method that can allow us to readily prepare long ssDNA with high efficiency and low cost. By combining DNA-hydrolyzing deoxyribozymes (enzymes made up of DNA) with the phage amplification system (Gu et al., 2013; Praetorius et al., 2017; Jia et al., 2021), we developed this protocol to biotechnologically amplify the chemically synthesized DNA as pseudogene fragments and robustly produce customized ssDNA. We focused on DNA nanostructures self-folded from a single long ssDNA to demonstrate the practicality and programmability of the method.

We use JM109 as the host bacteria, which serve as a factory to amplify the recombinant bacterial virus (phage) in large quantities. After collecting the supernatant (where the phages are) for PEG sedimentation and removal of protein coating, usually we can obtain several nanomoles of phage ssDNA per 300 mL of culture medium. By incubating phage ssDNA with Zn^{2+} to trigger the programmed DNA hydrolysis at specific sites by deoxyribozymes, we can separate the target ssDNA segment from the phage vector efficiently and cost-effectively. With this protocol, we (and others) have succeeded in the production of dozens of long ssDNAs of different sequence and with lengths ranging from a hundred to several thousand nucleotides (Praetorius et al., 2017; Engelhardt et al., 2019; Jia et al., 2021). In principle, sequence of the produced ssDNA is customizable according to the users' demands. As an example, here we programmed sequence of the produced ssDNA

to be able to self-fold into nanostructures for characterization. We expect that our protocol will promote the development of ssDNA applications in broad areas.

Prepare reagents and buffers

Timing: 1 day

1. Prepare the following solutions and buffers. See materials and equipment for the detailed recipes.
   a. 37% Acrylamide/Bis-acrylamide solution (29/1)
   b. 1 M HEPES (pH 7.0)
   c. 1 M NaCl
   d. 1 M $MgCl_2$
   e. 100 mM $ZnCl_2$
   f. 100 mM $NiCl_2$
   g. 20% HCl solution
   h. Deoxyribozyme reaction buffer 1 (pH 7.0)
   i. Deoxyribozyme reaction buffer 2 (pH 7.0)
   j. 2 M NaOH
   k. 10% SDS solution
   l. PPB2 solution
   m. PPB3 solution
   n. Acetic acid $\geq$99%
   o. TE buffer (10 mM Tris, pH 8.5)
   p. LB medium
   q. 2× YT medium (5 mM $MgCl_2$)
   r. Ampicillin solution (100 mg/mL)
   s. 100% ethanol
   t. 2× denaturing loading buffer
   u. 8% denaturing PAGE gel solution
   v. 50× TAE buffer
   w. 10× TBE buffer
   x. 2× YT (5 mM $MgCl_2$) upper agar (0.7%)
   y. 2× YT (5 mM $MgCl_2$) lower agar (1.5%)
2. Resuspend primer oligo stocks with ddH_2O to a concentration of 100 μM. See Table 1 for sequence. We ordered the oligos from Generay Biotech (Shanghai) with standard desalt purification.

Prepare helper phage stock

Timing: 3 days or more

3. Amplify and harvest vcsM13 helper phage
   DAY 0:
   a. Prepare a 100 mL sterile flask with 10 mL LB medium.
   b. Inoculate the medium with a scrape from JM109 competent cells (stored at −80°C), and culture the cells on a shaker at 220 rpm for 7 h at 37°C.

   Note: Keep the competent cells on dry ice to maintain the low temperature when they are taken out of the −80°C freezer.
   DAY 1:
   c. Prepare two 1 L sterile flasks with 300 mL 2× YT (5 mM $MgCl_2$) medium in each.

Table 1. Sequences of oligos and vectors used (Generay Biotech)

Oligo name	Oligo sequence (5′ to 3′)
ss-triangle	TTTTTTCTCAGGTCTAACAAGTCACATATCTGTGATGATCTAGTATTTTTACTAGGTCG CAACAGACACAATACTTGACCGATTTTTCGGTGCCTAATTGTGGCATTTGCGACAC CACTACATTCACTGTTAGTTCTGGCTTTAACACCTCTCTATCGCATCCAACCAAG ATCGCTGGAGACTATACAGTCGTATCTGAAGCTCGACATCTTTTGATGTCCGAAG TCAGATTGAGTTGTATAAGGAACAGCGATCTTGTTTTTTTGTTGGATGCGGTTGG TAGGTGCGTAGACCAGAGCCGAGTTTTCTCGGCAGCACTCTACGCTTGGACCAACCACCA TTTTTATGGTGATAGAGCCAAGTTAAAGGTGCTACTAACAGTGATTTTTTTATGTAGTGG TATCATCAATGCTATGTGTAGGCTTAGACCTGAGCAGTGCTCGCAGAGCTAACGACA CGACAATGTTGTCTCTCTCATTTTTATGAGATTCCTAACATTACTCAGTCGTTCTTCG TGCGAGCACTGTTTTTAGAGTTACGTATTCACTCGAAT
ss-triangle for recombination (Underlined are sequences coded for deoxyribozyme; "/" means the specific hydrolysis sites by deoxyribozyme; bold are homology arms)	AGGGAACAAAAGCTGGAGCTCACTGACTGACCTCAGCGTTGA/AGTCGCACGTTCGGTAGC TTTTTTCTCAGGTCTAACAAGTCACATATCTGTGATGATCTAGTATTTTTACTAGGTCGCAACAGAC ACAATACTTGACCGATTTTTCGGTGCCTAATTGTGGCATTTGCGACACCACTACATTCACTGTT AGTTCTGGCTTTAACACCTCTCTATCGCATCCAACCAAGATCGCTGGAGACTATACAGTCGTAT CTGAAGCTCGACATCTTTTGATGTCCGAAGTCAGATTGAGTTGTATAAGGAACAGCGATCTTGT TTTTTTGTTGGATGCGGTTGGTAGGTGCGTAGACCAGAGCCGAGTTTTCTCGGCAGCACTCTAC GCTTGGACCAACCACCATTTTTATGGTGATAGAGCCAAGTTAAAGGTGCTACTAACAGTGATTT TTTTATGTAGTGGTATCATCAATGCTATGTGTAGGCTTAGACCTGAGCAGTGCTCGCAGAGCTA ACGACACGACAATGTTGTCTCTCTCATTTTTATGAGATTCCTAACATTACTCAGTCGTTCTTCG TGCGAGCACTGTTTTTAGAGTTACGTATTCACTCGAATGCGGCTACGCGACAGCGTTGA/AGAGT GTAGTTACCTCAGAAGCTTGCGTGTGAATTCCCT
Primer1 fwd	AGTCGCACGTTCGGTAGCTTTTTTCTCAGGTCTAACAA
Primer1 rev	AACGCTGTCGCGTAGCCGCATTCGAGTGAATACGTAACT
Primer2 fwd	ACTGACTGACCTCAGCGTTGAAGTCGCACGTTCGGTAGC
Primer2 rev	CTGAGGTAACTACACTCTTCAACGCTGTCGCGTAGCCGCAT
Primer3 fwd	AGGGAACAAAAGCTGGAGCTCACTGACTGACCTCAGCGTTG
Primer3 rev	AGGGAATTCACACGCAAGCTTCTGAGGTAACTACACTCTTC
p3024 fwd	AAGCTTGCGTGTGAATTCCCT
p3024 rev	GAGCTCCAGCTTTTGTTCCCT
Primer4 fwd	CAGGAAACAGCTATGAC
Primer4 rev	GTAAAACGACGGCCAGT
E1	GCTACCGAACGTGCGATAGTTGAGCTGCTGAGGTCAGTCAGT
E2	CTGAGGTAACTACACTTAGTTGAGCTGCTGTCGCGTAGCCGC
E3	GCCAGCTTGGGTCTCTAGTTGAGCTGTGATCCATACGAATTC
E4	CACACGCCTCGAGATCTAGTTGAGCTGCAGAATTCCACCAC
p3024 vector (Sequence of the amplifiable strand of the phagemid is shown here. Bold sequence refers to the multiple cloning sites.)	CCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGT CGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTG CAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGC CCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGC ATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAG CGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCC CGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACC CCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCG CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTC AACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAA AAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTA GGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAA ATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAG TATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTT TGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTT ACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCC AATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAA GAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAG AAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTT TTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGC CATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAAC TATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGG ATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAG CCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAA ATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTA CTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACC CCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTTCTGCGCGTAATCTGCTGCTTGCA

(*Continued on next page*)

Table 1. ***Continued***

Oligo name	Oligo sequence (5′ to 3′)
	AACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTT CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTA GGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAG CGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTG AGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTAT CCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCC TGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCC TGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATA ACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGC AGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTC CCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCG GGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTA CACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACA GGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAA GCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCGTAAATCA ATGACTTACGCGCACCGAAAGGTGCGTATTGTCTATAGCCCCCTCAGCCACGAATTCG TCTGACGACGACAAGACAAGCTTGCGTGTGAATTCCCTGGCTTCTCCTGAGAAA
Recombinant p3024 carrying three ssDNA targets (The ssDNA targets encode a triangle (592 nt), a quadrangle (758 nt), and a rhombus (2395 nt); Underlined are sequences coded for deoxyribozyme; "/" means the specific hydrolysis sites by deoxyribozyme.)	CCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACG TCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGC CAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAA TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGC AGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCT CGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGAT TTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGG CCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGT GGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTA TAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAA TTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGTGGCACTTTTCGGGGAAA TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAG ACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCC GTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAA ACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATC GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTAT TGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAG TACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTG CCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGG AGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCT GAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGC AAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGG CGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTG GAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGAT CGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTA GATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCA AAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTT GAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTG TTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA ATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACAT ACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGT TGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGC ACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAA GCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACA GGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGC CACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAA CGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTC TTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTC GCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAA TACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTC CCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGC ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAA TTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAA AGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCGTAAATCAA TGACTTACGCGCACCGAAAGGTGCGTATTGTCTATAGCCCCCTCAGCCACGAATTCGTCTGACGAC GACAAGACCTCGAGATACTGACTGACCTCAGCGTTGA/AGTCGCACGTTCGGTAGCTTTTTTCTCAGGTC TAACAAGTCACATATCTGTGATGATCTAGTATTTTTACTAGGTCGCAACAGACACAATACTTGACCGAT TTTTCGGTGCCTAATTGTGGCATTTGCGACACCACTACATTCACTGTTAGTTCTGGCTTTAACACCT

(*Continued on next page*)

Table 1. ***Continued***

Oligo name	Oligo sequence (5′ to 3′)
	CTCTATCGCATCCAACCAAGATCGCTGGAGACTATACAGTCGTATCTGAAGCTCGACATCTTTTGATG
	TCCGAAGTCAGATTGAGTTGTATAAGGAACAGCGATCTTGTTTTTTTGTTGGATGCGGTTGGTAGGT
	GCGTAGACCAGAGCCGAGTTTTCTCGGCAGCACTCTACGCTTGGACCAACCACCATTTTTA
	TGGTGATAGAGCCAAGTTAAAGGTGCTACTAACAGTGATTTTTTTATGTAGTGGTATCATCAATGCT
	ATGTGTAGGCTTAGACCTGAGCAGTGCTCGCAGAGCTAACGACACGACAATGTTGTCTCTCTCATTTT
	TATGAGATTCCTAACATTACTCAGTCGTTCTTCGTGCGAGCACTGTTTTTAGAGTTACGTATTCACT
	CGAATGCGGCTACGCGACAGCGTTGA/AGAGTGTAGTTACCTCAGCTTGATGCTCACGTTTTTC
	TAGTGTGTCAGAGAGCTGAGTACGGATAGGTAGAAGGCTTTTGCCTTCACCTGATCCGTCGCAGT
	CTCTCCTCAGTTTTCTGAGTCATGACTGCGATGTGTCAGGTTCCAATGCACTCCTGTTGACGACTAG
	CGACACCAACGCACCTGTGTAACGATCGACTTCGGTCATCATGGACCAGTAGACGTACTAGTGTCTG
	ACTCTGAAGCTTAGCAGTCTCTATGCTGACAGCAGCAACTCGCTCGTTTTTACGAGCTGTACGCTGCTAT
	GGACATAGACGTTACTAAGCTTCAGTTTTTAGTCAGACACGTCTCAGTCTAACCTGTCATGAC
	AGTGGTTTTCCACTGTTGTCACAGGTCACGTTGAGACGAAGTCTTTTGACTTCTAGTACACGT
	GCTGGTCGACAATGACCGAAGTCTTTTTGATCGTTACAATGTAGCGTTGAGCACTCTAGTCCTG
	TGTTTTCACAGGTGCTAAGTGCTCTCGTCTACATCGCACGTTTTCGTGCGCAGGTGACGAGGT
	GTCGTAGCACGTCAACAGGATTTTTGTGCATTGGATACCTACACATACTCAGCATGATGACACACTAG
	GACTTCACGGTGAGTTATGACCGTCAGAGAGCTGACTGACCTCTTTTTAGAGGTTAACGAGC
	TCTTCCATGGTCATGTACAACCGTGAAGTCTTTTTCTTATACTCAGAGAATTCGTATGGAT
	CACGTTGA/AGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGTACCCCTCAGCGACGTT
	GAAGGATCAGTGGCAATCGTCATACGGCACGTAAGCCTTGCATTGACTAGCCCTCCCCCACAAC
	TGACTGATTGCTGAATCTTGCGGTGTGTGGAGTTCATCTGCATCCTGCCCAACTCCGGCGGCGGTT
	GCACGATCAACATTTTCTTTCTTTCTTTCTTTTATGAAAGGCAGTTGGGCCAGTAGGCGGTCCACCT
	ATGAGCACCAAAGGATCCTGGTCGTCGGGCCAGCCACCACGTATTGCTATTACGATTTTTCTTTCTTTCT
	TTCTTTTACGTATGCTAACTCATTGCTCCTAAGACCAGTATAAGTTCCATGGCTGGACTCCGGCAATG
	AGCGGGAACCGCTGATTCAACGACCAGCATTTTCTTTCTTTCTTTCTTTTACCATTCCGCGTCGAGGG
	ACGAATTGGATCTATCTCTGTGTCATTCTGGACCGTAAGCGCGCGTCAGAATTTGAAGAGGACAATCGA
	CGTTTTTCTTTCTTTCTTTCTTTTCGCTCTTCAAACGAGTCGGTTGCAATGTTGCGCCTCGGCGTGATCC
	TGCTTACTCGACTGCTCTAATGGACAAGGCTTAACCGCGCTTTCTTCTTTATCTCTCTTGTTAAGCAGTC
	TGCGAAATCGGGGTAGAACCAATGAATCCTCAAGCACTGCCCGGGTAGAGATGAGCAACCTTCTTGTCC
	TTTTTCTTTCTTTCTTTTTCTGAAGCAGACTATCTGACGCAGGGGTGGACGAAACCGAGGTGCCGAA
	GACAAGGGCTAGATCGGATTTCTCCGGTCCGGATCACGCGTTTTTCTTTCTTTCTTTCTTTTGGACAATC
	AGCAGGATTAAGTTCAACCTTGAATCGACTCCGGCAGCCACAGATCTCTACAGGACGAACGACACCT
	GAACTACGCTACCTCTTTTCTTTCTTTCTTTCTTTTGAAAGTGCTTATCTAGACGAAACATAGCCCGAGCC
	GAAGCGTATGTCGCGTGCCCCGAACGCGCCGTACAACCACAGGTTTTGGAAAATCTTTTCTTTCTTTCT
	TTCTTTTCCTTTCCTCAACTCGTGTACGGGTCTAAACCATCAGGACAGTATGAGTACAGGAAGGG
	CTCGAAGGCATGTTCAGTCAGTAGCCCCCGCAACAGCGACTAGATAATCGACCGCGTCCCA
	TACAGCGACTAGATAATCGACCGCGTCCCATTGCGGGGGCTAGTCTTTGAACATGCCCAATACCCC
	TTCCTGTGGGTGTACTGTCCTGATCCTCTAGACCCGTACCGTGGGTGAGGAAAGGTTTTCTTT
	CTTTCTTTCTTTTGATTTTCCAACGCAACTGGTTGTACGAAGTGCTCGGGGCACGACTGGTACG
	CTTCGGCGGTCTTTATGTTTCGTCAAGATAAGCACTTTCTTTTCTTTCTTTCTTTCTTTTGAGGTAGCGTTTT
	CCTGGTGTCGTTCCCTTTGTAGAGATCTGGCCAGACCGGAGTCGACAGAATGTTGAACTTACGCAT
	TCTGATTGTCCTTTTCTTTCTTTCTTTCTTTTACGCGTGATCAGAGCAGGAGAAATCCAATTCTGCCC
	TTGTCTGCTCATCCTCGGTTTCAGAATCCCCTGCGTCATTGGGCCTGCTTCAGATTTTCTTT
	CTTTCTTTCTTTTAGGACAAGAAACGGCTTCATCTCTACCCCAACAGTGCTTGAGCGGTAGTT
	GGTTCTACCTCGACTTCGCAGACTCACGCTCAAGAGAGATTTCTTTGAAAGCGCGGAGCGTGC
	TTGTCCATTCGGACCGTCGAGTAAGAATGCGCACGCCGAGGAACCTGATTGCAACCGCCCAC
	GTTGAAGAGCGTTTTCTTTCTTTCTTTCTTTTACGTCGATTGTGGTTTTCAAATTCTGGCACTTGC
	TTACGGTCTTCAAGGACACAGAGAAGAATTCAATTCGTCCCCCGATGCGGAATGGTTTTTCTTTCT
	TTCTTTCTTTTTGCTGGTCGTCTACCGAGCGGTTCCCTCGGCATGCCGGAGTCTCTGGCTGGAACTTATC
	GACATCTTAGGAGCAACACCCTAGCATACGTTTTTCTTTCTTTCTTTTATCGTAATAGTTC
	GAGGTGGTGGCTGAAGACCCGACCAGGATGTCCTGGTGCTCATAGGATTCTCGCCTACTGGC
	CGGGCTGCCTTTCATTTTTCTTTCTTTCTTTCTTTTTGTTGATCGTAGCCGTGCCGCCGGAGG
	ATAGTAGGATGCAGAAGGAAACCACACACCGCTAGATTCAGCAATCAAAGACTTGTGGGGGAG
	ATCCGGCATCGCGATTCATCTTCGCGAATCGGTACCGAGCTCGGATCCACTAGTCCAGTGTGG
	TGGAATTCTGCGTTGA/AGGATCTCGAGGCGTGTGAATTCCCTGGCTTCTCCTGAGAAA

d. Per 300 mL YT medium, inoculate with 3 mL of the JM109 cell culture from step 3b, and shake the flasks with a speed of 250 rpm at 37°C till the OD_{600} value reaches 0.4–0.5, which corresponds to an estimated cell concentration of about 3.2×10^8 – 4.0×10^8 mL^{-1}.

Note: Usually it takes about 2–3 h. After 1.5 h, the OD_{600} value of cell culture should be measured every 15–30 min. Time point by the culture reaches OD 0.4–0.5 can be estimated by fitting the plot of the measured OD values by an exponential function. These steps can be necessary as the culture can rapidly reach and surpass the desired OD values.

e. Add vcsM13 helper phage to each flask at MOI (multiplicity of infection) around 10.
Example:
vcsM13 helper phage is at a titer of 1.0×10^{15} pfu/mL (plaque forming unit per mL) according to the vendor's report. For every 300 mL JM109 culture with an OD_{600} value of 0.5, (4.0×10^8 cells $mL^{-1} \times 300$ mL) × 10 (MOI) / (1.0×10^{15} pfu/mL) = 1.2 μL helper phage should be added.

Note: a) vcsM13 helper phage is purchased from NEB. During transportation, temperature fluctuation may damage the phage's quality (ability to infect bacteria). Therefore, once the helper phage seeds are obtained from a seller, it is better to perform an in-house amplification to not only expand its quantity but also ensure its quality in infection. b) When there is a phagemid coexisting, the helper phage mainly assists the amplification of the phagemid DNA into the single-stranded form and helps to package the phagemid ssDNA into phagemid particles. When the phagemid is absent, the helper phage amplifies itself and packages itself into helper phage particles.

f. Shake the flasks with a speed of 250 rpm at 37°C for 4 h.
g. Transfer the cell culture in each flask to a 1-L centrifuge bottle and cap the bottles tightly.
h. Centrifuge the bottles at 4,000 rcf for 15 min at 4°C. Collect the supernatant containing phages by gently pouring it into a 1-L glass beaker, and discard the pellet (cells).
i. Add PEG8000 and NaCl (40 g PEG8000 and 30 g NaCl per 1 L medium) to the collected supernatant. Stir the mixture on a magnetic stirrer until the solids are completely dissolved. Then bath the mixture on ice for 30 min.
j. Transfer the mixture to two 1-L centrifuge bottles with equal volume in each and cap the bottles tightly.
k. Centrifuge the bottles at 5,000 rcf for 30 min at 4°C. Discard the supernatant and collect the pellet (phages).
l. Add 6 mL TE buffer to each bottle to resuspend the phage pellet. Use a pipette to blow the pellet multiple times until the pellet is completely dissolved.
m. Centrifuge the bottles at 16,000 rcf for 10 min at 4°C to further remove the bacteria residue.
n. Pipette the supernatant carefully into a 15 mL tube.
o. Store the tube at −80°C.

4. Measure the titer of vcsM13 helper phage
DAY 2:
a. Inoculate with a scrape from the stored JM109 competent cells (at −80°C) into 5 mL 2× YT (5 mM $MgCl_2$) medium, and culture the cells on a shaker with a speed of 220 rpm at 37°C until the OD_{600} reaches 0.4–0.8.

Note: Usually it takes about 6–8 h. After 5.5 h, the OD_{600} value of cell culture should be measured every 15–30 min. Similar to the Note in step 3d, time point by the culture reaches OD 0.4–0.8 can be estimated by fitting the plot of the measured OD values by an exponential function.

b. Microwave the upper agar (0.7%) and lower agar (1.5%) until they are melted. Place the upper and lower agar in water bath at 45°C and 37°C, respectively, to maintain their fluid property.
c. Cast 7–10 mL of the melted lower agar into each of the six 5-cm round plates. Cover the plates and leave them at 23°C for 30–60 min until the agar solidifies to form an agar layer. Keep the plates in an incubator at 37°C.
d. Thaw the vcsM13 helper phage stock in step 3o on ice-water bath.
e. Pipette 1 μL helper phage stock and make a series of dilution sequentially (10^8, 10^9, 10^{10}, and 10^{11} times) with 2× YT medium (5 mM $MgCl_2$). For each diluted phage sample, pipette 10 μL and mix it with 100 μL of bacterial cell culture from step 4a in a 1.5-mL Eppendorf tube. Gently shake the mixture by hands. Then leave them at 23°C for 5 min.

Note: The stocked vcsM13 helper phage in step 3o is too concentrated for titer measurement. A series of dilution help to find out the proper concentrations that can lead to countable plaques formed later on the agar plates.

f. Prepare six 10-mL glass bottles. Four of them will be used to continue the experiments with the four samples in step 4e. The other two will be used in control experiments.

g. Take the agar plates in step 4c out of incubator. For each experimental group, aliquot 4–5 mL of the melted upper agar in step 4b and 110 μL of the bacteria-phage mixture in step 4e into the glass bottle in step 4f. Gently shake the bottle by hands. Then immediately cast the sample onto the solidified lower agar. Rotate the plates to make the upper agar well distributed. Cover the plates and leave them at 23°C for 30–60 min until the upper agar solidifies to form a thin layer on top of the lower agar.

Number of plaques	Dilution factor	Volume of the diluted phages (μL)	Titer (pfu/mL)
200	10^9	10	2×10^{13}

h. Two control groups are conducted at the same time. In one, the melted upper agar is not supplemented with the bacteria-phage mixture in step 4e; in the other, the melted upper agar has only the supplementation of the bacteria in step 4a.

i. Place the agar plates upside down in an incubator at 37°C for 10–12 h.
DAY 3:

j. Estimate the phage's titer based on the number of plaques counted on the plates. An agar plate with clear and countable plaques is also shown as an example in Figure 1.
Titer = (Number of plaques × Dilution factor)/Volume of dilution used = 200 pfu × 10^9/10 μL = 2×10^{13} pfu/mL

k. According to the estimated titer in step 4j, dilute the helper phage solution in step 4d with TE buffer to a final titer of 5×10^{12} pfu/mL. Prepare multiple 500-μL Eppendorf tubes, and aliquot 100 μL of the diluted helper phage into each tube for storage at −80°C.

Note: Aliquoting of diluted helper phage will make the future work more convenient. For every 1-L flask containing 300 mL bacterial cell culture with an OD_{600} value of 0.5, an MOI value of 1–10 is recommended when the cells are inoculated with the helper phage. Following our protocol, one can just take one stored helper phage sample (100 μL, 5×10^{12} pfu/mL) and supplement all (100 μL) of it for every 300 mL cell culture. That corresponds to an MOI value of 4.2, which falls into the recommended range.

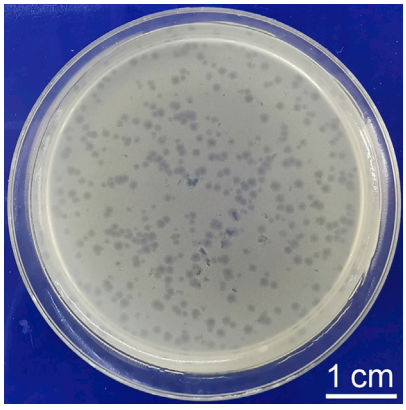

Figure 1. An example agar plate with clear and countable phage plaques

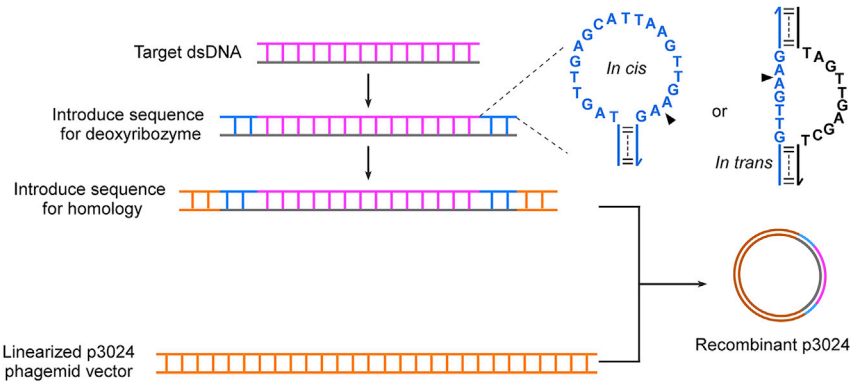

Figure 2. Programming sequence of the deoxyribozyme and homology arm into the target dsDNA for phagemid recombination

MOI = 100 μL × (5 × 10^{12} pfu/mL) / (300 mL × (4.0 × 10^{8} cells/mL)) = 4.2

Design and prepare target DNA into (pseudo)gene segments for recombination

Timing: 1 day

Basically, target DNA with any sequence can be amplified and produced in the single-stranded form by this protocol. To build a dsDNA template that can be inserted into a phagemid vector for amplification and deoxyribozyme cutting, sequence that encodes the DNA-hydrolyzing DNAs and the homology arms needs to be designed flanking around the target DNA fragment as shown in Figure 2. Details on the sequence selection of the deoxyribozymes can be found in Jia et al. (2021). When the target DNA is not amplifiable from any known DNA templates and requires completely chemical synthesis, sequence of the flanking deoxyribozymes and homology arms can be synthesized together in one fragment with the target DNA. If the target DNA is a gene fragment in an attainable template, step-by-step PCR can be used to add sequence that encodes the deoxyribozymes and homology arms to both ends of the target. For example, in this protocol we chose to produce ssDNA that can self fold into a triangle (Jia et al., 2021). We have chemically synthesized the dsDNA template that encodes the triangle. In other words, we have a pseudogene fragment that is amplifiable by PCR (with Primer 1 fwd & rev for amplification of the target, Table 1). After PCR amplification with a new set of primers programmed with the addition of deoxyribozyme sequence in them (Primer 2 fwd & rev, Table 1), the dsDNA products will carry deoxyribozyme sequence on both ends. These products can further serve as templates for another round of PCR amplification, with another new set of primers programmed with the addition of homology arm sequence (Primer 3 fwd & rev, Table 1). After these amplifications, eventually obtained are the target dsDNA fragments flanked by deoxyribozyme and homology arm sequence (Table 1, Figure 2).

After homology-guided recombination, the recombinant phagemid is subjected to in vivo rolling circle replication in E. coli. Only one of the two strands of the dsDNA is amplified and produced in the end as ssDNA (Figure 3). Thus one should pay attention to the direction of the dsDNA insert in relation to the dsDNA vector of the phagemid, and make sure that the to-be-produced ssDNA strand

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacteria and virus strains		
vcsM13 helper phage	New England Biolabs	Cat# N0315S
JM109 competent cell	Zymo Research	Cat# T3003
p3024 phagemid	Lin et al., 2012	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
$ZnCl_2$	Sigma-Aldrich	Cat# 429430
Hepes	Sigma-Aldrich	Cat# H3375
NaCl	Sigma-Aldrich	Cat# S7653
$MgCl_2$	Sigma-Aldrich	Cat# M8266
LiCl	Sigma-Aldrich	Cat# L9650
$NiCl_2$	Sigma-Aldrich	Cat# 339350
KOAc	Sigma-Aldrich	Cat# P1190
Acrylamide	Sigma-Aldrich	Cat# A8887
Bis-acrylamide	Sigma-Aldrich	Cat# 146072
Tris base	Sigma-Aldrich	Cat# 93352
SDS	Sigma-Aldrich	Cat# L3771
Boric acid	Sigma-Aldrich	Cat# B7660
Glacial acetic acid	Sangon Biotech	Cat# A501931
Sodium acetate	Sigma-Aldrich	Cat# S2889
100% ethanol	Sangon Biotech	Cat# A500737
EDTA	Sigma-Aldrich	Cat# EDS-100G
Urea	Sangon Biotech	Cat# A610148
Agarose LE	Tiangen Biotech	Cat# RT101
Tryptone	Millipore	Cat# T7293
Yeast extract	Millipore	Cat# Y1625
Ampicillin trihydrate	Sangon Biotech	Cat# A100741
Kanamycin sulfate	Sangon Biotech	Cat# A100408
Agar	Sangon Biotech	Cat# A100637
SYBR Gold nucleic acid gel stain	Invitrogen	Cat# S11494
Gel-red	Beyotime Biotechnology	Cat# D0139
PEG8000	Sigma-Aldrich	Cat# 89510
Glycogen	Sangon Biotech	Cat# A620301
Sucrose	Sangon Biotech	Cat# A502792
Bromphenol blue	Sigma-Aldrich	Cat# B0126
Xylene Cyanol FF	Sigma-Aldrich	Cat# 335940
NaOH	Sangon Biotech	Cat# A100583
TE buffer	Sangon Biotech	Cat# B548106
Critical commercial assays		
Phanta Max Super-Fidelity DNA Polymerase	Vazyme Biotech	Cat# P505-d1
High-Fidelity 2× PCR Master Mix	New England Biolabs	Cat# M0541
Gel DNA Recovery Kit	Zymo Research	Cat# D4001
ClonExpress II One Step Cloning Kit	Vazyme Biotech	Cat# C112-01
Plasmid Mini Kit I	Omega Bio-tek	Cat# D6943
Oligonucleotides		
ss-triangle (see Table 1)	Generay Biotech	N/A
ss-triangle for recombination (see Table 1)	Generay Biotech	N/A
Primer1 fwd (see Table 1)	Generay Biotech	N/A
Primer1 rev (see Table 1)	Generay Biotech	N/A
Primer2 fwd (see Table 1)	Generay Biotech	N/A
Primer2 rev (see Table 1)	Generay Biotech	N/A
Primer3 fwd (see Table 1)	Generay Biotech	N/A
Primer3 rev (see Table 1)	Generay Biotech	N/A
Primer4 fwd (see Table 1)	Generay Biotech	N/A
Primer4 rev (see Table 1)	Generay Biotech	N/A
p3024 fwd (see Table 1)	Generay Biotech	N/A
p3024 rev (see Table 1)	Generay Biotech	N/A
E1 (see Table 1)	Generay Biotech	N/A
E2 (see Table 1)	Generay Biotech	N/A
E3 (see Table 1)	Generay Biotech	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
E4 (see Table 1)	Generay Biotech	N/A
Recombinant DNA		
p3024 vector (see Table 1)	This paper	N/A
Recombinant p3024 carrying three ssDNA targets (see Table 1)	This paper	N/A
Other		
SpectraPor biotech dialysis membrane	Repligen	Cat# 132478
Parafilm	Heathrow Scientific	Cat# 291-1214
Molecular Imager Gel Doc XR+ with Image Lab software	Bio-Rad	Cat# 170-8195
Benchtop UV transilluminators	Tiangen Biotech	Cat# OSE-470L
Electrochemistry benchtop pH meter (Orion Star A211)	Thermo Scientific	Cat# STARA2110
Horizontal electrophoresis apparatus	Bio-Rad	Cat# 1704466
Vertical electrophoresis apparatus	CBS Scientific	Cat# SG-125
Power supplies	CBS Scientific	Cat# EPS-300X
Stackable incubated shaker	Thermo Scientific	Cat# SHKE4000-1CE
Benchtop centrifuge	Eppendorf	Cat# 2231000656
Metal bath incubator	Tiangen Biotech	Cat# OSE-DB-01
Atomic force microscope (MultiMode 8)	Bruker	https://www.bruker.com/en/products-and-solutions/microscopes/materials-afm/multimode-8-hr-afm.html?q1=cat
PCR thermal cycler	Eppendorf	Cat# 6331000076
NanoDrop 2000/2000c	Thermo Scientific	Cat# ND2000-EU
Avanti J-E BioSafe high performance centrifuge	Beckman Coulter	Cat# 95-176

(not its complementary strand) is connected with the amplifiable ssDNA vector of the phagemid. For design convenience, sequence of the p3024 ssDNA vector is shown in Table 1 with the multiple cloning sites (MCS) marked in red.

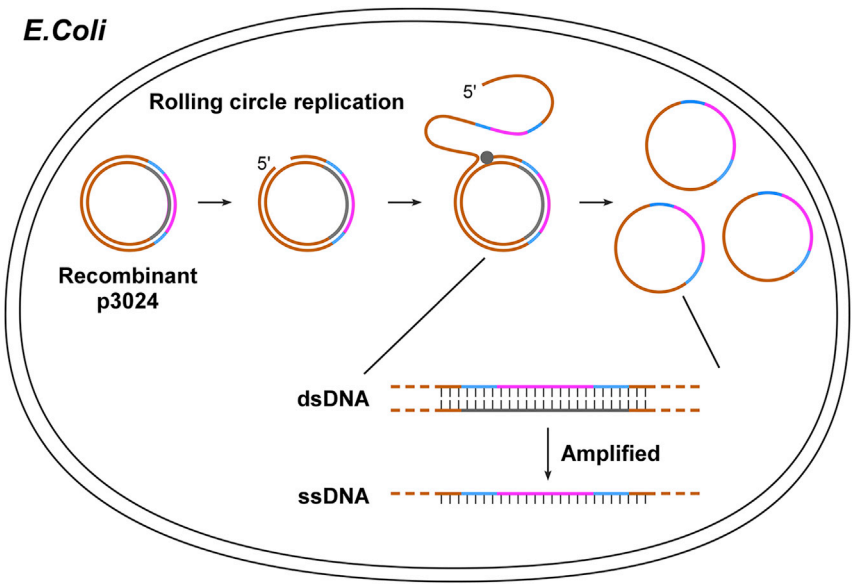

Figure 3. Schematic drawing of the amplification process of the recombinant phagemid in *E. coli*

KEY RESOURCES TABLE

Deoxyribozyme reaction buffer 1 (pH 7.0, store at 23°C with a shelf-life of ~6 months)		
Reagent	Final concentration	Amount
1 M Hepes	50 mM	2.5 mL
1 M NaCl	100 mM	5 mL
ddH_2O	N/A	To 50 mL
20% HCl solution (v/v)	Used for pH adjusting to 7.0	X mL
Total	**N/A**	**50 mL**

Alternatives: Other equipment with the similar function can be used instead, including gel imaging systems with general imaging function, pH meters with an accuracy to 0.01 unit, electrophoresis apparatus and power supplies with a voltage range of 0–2,000 v, incubators with a temperature range of 0°C–100°C, centrifuges with a maximum speed up to 16,000 rcf, as well as microscopes with the ability to characterize DNA nanostructures.

MATERIALS AND EQUIPMENT

Deoxyribozyme reaction buffer 2 (pH 7.0, store at 23°C with a shelf-life of ~3 months)		
Reagent	Final concentration	Amount
1 M Hepes	50 mM	2.5 mL
1 M NaCl	100 mM	5 mL
1 M $MgCl_2$	20 mM	1 mL
100 mM $ZnCl_2$	4 mM	2 mL
ddH_2O	N/A	To 50 mL
20% HCl solution (v/v)	Used for pH adjusting to 7.0	X mL
Total	**N/A**	**50 mL**

Buffer

⚠ CRITICAL: The DNA-hydrolyzing deoxyribozymes are very sensitive to pH fluctuations. To maintain their robust activity, it is better to keep the pH value of the reaction buffer 1 around 7.0, with a fluctuation of $\pm$0.1 unit.

PPB2 solution (store at 23°C with a shelf-life of ~3 months)		
Reagent	Final concentration	Amount
2 M NaOH	200 mM	10 mL
10% SDS solution (w/v)	1% (w/v)	10 mL
ddH_2O	N/A	80 mL
Total	**N/A**	**100 mL**

PPB3 solution (store at 23°C with a shelf-life of ~3 months)		
Reagent	Final concentration	Amount
KOAc	3 M	29.442 g
Glacial acetic acid	Used for pH adjusting to 5.50	X mL
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

2× YT medium containing $MgCl_2$ (store at 4°C with a shelf-life of ~6 months)		
Reagent	Final concentration	Amount
Tryptone	16.0 g/L	16 g
Yeast extract	10.0 g/L	10 g
NaCl	5.0 g/L	5 g
$MgCl_2$	5 mM	4.7 g
ddH_2O	N/A	To 1,000 mL
Total	**N/A**	**1,000 mL**

2× denaturing loading buffer (store at 4°C with a shelf-life of ~3 years, warm it at 80°C before use)		
Reagent	Final concentration	Amount
Sucrose	20% (w/v)	4 g
Bromphenol blue	0.05% (w/v)	10 mg
Xylene cyanole FF	0.05% (w/v)	10 mg
10% SDS solution(w/v)	0.1% (w/v)	0.2 mL
10× TBE	1× TBE	2 mL
Urea	16 M	19.2 g
ddH_2O	N/A	To 20 mL
Total	**N/A**	**20 mL**

8% denaturing PAGE gel (store at 23°C with a shelf-life of ~1 year)		
Reagent	Final concentration	Amount
37% Acrylamide/Bis-acrylamide (29/1)	8% (w/v)	216 mL
10× TBE	1× TBE	100 mL
Urea	8 M	480 g
ddH_2O	N/A	To 1,000 mL
Total	**N/A**	**1,000 mL**

1% denaturing agarose gel (fresh)		
Reagent	Final concentration	Amount
Agarose LE	1% (w/v)	1 g
50× TAE	1× TAE	2 mL
Urea	1 M	6 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

1% agarose gel (fresh)		
Reagent	Final concentration	Amount
Agarose LE	1% (w/v)	1 g
50× TAE	1× TAE	2 mL
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

LB agar medium (sterilize, then store at 4°C with a shelf-life of ~6 months)		
Reagent	Final concentration	Amount
Tryptone	10 g/L	1 g
Yeast extract	5 g/L	0.5 g
Agar	15 g/L	1.5 g
NaCl	10 g/L	1 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Alternatives: LiCl can replace NaCl in the deoxyribozyme reaction buffer 1.

⚠ CRITICAL: The DNA-hydrolyzing deoxyribozymes are very sensitive to pH fluctuations. To maintain their robust activity, it is better to keep the pH value of the reaction buffer 2 around 7.0, with a fluctuation of ±0.1 unit.

Alternatives: LiCl can replace NaCl in the deoxyribozyme reaction buffer 2.

Prepare LB agar plates:

- Microwave 100 mL LB agar medium prepared in a 250-mL bottle according to the above recipe. Every 2–3 min, take the medium out of microwave and shake it gently by hands. Continue doing this until all contents are dissolved and the medium is completely clear. This may take 15 min or longer.
- Place the medium at 23°C to cool it down until it can be handled by hands (40°C–50°C). This step takes about 20–30 min.
- Add 100 μL ampicillin (100 mg/mL) into the LB agar medium, and shake the bottle by hands to mix.
- Meanwhile, prepare twenty 5-cm plates.
- Pour the LB agar medium into the 5-cm plates until it spreads over the bottom. Approximately 5 mL medium is used for each plate.
- Cover the plates. Leave them at 23°C for solidification. Usually it takes 1 or 2 h.
- Label the plates and store them upside down in a 4°C refrigerator.

STEP-BY-STEP METHOD DETAILS

Recombination and transformation with the phagemid

Timing: 2 days

Day 1:

1. Prepare the target dsDNA and linearized phagemid vector for cloning
   a. Determine sequence of the target DNA for ssDNA preparation. As we described in the section of "before you begin", the target DNA sequence can be fully chemically synthesized or come

Reagent	Final concentration	Volume
2× Phanta Max buffer	1×	25 μL
dNTP mix (10 mM each)	0.2 mM	1 μL
Primer1 fwd (10 μM)	0.4 μM	2 μL
Primer1 rev (10 μM)	0.4 μM	2 μL
Phanta Max Super-Fidelity DNA Polymerase (1 U/μL)	0.02 U/μL	1 μL
Plasmid DNA (10 ng/μL, after dilution)	0.2 ng/μL	1 μL
ddH_2O	N/A	18 μL
Total	**N/A**	**50 μL**

Step	Temp	Time	Cycle
Initial denaturation	95°C	30 s	1
Denaturation	95°C	15 s	35
Annealing	60°C	15 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Holding	4°C	N/A	1

from genomic DNA. For the former, during chemical synthesis sequence of the flanking deoxyribozymes and homology arms can be directly added to the ends of the target dsDNA (Figure 2), thus the following steps (b(i-III)) are not needed. For the latter, please refer to steps (b(i-III)) for the construction of dsDNA that can be inserted into a phagemid vector.

b. Basically, we use the chemically synthesized primers to PCR amplify the target DNA in a step-by-step way for DNA reconstruction. Take the pseudogene fragment that encodes an ssDNA triangle (Table 1, Jia et al., 2021) as an example,
   i. First, employ primer1 fwd & rev as the initial pair of primers to amplify the pseudogene template as following:
   Thermo-cycling condition of first round PCR
   Use agarose gel electrophoresis and DNA recovery kit to purify the first round of PCR products.
   ii. Second, treat the purified products as templates, and use primer2 fwd & rev as the second pair of primers for the second round of PCR amplification. Use agarose gel electrophoresis and DNA recovery kit to purify the second round of PCR products.
   iii. Third, similarly use primer3 fwd & rev as the third pair of primers for the third round of PCR amplification. After this operation, sequence of the deoxyribozyme and homology arm has been added to the ends of the target dsDNA. Use agarose gel electrophoresis and DNA recovery kit to purify the products. Use Nanodrop to quantify the concentration of collected dsDNA. Store the sample at 4°C or −20°C.

c. Prepare the linearized vector of p3024 phagemid (3024 bp, a pBluescript variant). In the multiple cloning site, set up the primer binding regions for PCR amplification to generate the full-length linear phagemid, using the circular p3024 phagemid as the template. A pair of primers (p3024 fwd & rev) has been shown in Table 1 as an example. PCR protocols are similar to that used in step b.i., except that the time used for extension is increased from 30 sec to 3 min in each PCR cycle. Purify the linearized phagemid vector by agarose gel electrophoresis and recover the products with the DNA Recovery Kit.

Alternatives: Digest the circular p3024 phagemid with restriction enzymes, and isolate the linearized products by agarose gel electrophoresis.

2. Clone the recombinant p3024 phagemid
   a. Insert the target dsDNA fragment into the p3024 phagemid backbone by homologous recombination. Prepare two clean Eppendorf tubes, one for the experimental group and the other as a negative control. Refer to Table 2 to add the components for the recombination reaction. Use a pipette to gently mix the reactant (Do not shake), and briefly centrifuge the tubes to let the samples set at the bottom. Details about the ClonExpress II One Step Cloning Kit can be further found on its standard protocol.
   b. Incubate the samples at 37°C for 30 min. Then chill the tubes on ice immediately for 10 min.
   c. Meantime, thaw two tubes of JM109 competent cells (100 μL in each) on ice. Pipette 10 μL of the recombinant products and mix it with the competent cells in one tube. Tap the tube gently for a thorough mixing and leave the tube on ice for 30 min. Do the same to the negative control sample.

Table 2. Recombination with the ClonExpress II One Step Cloning Kit

	Experimental group	Negative control
Linearized p3024 vector	(0.02 × number of base pairs of the linearized vector) ng	(0.02 × number of base pairs of the linearized vector) ng
Target dsDNA	(0.04 × number of base pairs of the target dsDNA) ng	0 μL
5× CE II Buffer	4 μL	4 μL
Exnase II	2 μL	2 μL
ddH_2O	To 20 μL	To 20 μL

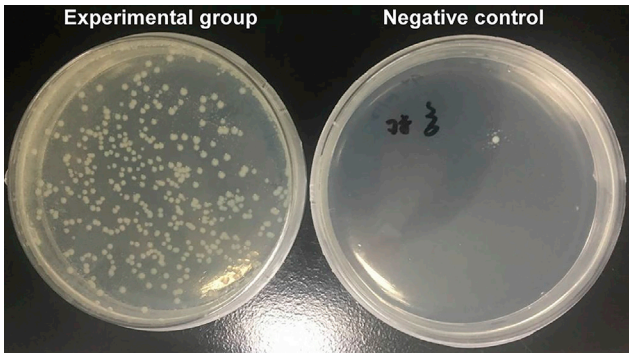

Figure 4. Example agar plates showing the successful transformation and cloning

d. Heat-shock the samples at 42°C for 45 sec. Then immediately chill them on ice for 2–3 min. After that, add 900 μL of the LB medium to the samples, and shake the samples with a speed of 220 rpm at 37°C for 1 h.
e. Take the samples off the shaker. Centrifuge the tubes at a speed of 5,000 rcf for 5 min. Pipette 900 μL of the supernatant and discard. Resuspend cell pellet at the bottom of the tubes in the left-over medium by vigorous tapping.
f. Warm two LB agar plates supplemented with ampicillin (100 μg/mL) at 37°C for 30 min. Plate 100 μL of each transformation (including the negative control) on the plates. Continue to incubate the plates at 37°C for 12 h. Multiple colonies should be viewable for the experimental group, and close-to-zero colonies should be identified in the negative control (Figure 4).

Day 2–4:

3. Screen for colonies with the correct insertion of the target dsDNA
   a. Prepare multiple 5-mL sterile tubes. Add 2 mL LB medium supplemented with ampicillin (100 μg/mL) into each tube. In our experiments, we used 10 tubes to examine the insertions in 10 colonies.
   b. Prepare the same number of 200-μL Eppendorf tubes. To each tube, add 30 μL of the PCR reaction solution prepared as above (see step 1.b.i.) including all components but the template. The pair of primers (primer4 fwd & rev, Table 1) is designed to complement with the regions on the p3024 vector nearby the insertion. Suggested concentrations of the primers in the reaction buffer are around 0.5–1 μM.
   c. Use the 200-μL pipette tip to pick a single colony from the agar plate. Dip the tip firstly into the LB medium for inoculation, then into the PCR solution for providing templates for amplification. Repeat the operation until all 10 colonies are picked.

   Note: a) Keep the tip dipping in the medium for 5–10 sec to ensure inoculation; b) For individual colonies, remember to label the 5-mL tube and 200-μL tube correctly, and make sure their one-to-one correspondence.
   d. Cultivate the colonies in the medium at 37°C for 8 h, agitating at a speed of 220 rpm.
   e. Meanwhile, run standard PCR amplification (25–30 cycles) on a thermocycler. Then exploit agarose gel (1%) electrophoresis to check which colonies contain the right length of the insertion of the target DNA. An example gel is shown in Figure 5.
   f. According to the electrophoresis data (Figure 5), discard the culture inoculated with colonies (1 & 9) carrying wrong insertions, and collect the cultures inoculated with the colonies with correct insertion (2–8, & 10).
   g. From the culture, extract the recombinant p3024 phagemid dsDNA using the Plasmid mini kit I. Pick three out of eight phagemid samples and send them for sequencing.

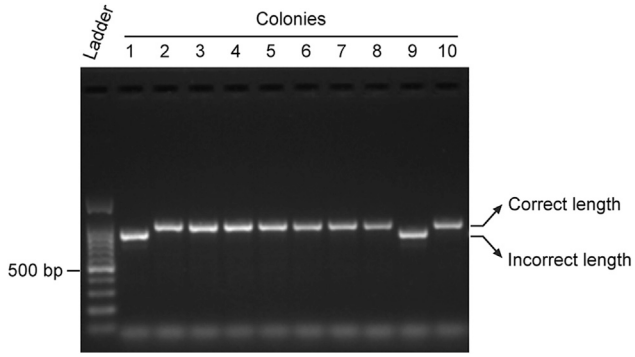

Figure 5. Screening for colonies with the correct length of the insertion by agarose gel electrophoresis

Note: Normally the sequencing data show that, at least one among three samples contains the insertion of target DNA without a mutation. If all three samples contain mutations, check the other samples by sequencing.

h. While waiting for the sequencing data, keep the DNA samples at −20°C for later use.

Note: The sequencing process takes about 2 days.

Amplification of phagemid and extraction of ssDNA from phagemid particles

Timing: 3 days

Day 5:

4. Transformation with the recombinant p3024 phagemid carrying the correct insertion
   a. Thaw JM109 competent cells (100 μL in a tube) on ice. Pipette 1 μL of the recombinant phagemid from step 3.h (at a concentration of around 100 ng/μL), and mix with the competent cells. Tap the tube gently for a thorough mixing and leave the tube on ice for 30 min.
   b. Heat-shock the sample at 42°C for 45 sec. Then immediately chill it on ice for 2–3 min. After that, add 900 μL of the LB medium to the sample, and shake the sample with a speed of 220 rpm at 37°C for 1 h.
   c. Take the sample off the shaker. Centrifuge the tube at a speed of 5,000 rcf for 5 min. Pipette 900 μL of the supernatant and discard. Resuspend cell pellet at the bottom of the tube in the left-over medium by vigorous tapping.
   d. Warm a LB agar plate supplemented with ampicillin (100 μg/mL) at 37°C for 30 min. Plate 100 μL of the transformation sample on the agar. Cover the plate and place it upside down. Continue to incubate the plate at 37°C for 12 h or longer, until colonies come out.

Day 6:

   e. Prepare a 50 mL sterile centrifuge tube with 20 mL LB medium supplemented with ampicillin (100 μg/mL).
   f. With a 200-μL pipette tip, inoculate a single bacterial colony from the agar plate in step 4.d into the 20 mL LB medium.
   g. Incubate the medium culture at 37°C and shake it at a speed of 220 rpm for about 10–12 h.
   h. Meantime, seal the agar plate with parafilm and keep it at 4°C.

Note: This is to store the colonies in case more phagemid needs to be amplified later. With the sealing, the colonies can be still effective after 6 months.

Day 7:

5. Amplification of the recombinant phagemid
   a. Prepare two 1-L sterile flasks with 300 mL 2× YT medium (5 mM $MgCl_2$) in each.
   b. For each flask, inoculate with 3 mL of the JM109 cell culture from step 4.g.
   c. Shake the flasks with a speed of 200–220 rpm at 37°C until the OD_{600} of the culture reaches about 0.4–0.5.

   Note: This usually takes about 2–3 h. After 1.5 h, the OD_{600} value of cell culture should be measured every 15–30 min. Time point by the culture reaches OD 0.4–0.5 can be estimated by fitting the plot of the measured OD values by an exponential function. These steps can be necessary as the culture can rapidly reach and surpass the desired OD values.
   d. Thaw the vcsM13 helper phage (prepared in the step 4.k of "before you begin", 100 μL, 5 × 10^{12} pfu/mL)) at 23°C.
   e. At an OD_{600} value of 0.4–0.5, add 100 μL vcsM13 helper phage into each flask. Continue to shake the flasks with a speed of 200–220 rpm at 37°C for 30 min.
   f. Add 210 μL kanamycin (100 mg/mL) into each flask to select for cells that have been infected by the helper phage. Continue to shake the flasks with a speed of 200–220 rpm at 37°C for 3.5 h.

6. Harvesting the phagemid particles
   In the presence of the vcsM13 helper phage, the recombinant p3024 phagemid is amplified in the single-stranded form and packed into phagemid particles, the majority of which are secreted into the medium.
   a. Prepare two clean 1-L centrifuge bottles.
   b. Transfer the medium culture in step 5.f to the two bottles. Balance, and then centrifuge at 4,000 rcf for 15 min at 4°C.
   c. Prepare a clean 1-L glass beaker.
   d. Collect the supernatant (where the phagemid particles are) from step 6.b by pouring it into the beaker. Discard the pellet containing bacteria.

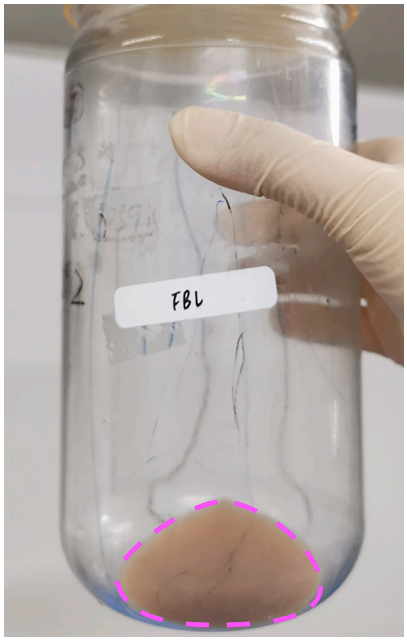

Figure 6. Normal bacterial pellet after centrifugation
The pellet is highlighted with magenta dashed-lines.

Figure 7. Semitransparent solution after dissolving of PEG8000 and NaCl

Note: The shape, color, and cleanness of the pellet can be used to judge the quality of the phage amplification process. An example with normal bacterial pellet after centrifugation is shown in Figure 6. See troubleshooting, problem 1 for abnormal pellet.

e. Weigh 24 g PEG8000 and 18 g NaCl on a balance, and add them into the supernatant. (Per 1 L supernatant, add 40 g PEG8000 and 30 g NaCl.)
f. Place the mixture on a magnetic stirrer for 30-min agitating at 23°C. After the solute (PEG8000 and NaCl) is completely dissolved, an example of normal solution (semitransparent) is shown in Figure 7. See troubleshooting, problem 2 for abnormal solution.

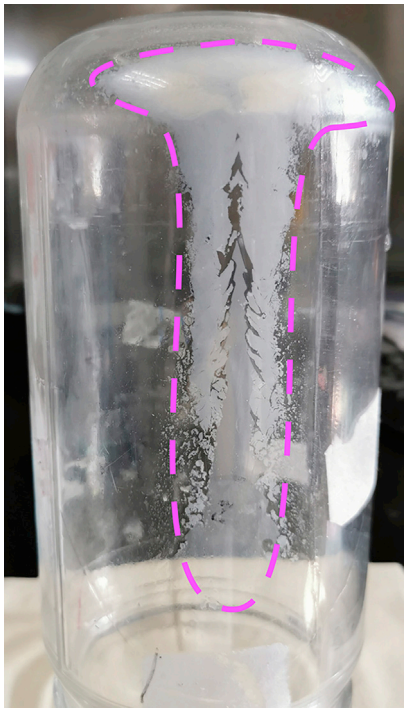

Figure 8. An example showing the pellet of phagemid particles
The pellet is highlighted with magenta dashed-lines.

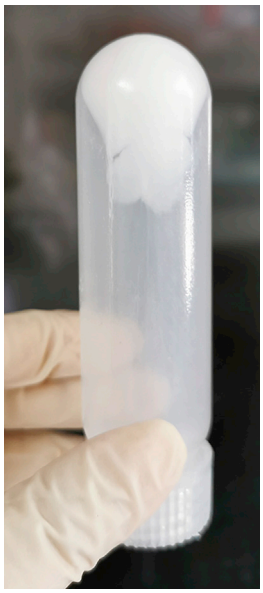

Figure 9. An example showing a lipid/protein pellet

g. Prepare two clean 500-mL centrifuge bottles.
h. Transfer the solution in step 6.f to the two bottles. Balance the bottles, incubate them in ice-water bath for 30 min, and centrifuge at 5,000 rcf for 30 min at 4°C. The recombinant p3024 phagemid particles are precipitated in this step. After discarding the supernatant, an example of the pellet of phagemid particles is shown in Figure 8.
i. Resuspend the pellet in TE buffer. Usually for the pellet generated from every 300 mL culture, we use 4 mL TE buffer.

Note: a) Using the 1-mL pipette to repeatedly blow the buffer onto the pellet can facilitate its dissolving. b) More buffer solution can be used to dissolve the pellet of larger size.

j. Centrifuge the suspension at 16,000 rcf for 10 min at 4°C to remove undissolved substances.
k. Prepare two sterile 50-mL centrifuge tubes.
l. Pipette the supernatant in step 6.j carefully and transfer into the 50-mL tubes for storage at 4°C.

Day 8:

7. Stripping proteins off the p3024 recombinant phagemid particles
   a. For every 4 mL TE buffer (containing recombinant phagemid particles) in a 50-mL centrifuge tube, add 8 mL PPB2 solution and gently shake the mixture by hands at 23°C for 3 min.
   b. For each tube, add 6 mL PPB3 solution to the mixture. Screw up the cap and turn the tubes upside down a few times to thoroughly mix the solution.
   c. Incubate the tubes in an ice-water bath for 10 min.
   d. Balance the tubes, and centrifuge them at 16,000 rcf for 30 min at 4°C.

Note: The denatured proteins and lipids are precipitated during this step, while the phagemid ssDNAs are left in the supernatant. An image of a lipid/protein pellet is shown in Figure 9.

   e. Prepare another two 50-mL centrifuge tubes.

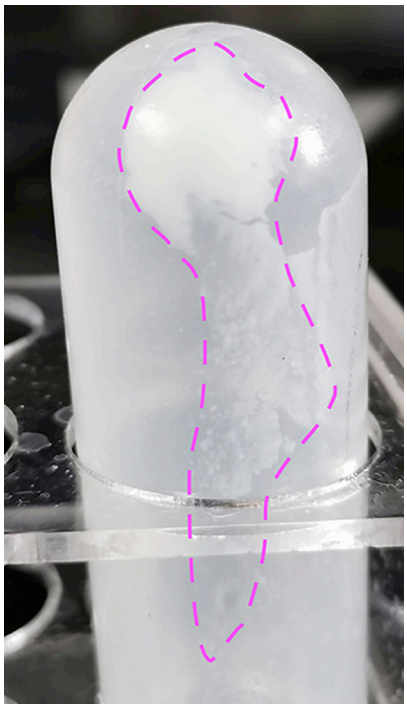

Figure 10. An example showing the phagemid ssDNA pellet
The pellet is highlighted with magenta dashed-lines.

f. After centrifugation in step 7.d, pipette the supernatant carefully to transfer to the new 50-mL centrifuge tubes, and discard the sediment. The volume of supernatant in each tube should be around 18 mL.

Note: Do not suck the sediment into the supernatant. Otherwise the purity of the phagemid ssDNA products would be affected. In case the supernatant is contaminated with the sediment after transferring, repeat steps 7.d–f.

8. Recovering the p3024 recombinant phagemid ssDNA
   a. For each tube from step 7.f (18 mL solution), add 18 mL 100% ethanol (v/v: 1/1).
   b. Screw up the cap, and vortex the tubes vigorously for thorough mixing.
   c. Incubate the tubes in an ice-water bath for 30 min.
   d. Centrifuge the tubes at 16,000 rcf for 30 min at 4°C.
   e. Discard the supernatant. For each tube, wash the pellet (phagemid ssDNA) with 20 mL 75% ethanol (ice-cold) by slow pipetting to further remove salts that are co-precipitated with the DNA.
   f. Centrifuge the tubes at 16,000 rcf for 15 min at 4°C.
   g. Discard the supernatant.
   h. Place the centrifuge tubes upside down on a tube holder to remove the supernatant residue and allow the ssDNA pellet air dry. This takes about 1 h. Apparent white pellet should be visible at the bottom and inner wall of the tube as shown in Figure 10.
   i. For each tube, dissolve the dried products (recombinant p3024 phagemid ssDNA) with 2 mL Deoxyribozyme reaction buffer 1.
   j. Combine solution from two tubes (2 × 2 mL) and transfer into a 15-mL Eppendorf tube.
   k. Use Nanodrop to quantify the ssDNA concentration. Keep the sample at 4°C for short-term (days) storage or at −20°C for long-term (years) storage. See troubleshooting, problem 3 for the potential problem.

Note: Usually a concentration of several hundred to over a thousand ng/μL is read out by Nanodrop. In other words, the yield of phagemid ssDNA is commonly around 1–10 pmol per 1 mL cell culture.

Isolation of target ssDNA from the recombinant phagemid ssDNA by deoxyribozyme-guided site-specific hydrolysis

Timing: 2 days

Day 9:

9. Cleavage of recombinant p3024 phagemid ssDNA by deoxyribozymes to release the target ssDNA
   a. Convert the concentration (C) of phagemid ssDNA from ng/μL to nM. This is calculated with the following equation: $C_{nM} = 10^6 \times C_{ng/\mu L} \div$ Molecular weight of phagemid ssDNA, wherein the Molecular weight of phagemid ssDNA roughly equals to (330 × the number of bases) g/mol.
   b. Resuspend the pre-ordered DNA enzyme strands (**E1&2** in Table 1) in ddH_2O to a concentration of 100 μM each.

Note: To cleave ssDNA at a specific site, the deoxyribozymes can be designed to attack either *in cis* or *in trans* (Figure 2). In this protocol, we choose the trans-attacking as an example, in which short, synthetic DNA enzyme strands must be added to hybridize to the deoxyribozyme substrate sites for DNA cleavage (Figure 11). If the cis-attacking mode was chosen, then the entire deoxyribozyme sequence would be programmed in the recombinant phagemid ssDNA, and no extra DNA enzyme strands would be needed (Praetorius et al., 2017). The pros and cons of the cis- and trans-attacking can be found in Praetorius et al. (2017) and Jia et al. (2021).

Reagent	Original concentration	Volume	Quantity
Phagemid ssDNA	700 nM	4 mL	2800 pmol
E1	100 μM	84 μL	8400 pmol
E2	100 μM	84 μL	8400 pmol
Deoxyribozyme reaction buffer 1	N/A	23.832 mL	N/A
Total	**N/A**	**28 mL**	**N/A**

   c. Use a molar ratio of 3:1 for enzyme vs substrate to construct the reaction system. The enzyme refers to the DNA enzyme strand (**E1&2** in Table 1), while the substrate refers to the recombinant p3024 phagemid ssDNA (Figure 11). For example, the 4 mL phagemid ssDNA solution from step 8.k was measured with a concentration of ~700 nM. That means totally we have 2.8 nmol phagemid ssDNA. It requires 8.4 nmol E1 and 8.4 nmol E2, or 84 μL of each synthetic DNA enzyme strands (100 μM), for digestion at the specific sites.
   d. Prepare a 100 mL glass beaker.
   e. Pour 4 mL phagemid ssDNA solution into the beaker. Add 84 μL of E1 and 84 μL of E2 to the beaker. Adjust the total volume of the sample to 28 mL with the Deoxyribozyme reaction buffer 1, which corresponds to a concentration of phagemid ssDNA of 100 nM.
   A recipe for digestion of phagemid ssDNA with deoxyribozymes

⚠ CRITICAL: The deoxyribozymes we used here are Zn^{2+} dependent and have a sharp Zn^{2+} range (Gu et al., 2013). This metal ion is included in the Deoxyribozyme reaction buffer 2. High concentration of DNA is known to reduce the free Zn^{2+} concentration in the solution

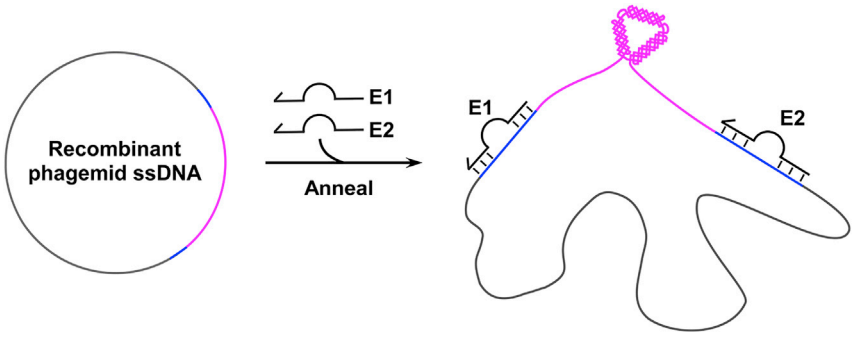

Figure 11. An example showing the trans-attacking by synthetic DNA enzyme strands
The target ssDNA (magenta) produced here is programmed to be able to self-fold into a triangular shape.

due to the non-specific binding of Zn^{2+} to DNA backbone. In our case, this will lead to the inefficiency of deoxyribozyme-guided hydrolysis of DNA. To avoid this issue, it is necessary to control the concentration of phagemid ssDNA in the deoxyribozyme-digestion system. Based on our experience, it is better to keep the concentration of phagemid ssDNA in the Deoxyribozyme reaction buffer 1 no more than 100 nM.

f. Place the beaker on a heater, and heat sample near to boiling (with a couple of bubbles generated from the bottom of the solution).
g. Take the beaker off the heater. Cover the beaker with Saran wrap and leave it at 23°C for cooling down. It usually takes about 1 h.

Note: The steps 9.f–g are annealing steps to allow the deoxyribozyme enzyme strands (E1&2) to hybridize to the corresponding substrate locations on the recombinant p3024 phagemid ssDNA, getting ready for the DNA-catalyzed DNA hydrolysis.

h. Add an equal volume (28 mL) of the Deoxyribozyme reaction buffer 2 to the beaker. Place the sample in a thermostatic chamber with the temperature setting at 37°C for ~12-h incubation.

Note: The target ssDNA fragment is released from the recombinant p3024 phagemid ssDNA by the DNA-catalyzed DNA hydrolysis in this step.

Day 10:

10. Purification of the target ssDNA
    a. Prepare a 250-mL centrifuge tube.
    b. Pour the sample from step 9.h into the tube. Add 3 volumes (168 mL) of 100% ethanol, and vortex to mix the solution thoroughly.
    c. Bury the tube in dry ice for 30 min.
    d. Balance the rotor and centrifuge the tube at 16,000 rcf for 30 min at 4°C.
    e. Discard the supernatant.
    f. Wash the white precipitate with 50 mL 75% ethanol (pre-cooled at 4°C).
    g. Balance the rotor and centrifuge the tube again at 16,000 rcf at 4°C for 10 min.
    h. Discard the supernatant.
    i. Place the 250-mL centrifuge tube upside down to remove the supernatant residue and allow the ssDNA pellet air-dried. This takes about 1 h.
    j. With 8 mL ddH_2O, rinse the inner wall of the tube where the DNA pellet is. Use 1 mL pipette to repeatedly blow water onto the wall to completely dissolve the DNA.
    k. Transfer 8 mL DNA solution into a 50-mL Eppendorf tube.
    l. Prepare 1% denaturing agarose gels. See the "prepare reagents and buffers" section for the detailed recipe.

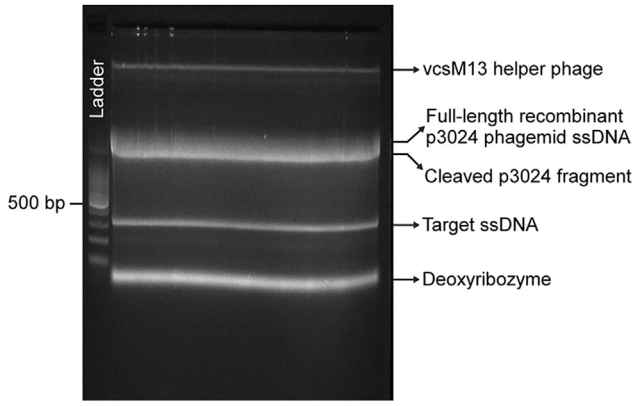

Figure 12. A typical gel image for deoxyribozyme-assisted digestion of phagemid ssDNA

Alternatives: a) A general 1% agarose gel without the denaturing content (urea) also works for the purification. However, its resolution will not be as good as that of the 1% denaturing agarose gel, because the hybridization between the deoxyribozyme enzyme and substrate strands will lead to smearing of the cleaved ssDNA fragments under non-denaturing conditions. b) For milligram level of DNA, anion exchange chromatography can be used to separate the cleaved target ssDNA from the phagemid ssDNA vector.

m. Add 8 mL 2× denaturing loading buffer to the DNA sample in step 10.k. Shake the sample by hands for thorough mixing.

n. Load the sample on denaturing agarose gels. Run the gels at 6 volts/cm at 4°C for ~2 h.

Note: a) Normally on a 12 cm × 8 cm × 0.8 cm (length × width × height) agarose gel, about 1 mL of the DNA sample in step 10.m can be loaded. Therefore to purify all 16 mL of the sample, multiple gels are required. Based on our experience, loading less than 200 pmol of the recombinant p3024 phagemid ssDNA on one agarose gel can guarantee the separation quality. Over-loading of DNA will decrease the resolution of electrophoresis. b) Agarose gels of a large size can be used to minimize the working load. c) Running the gels at low temperatures is suggested. High temperatures tend to cause melting of urea-containing gels.

o. Scan the gels using the Molecular Imager Gel Doc XR+ (Bio-Rad). An example gel image is shown in Figure 12. See troubleshooting, problem 4 for the potential problem.

⚠ CRITICAL: There should be 4–5 visible bands on the gel. The vcsM13 helper phage ssDNA (~8,600 nt) usually displays on the top. Depending on the size of the target ssDNA, it can be shown above or below the recombinant p3024 phagemid ssDNA. In our experiment, the target ssDNA is ~600 nt. Therefore its corresponding band is below the recombinant p3024 (3,600 nt) and the cleaved p3024 fragment (~3,000 nt). Because the lengths of the recombinant p3024 and the cleaved p3024 fragment are too close, they are not separated very well on the gel and presented as one broad band. The deoxyribozyme enzyme strands usually run to the bottom of the gel. Loading a DNA ladder into the gel can help to judge which band is supposed to be the target ssDNA.

p. Using razor or scalpel, excise gel containing a target DNA fragment under UV irradiation, and recover the ssDNA with the DNA recovery kit (see key resources table).

Alternatives: Place the gel band into a dialysis pocket (SpectraPor biotech dialysis membrane, MWCO: 8–10 kD) with 1× TAE buffer. Seal the pocket and place it in an agarose gel-running chamber filled with 1× TAE buffer. Slowly run DNA out of the gel into the buffer in the pocket for ~1 h. Open the dialysis pocket and pipette the TAE buffer out. Precipitate DNA with ethanol to collect the purified target ssDNA products.

Note: The deoxyribozyme-assisted excision of the target ssDNA off the recombinant phagemid is based on complementation of the DNA enzyme strands to the substrate regions on phagemid DNA. This complementation still exists after DNA cleavage, thus could result in the contamination of the target ssDNA with DNA enzyme strands if denaturation is incomplete. A second-time gel purification of the collected target ssDNA helps to further remove the potential contaminants of DNA enzyme strands, if high-quality of target ssDNA is critical to the downstream applications.

q. Use Nanodrop to measure the concentration of the target ssDNA. Store the sample at −20°C for later use.

Note: a) Usually we obtain yields of 10% to 20% after deoxyribozyme digestion and target-DNA purification. For the 2.8 nmol recombinant p3024 phagemid ssDNA extracted from 600 mL cell culture, eventually we collected 587 pmol (~0.1 mg) of the target ssDNA (~600 nt in length), which corresponds to an ~21% yield. b) To produce more quantities of target ssDNA, one can simply start with more shake flasks of cell culture, or transfer to fermenter for more efficient culturing and amplification of the recombinant phagemid. c) The cost of ssDNA production with this approach has been estimated to be ~$100 per 1 mg ssDNA with shake flask, and ~$200 per 1 g ssDNA with fermenter (Jia et al., 2021).

Quality check of the target ssDNA

Timing: 1 day

The target ssDNA we prepared was designed to be able to self-fold into a triangular shape. Therefore its quality can be readily checked by atomic force microscopy (AFM). We conducted the characterization with a Multimode VIII microscope with ScanAsyst-Fluid + tips in the ScanAsyst mode in fluid. Users can design the sequence of target ssDNA for other purposes, including ssDNA-mediated targeted gene knockin and so on, with other characterization methods to check its quality. For example, one can use the next-generation sequencing (NGS) technology to evaluate the error/mutation rates of the produced ssDNA target, or use the chromatography-based separation method to check the purity of the produced ssDNA target. In this protocol, we focus on AFM characterization of the shapes formed by the target ssDNA in the following steps.

Reagent	Final concentration	Volume added (μL)
Target ssDNA (139.57 ng/μL)	50 nM	7
10× TAE (125 mM $MgCl_2$)	1× TAE (12.5 mM $MgCl_2$)	10
ddH_2O	N/A	83
Total /μL	**N/A**	**100**

Day 11:

11. Characterization of the ssDNA triangle by AFM
    a. Prepare a 0.6 mL Eppendorf tube.
    b. Add each component into the tube according to the table below:
    c. Use a PCR thermal cycler to perform fast annealing of the sample: 90°C for 3 min, 75°C for 5 min, 60°C for 5 min, 45°C for 5 min, and 22°C for 5 min. Store the sample at 4°C.
    d. Do a 10× dilution with the 1× TAE buffer (12.5 mM $MgCl_2$) to adjust sample's concentration to 5 nM.
    e. Deposit 5 μL of the 5 nM sample onto the freshly cleaved mica surface and leave to adsorb for 5 min.
    f. Add 25 μL of the 1× TAE buffer (12.5 mM $MgCl_2$) and 5 μL of 100 mM $NiCl_2$ onto the mica.

Table 3. Recovery rate of ssDNA after purification

	Recombinant p3024 ssDNA	Target ssDNA after 1st purification	Target ssDNA after 2nd purification
Length of ssDNA (nt)	3538	592	592
Concentration (ng/μL)	827.25	286.83	139.57
Concentration (μM)	0.7085	1.468	0.7144
Volume (mL)	4	0.4	0.4
Total amount (pmol)	2834.1	587.28 (20.7% yield)	285.76 (10.1% yield)

g. Scan the sample with a peak force set point of 0.01 N, peak force amplitude of 50 nm, and peak force frequency of 2 kHz. A representative image is presented in the section "expected outcomes".

EXPECTED OUTCOMES

We normally gain 1–10 pmol of the recombinant p3024 phagemid ssDNA from per 1 mL cell culture. In the example shown in this protocol, we obtained 2834.1 pmol of the recombinant p3024 phagemid ssDNA from the total 600 mL cell culture. After deoxyribozyme digestion and gel purification, about 10%-to-20% of the target ssDNA fragments were recovered. Details can be found in the following table (Table 3):

We designed our target ssDNA to be able to self-fold into a triangle. Under AFM, triangular shapes are expected for visualization, which is shown in Figure 13. More than 95% of the products display as triangles. See troubleshooting, problem 5 for the potential problem.

For the trans-acting deoxyribozyme, targeting of the DNA enzyme strands to their recognition sites is achieved through hybridization to the flanked programmable sequence. By simply varying the sequence, we could in principle have numerous DNA enzymes, with each of them designed to target a specific site. Thus we can expect to harness this feature to excise individual ssDNA segments from the tandemly arranged ssDNA amplicons in a controllable fashion, by using the corresponding set of

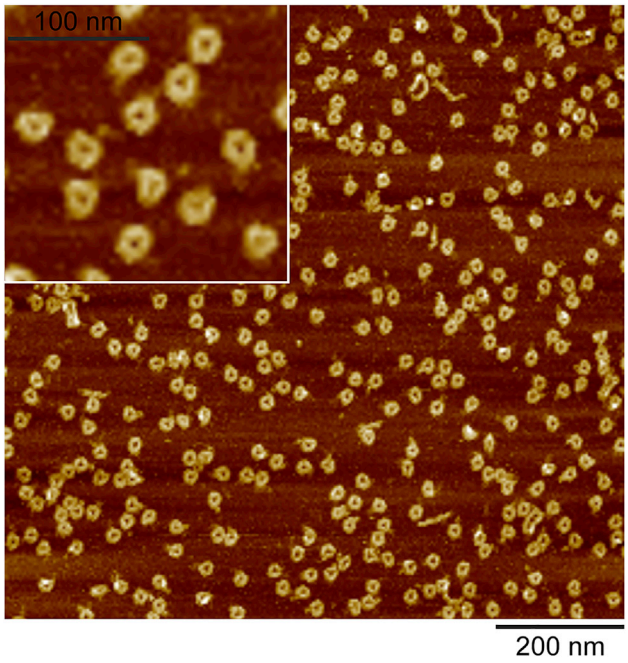

Figure 13. AFM imaging of ssDNA triangles

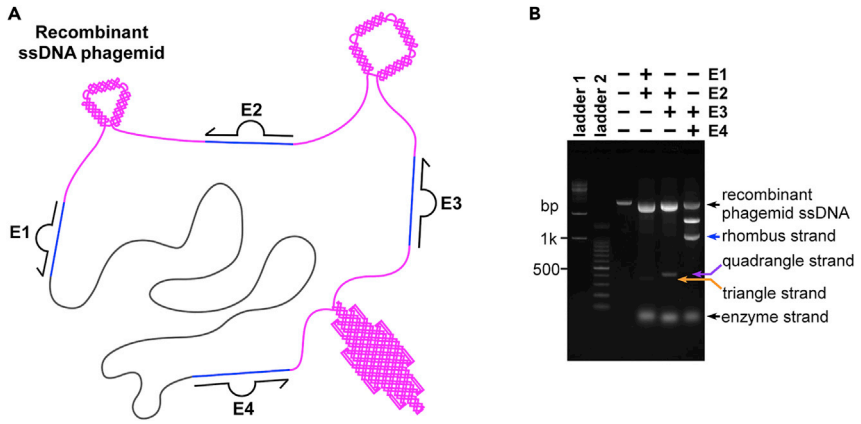

Figure 14. Controlled production of the ssDNA target from tandemly arranged ssDNA amplicons

(A) Tandem arrangement of ssDNA targets in the recombinant ssDNA phagemid. The three ssDNA targets are designed to be able to self-fold into a triangle (592 nt), a quadrangle (758 nt), and a rhombus (2395 nt). They are connected one by one, with the interconnection sequences programmed to be targeted by the *trans*-cleaving deoxyribozymes E1-E4.

(B) Selective processing of the individual ssDNA targets shown by denaturing agarose gel electrophoresis. Excision of specific ssDNA targets from the recombinant phagemid is achieved by using different combinations of the deoxyribozymes E1-E4.

DNA enzyme strands (Figure 14). When several ssDNA targets need to be prepared, amplifying them in tandem and selectively processing them should save time and cost. In addition, controlled release of ssDNA target one at a time avoids the co-existing of two targets with similar molecular weights in a system, which would be hardly separable by any DNA-purification method.

Besides the triangles, the quadrangles and rhombuses produced by selective processing of the tandemly arranged ssDNA amplicons (Figure 14) are expected for visualization under AFM (Figure 15).

LIMITATIONS

The helper phage-phagemid amplification system has an ssDNA-length capacity limitation. It has been reported that the phagemid can carry inserted DNA fragments with a length up to ~30,000 nt (Chen et al., 2018). Thus the current system likely can produce ssDNA with a length of no more than 30,000 nt.

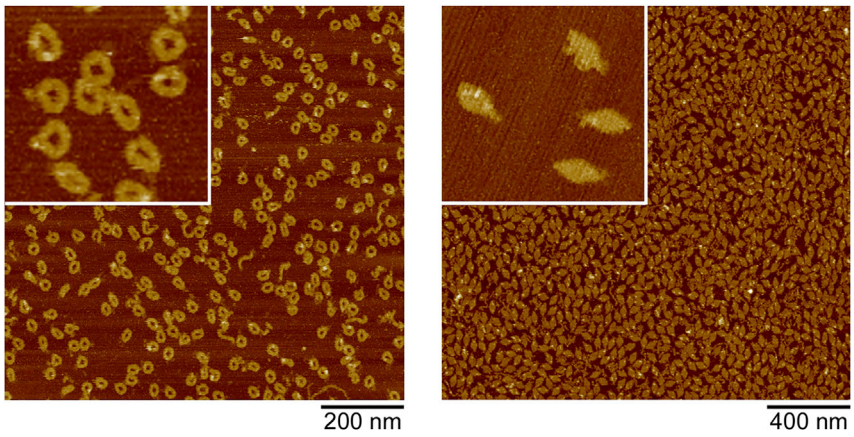

Figure 15. AFM imaging of ssDNA quadrangles (left) and rhombuses (right)

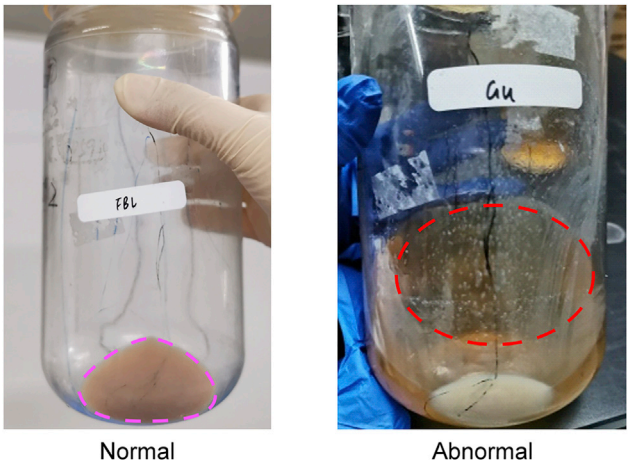

Figure 16. Comparison between the normal and abnormal bacterial pellet
The noteworthy areas are highlighted with colored dash-lines.

Although very robust, the deoxyribozymes do not possess decent sequence generality, which leads to "scar" sequence at the 5′ and 3′ ends of the target ssDNA. For example, using the robust class I deoxyribozyme (Gu et al., 2013) to hydrolyze DNA will leave at least 2 scar nucleotides at the 5′ end and 5 scar nucleotides at the 3′ end. The scars will not affect the nanostructure folding displayed in this protocol. But it may limit other applications that rely on the identities of the terminal sequence. We expect to overcome this limitation by identifying DNA-hydrolyzing deoxyribozymes with better sequence generality.

A separate room and circulatory system for phage/cell culturing is highly recommended. Phage contamination can be a critical issue for labs working with bacteria.

TROUBLESHOOTING

Problem 1

During the harvesting of the phagemid particles (step 6), the bacterial pellet seems abnormal. Normal pellet has a clear edge and sits at the bottom (Figure 16). If a fuzzy edge was displayed or the pellet spread over the tube wall, it refers to a bacteriolytic phenomenon.

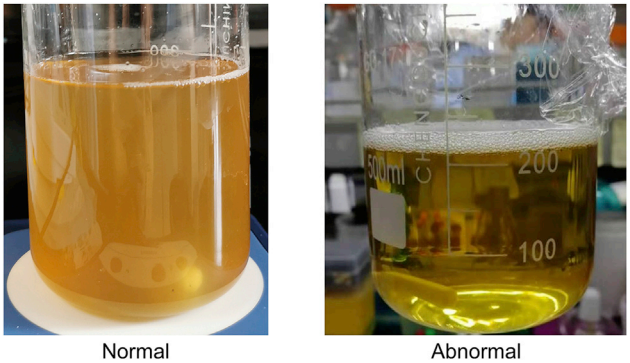

Figure 17. Comparison between the normal and abnormal solution after dissolving of PEG 8000 and NaCl

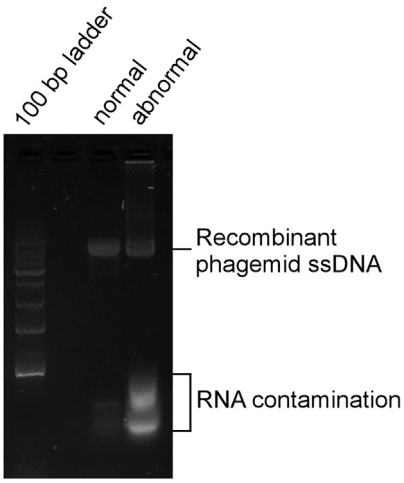

Figure 18. Comparison between the normal and abnormal phagemid ssDNA

Potential solution

The cell medium may be contaminated during inoculation and culturing. Eliminate potential contaminants. Execute the UV disinfection to ensure a clean culturing environment. Meantime, decrease the dosage of the vcsM13 helper phage to one-third or one-fifth of previously suggested.

Problem 2

The amplification of phagemid particles is unsuccessful (step 5). After the removal of bacterial cells from the culture, the supernatant is supplemented with PEG8000 and NaCl for the precipitation of phagemid particles. Once the two chemicals are completely dissolved, the solution should look semitransparent. If it was completely transparent (Figure 17), it is abnormal and means a failure of the amplification.

Potential solution

Follow the steps in ''Amplify and harvest vcsM13 helper phage''. Prepare fresh vcsM13 helper phage stock, and determine its titer according to the instructions in the section of ''Prepare helper phage stock''. In addition, make sure the correct antibiotics are used during (helper) phage amplification and bacterial cell culturing.

Problem 3

There are plenty of short nucleic acid fragments in the collected recombinant p3024 phagemid ssDNA (Figure 18), resulting in the inaccuracy of the estimated concentration of phagemid ssDNA and low yields of the recovered target ssDNA fragment eventually (step 8). The short nucleic acids mainly refer to RNAs that are released from the lysis of bacterial cells. High level of RNA contamination induced by cell lysis is considered as abnormal.

Potential solution

During cell culturing, reduce the shaking speed from 250 rpm to 220–200 rpm or even lower. Make sure the temperature is maintained at no more than 37°C. Decrease the dosage of the vcsM13 helper phage to one-third or one-fifth of previously suggested. Meantime, eliminate potential contaminations that could lead to cell lysis.

Problem 4

During the purification of ssDNA target, the corresponding DNA band is hardly visible, while the band intensity of the recombinant phagemid ssDNA precursor is very strong. This refers to an incomplete DNA cleavage by the deoxyribozymes (step 10).

Potential solution

Check the sequence of deoxyribozymes, make sure at least 10 base-pairs are designed in the complementary region, and try to extend the length of complementation to 15 bp or even more. Check the pH values of Deoxyribozyme reaction buffer 1 & 2, make sure they are around 7.0 with a fluctuation of less than 0.1 unit, because these deoxyribozymes are very pH sensitive. Also check the concentration of the recombinant phagemid ssDNA in the reaction buffer, make sure its final concentration is no more than 50 nM. DNA is known to bind Zn^{2+} non-specifically. High concentration of DNA will lead to significantly decrease in free Zn^{2+} level, resulting in the inefficiency of the Zn^{2+}-dependent deoxyribozymes.

Problem 5

The purity of the ssDNA target is less than 90%. Besides the ssDNA target, there seems to be some other nucleic acids in the purified sample. This means that very likely there is a purification issue (step 10).

Potential solution

After the cleavage of DNA by deoxyribozymes, remove the majority of metal ions in the buffer by precipitating DNA with ethanol. Make sure the DNA pellet is resuspended in a denaturing buffer to separate the excised ssDNA target from the phagemid precursor. Run DNA sample on a denaturing gel, e.g., an agarose gel with 1–2 M urea or a PAGE gel with 8 M urea, to purify the target ssDNA. During gel purification, do not overload DNA samples, which will decrease the gel's resolution. If all of those had been tried and the purity had not been improved, a second round of purification is recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hongzhou Gu, hongzhou.gu@fudan.edu.cn

Materials availability

The engineered DNA templates, including the recombinant phagemids, for deoxyribozyme-assisted ssDNA nanostructure production in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if it is potentially used for commercial production.

Data and code availability

This study did not generate datasets or codes.

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AUTHOR CONTRIBUTIONS

J.L. performed all of the experiments to support the protocol, analyzed the data, and prepared the manuscript. H.G. initiated the project, conceived the method principle and design, and prepared the manuscript.

DECLARATION OF INTERESTS

A China patent (ZLYY239-201032A) on large-scale production of ssDNA nanostructures with DNA-cleaving DNAs has been filed.

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