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Modular System for the Construction of Zinc-Finger Libraries and Proteins

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Abstract

Engineered zinc-finger transcriptional factors (ZF-TF) are powerful tools to modulate the expression of specific genes. Complex libraries of ZF-TF can be delivered into cells to scan the genome for genes responsible for a particular phenotype or to select the most effective ZF-TF to regulate an individual gene. In both cases, the construction of highly representative and unbiased libraries is critical. In this work we describe a novel, user-friendly, zinc-finger technology suitable for the creation of complex libraries and the construction of customized ZF-TFs. The novel technology described here simplifies the building of zinc-finger libraries, avoids PCR-introduced bias, and ensures equal representation of every module. We also describe the construction of a customized ZF-TF that can be transferred to a number of expression vectors. This protocol can be completed in 9 to 11 days.

Introduction

Zinc-finger (ZF) domains of the Cys₂-His₂ class are among the most common DNA-binding motifs found in eukaryotes. Each 30-amino acid domain contains a single amphipathic helix responsible for binding three base pairs of DNA through the formation of specific contacts^{1, 2}. These domains can be covalently linked into multi-modular proteins to recognize longer DNA sequences. When fused to effector domains such as transcriptional activators and repressors³⁻¹⁷, methylases¹⁸⁻²¹, recombinases^{22,23}, transposases²⁴, integrases²⁵, nucleases²⁶⁻³⁶, or other domains³⁷⁻⁴⁰, ZF domains can be used to direct transcription (up-regulation or down-regulation of specific genes), modify genes (targeted mutations, gene repair, epigenetic modification, or gene replacement), or as serve as novel diagnostic tools. Six-finger proteins specifically recognize 18 base pair DNA sequences, in theory, a long enough region to be unique in the human or any other genome^{11,41}. This specificity has been demonstrated in transgenic plants and human cells using array analysis^{8,42,43}. Zinc-finger directed proteins have potential applications for the study of gene expression and function in normal and disease processes as well as for gene therapy for treatment of cancer and genetic disorders.

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Author Contributions B.G. and L.J.S. contributed equally to this work. C.F.B. conceived of the SuperZif library construction concept and directed the research. Y.Y., B.G. and C.F.B. designed the SuperZif vectors. R.P.F. designed and constructed the pSCV vector, modified the SuperZiFCNN vector and aided in library construction. B.G., L.J.S., and L.A. constructed and tested the libraries. The paper was written by L.J.S. with assistance from B.G., C.F.B. and R.F.

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Polydactyl Zinc-Finger Proteins and Libraries

We have developed a simple system for creating zinc-finger libraries and specific proteins based on three unique vectors containing all of the GNN³, ANN⁴, or CNN⁵ domains (Fig. 1, Supplementary Fig. 1, and Supplementary Sequence Archive (SSA)). This system will enable researchers who are not well versed in zinc-finger technology to easily assemble libraries and designed polydactyl zinc-finger proteins. The availability of these tools will greatly enhance the ability of scientists in the areas of gene therapy and gene regulation to specifically target a genomic site of interest.

Libraries of zinc-finger proteins have proven to be useful for selection of endogenous gene regulators⁴⁴⁻⁵⁴. Zinc-finger domains designed to bind a specific target may do so *in vitro*, but may not be able to bind the genomic target *in vivo*. Factors such as secondary structure, chromatin structure, or binding of other proteins may prevent the designed zinc-finger from binding to its intended genomic target. Using a library frees the researcher from choosing a predefined target site within a promoter. When an appropriate selection system is used in conjunction with the library, the zinc-finger transcriptional regulator that binds to the optimal region in the promoter can be discovered^{44,45,54}. This strategy may also result in the identification of novel, indirect gene regulators that may be useful for pathway discovery. Libraries can also be used for selection of specific phenotype changes to identify genes involved in normal and/or disease processes^{46,48,51,52}. The libraries created with this protocol can also be used in bacterial^{55,56}, yeast^{57,58}, and cell-free^{59,60} zinc-finger selection systems.

Polydactyl Zinc-Finger Construction Methods

Two protocols describing creation of polydactyl zinc-fingers have been published^{61,62}. The Wright *et al.* protocol⁶² describes modular assembly of zinc-finger proteins from a set of 140 zinc-finger modules utilized by the Zinc Finger Consortium Modular Assembly Kit. Most of the zinc finger modules used in this protocol and others are actually derived from zinc finger modules published by the Barbas laboratory. The Carroll *et al.* protocol⁶¹ describes a PCR-based method for constructing multi-finger proteins that are again larger derived from our reported domains. Both of these systems are good for constructing individual and designed zinc fingers. However, libraries created with these systems would probably not be representative of all possible zinc-finger domains (note: the published manuscripts do not claim that these systems can be used to make libraries). One of the potential pitfalls of the Wright *et al.* protocol is that each zinc-finger must be carefully mixed in equal molar quantities to ensure that the library is not biased and that numerous helices of redundant binding specificity are present. The Carroll *et al.* protocol utilizes PCR and thus mutations introduced due to polymerase fidelity and differential annealing of degenerate primers would bias libraries.

Maeder *et al.*⁶³ have used OPEN (Oligomerized Pool ENGINEering) to create pools of three-finger proteins that target a defined 9 bp site. These pools can then be combined to select zinc-finger proteins that bind a 9 bp target sequence in a bacterial two-hybrid system. This system was successful in selecting three-finger zinc-finger proteins that when fused to nucleases were functional in human and plant cells. However, this system hinges on the construction of hundreds of pools of zinc fingers and the selection of the correct pools. The pools are also limited to GNNs and some TNNs, which limits the available target sites.

The SuperZiF System

The system described in this protocol is ideal for creating zinc-finger libraries and designed polydactyl zinc-finger proteins. Three SuperZiF plasmids were synthesized in the pUCminusMCS vector (Blue Heron) containing all zinc-finger domains previously published and characterized; one SuperZiF plasmid each for the GNN³, ANN⁴, and CNN⁵ zinc-finger domains (Fig. 1, Supplementary Fig. 1, and SSA). For library assembly, *XhoI* and *XmaI*

restriction sites are 5' to each finger and *AgeI* and *SpeI* sites are 3' to each finger (Fig. 2, Supplementary Fig. 1, and SSA). When creating zinc-finger libraries, the SuperZiF plasmids can be cut with *XhoI* and *SpeI* to release each finger's coding sequence in equal molar quantities, thereby reducing library bias. When assembled into polydactyl zinc-finger proteins, each finger from the SuperZiF plasmid is equally represented at each position. If more than one SuperZiF plasmid is used to generate individual fingers, then the diversity of the potential binding sites for the library is increased accordingly. The SuperZiF zinc-fingers were built on the Sp1C backbone⁶⁴, which has shown enhanced stability, increased affinity, and better expression¹⁰ than the Zif268 backbone². To minimize repeated sequences, which may cause problems with recombination, plasmid propagation, and sequencing, the nucleotide sequence for the Sp1C backbone was varied without affecting the amino acid sequence (see SSA).

The SuperZiF system is also useful for engineering proteins to target specific DNA sequences. The Zinc Finger Tools⁶⁵ website (www.zincfingertools.org) can be used to identify potential zinc-finger target sites and to design polydactyl zinc-fingers that target those specific sites. A scoring function is incorporated to help the user choose which of the proposed zinc-finger proteins are likely to be the most specific for the selected DNA target. For constructing designed proteins, unique restriction sites were placed between each finger, enabling the isolation of any individual finger from the appropriate SuperZiF vector (Fig. 1). Individual fingers can also be isolated and saved during the first step of the library construction process for future use when assembling designed proteins.

First we describe the method used to create a standard six-finger library from a single SuperZiF plasmid (SuperZiFGNN). Next, we describe a modified version of that method to create a five-finger library with 12 GNN (no GNGs) and 15 ANN zinc-fingers, which covers more sequence space than a library where every fourth base is the same. This GNN/ANN five-finger library was successfully used to select zinc-finger proteins that were able to bind the γ -globin promoter and up-regulate the transcription of this gene⁵⁴. We have also included a method to create designed zinc-finger proteins from either the SuperZiF vectors or from preselected one-finger clones, which can easily be recovered from the first steps in the construction of a standard six-finger library. This method has been successfully used for the rapid assembly of chimeric zinc finger recombinases capable of transgene integration into the human genome with more than 98% accuracy⁶⁶.

Once the zinc-finger libraries or designed clones are constructed, they can be cloned into a variety of expression vectors using the *SfiI* restriction sites that flank the zinc-finger cassettes. Our group has constructed retroviral, lentiviral, and transient expression vectors with both transcriptional activators and repressors (Table 1, Supplementary Fig. 2 and SSA) designed for *SfiI* cloning of polydactyl zinc-fingers which are available upon request^{10,11,54,67}. An adapted pMAL-c vector (New England Biolabs)⁴¹ is also available for bacterial expression and purification via maltose-binding protein fusion. Using these vectors, selection methods can be employed to isolate zinc-finger transcription factors with the desired target specificity^{44,45,54} or phenotype change^{46,48,51,52}.

Materials

Reagents

SuperZiFGNN (Blue Heron, see Supplementary Fig. 1 and SSA)

SuperZiFANN (Blue Heron, see Supplementary Fig. 1 and SSA)

SuperZiFCNN (Blue Heron, see Supplementary Fig. 1 and SSA)

pSCV(see Supplementary Fig. 1 and SSA)

Expression vectors (see Table 1) (see Supplementary Fig. 2 and SSA)

Bacteria strain SS320 (F^- *lacI22 lacZ pro-48 met-90 trpA trpR his-85 rpsL azi-9 gyrA* λ^- *PI^s*; a gift from Dr. Sachdev S. Sidhu at Genentech⁶⁸. Electrocompetent SS320 cells are available from Lucigen, 60512-1)

Bacteria strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'**proAB lacI^qZAM15 Tn10* (Tet^r)]; Stratagene, 200228)

Restriction enzymes (from New England Biolabs): *XhoI* (R0146L), *Spe*(R0133L)*I*, *SfiI* (R0123L) (20 U/ μ L), *AgeI* (R0552L), *XmaI*(R0180L), *NheI*(R0131L), *AccI*(R0161S), *AflIII*(R05205)

Restriction enzymes (from Roche): *EcoRI* (1175084), *SfiI*(1288059) (40 U/ μ L)

10 $\times$ restriction enzyme buffers (New England Biolabs)

T4 DNA Ligase and T4 DNA Ligase buffers (Invitrogen, 15224090)

Calf intestinal phosphatase (CIP, New England Biolabs, M0290L))

PureLink PCR Purification kit (Invitrogen, K310002)

QIAquick Gel Extraction kit (Qiagen, 28704)

Ultrafree-MC centrifuge filter units, 0.4 μ m (Millipore, UFC30HNVB) (For “Freeze and Squeeze”⁶⁹)

HiPure Plasmid Filter Maxiprep kit (Invitrogen, K210017)

pSCVseqF: 5'-GTA AAA CGA CGG CCA GTG AGC GC-3'

pSCVseqB: 5'- GAT ACC GCT CGC CGC AGC CGA AC-3'

100% ethanol

3 M Sodium acetate

Glycogen (Roche, 901393)

SB medium (see Reagent Setup)

SOC medium (see Reagent Setup)

Carbenicillin (Omega Scientific)

UltraPure agarose (Invitrogen, 16500500)

Tris-acetate EDTA (TAE)

Ethidium Bromide 10 mg/mL solution (EtBr) (Sigma-Aldrich, E1510) CAUTION – carcinogen, use proper personal protective equipment including gloves.

Equipment

Electroporation cuvettes, 2 mm gap (VWR, 89047-208)

Electroporator (BioRad)

Sterile microcentrifuge tubes

Sterile 14-mL culture tubes (Falcon)

Standard orbital shaker for growing bacterial cultures

Heating block or water bath for enzyme digestions

Sterile 250 mL centrifuge bottles
Electrophoresis system (Fisher Biotech)

Reagent Setup

SB medium (1 L):

10 g MOPS (hemisodium salt)
30 g Tryptone Peptone
20 g Yeast Extract

Dissolve in dH₂O, adjust volume, autoclave

Store at 4°C for up to 6 months

SOC medium (1 L):

20 g Tryptone peptone
5 g Yeast extract
0.5 g NaCl

Dissolve in 800 mL dH₂O

Add 10 mL of 0.25 M KCl

Adjust pH to 7.0

Add 10 mL of 1 M MgCl₂

Adjust volume to 1 L and autoclave

Add 1 mL of 1 M glucose

Store at 4°C for up to 6 months

The SuperZiF, pSCV, and expression plasmids listed in Table 1 are available from our laboratory upon request.

Procedure

Construction of a six-finger GNN library. Timing: 9 days

1| Create a one-finger library from the SuperZiFGNN plasmid. Digest 10 µg SuperZiFGNN with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours to create a 1ZF insert.

Component	Amount
SuperZiFGNN	10 µg
<i>XhoI</i> (20 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 2)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 5 µg pSCV with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV	5 µg
<i>XhoI</i> (20 U/µL)	1 µL
<i>SpeI</i> (10 U/µL)	1 µL
10× Buffer (NEB 2)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

2| Purify the digested SuperZiFGNN DNA fragment by electrophoresis on 2% agarose gel (1× TAE with EtBr). Cut out the 100-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

Purify the pSCV vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

3| Ligate purified pSCV vector and the 1ZF GNN insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess the library background (i.e., efficiency of digestion and dephosphorylation). Allow the ligation reaction to proceed overnight at room temperature (approximately 25°C).

Component	Ligation	Control
pSCV vector	100 ng	100 ng
1ZF GNN insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase (1 U/µL)	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL
Total	20 µL	20 µL

4| Ethanol precipitate the ligation reactions. Add 2 µL 3 M sodium acetate (NaOAc, pH ~5.2-6.0), 1 µL glycogen (1 mg/mL) (optional), and 50 µL of absolute ethanol. Let stand at -20°C for at least 1 hour. Centrifuge at ≥ 15,000 xg for 30 minutes. Remove supernatant and wash with 500 µL 70% ethanol. Centrifuge at ≥ 15,000 xg for 15 minutes. Remove supernatant and allow pellet to air-dry for 15 minutes. Resuspend DNA pellet in 10 µL nuclease-free water.

PAUSE POINT Ligations can be stored in the NaOAc/ethanol solution or after resuspension in water at -20°C indefinitely.

5| Transform 5 µL each of the ligation and control in 75 µL competent cells via electroporation in a 2-mm gap cuvette (2.5 kV, 15.0 µF, 200 Ohms). We recommend SS320⁶⁸ cells due to high competency needed for later stages, however at this stage, XL1Blue cells are acceptable.

6| After electroporation, add 1 mL SOC media each to the transformed ligation and control and transfer to 14-mL culture tubes. Incubate with agitation (250 rpm) at 37°C for 1 hour.

7| Plate 0.1 µL and 1 µL of the transformed ligation and 1 µL and 10 µL of the transformed control onto SB plates containing 100 µg/mL carbenicillin. Allow these to grow overnight at 37°C. Mix the remaining transformed ligation with 100 mL SB with 50 µg/mL carbenicillin. Incubate the culture with agitation (250 rpm) overnight at 37°C.

8| Count colonies on the transformed ligation and control plates. To ensure 10× coverage of the library 160 colonies per 1000 µL of SOC culture are required. The transformed ligation plates should have at least 10-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

9| Do a diagnostic digest for quality assurance of pSCV-1ZFGNN. Use the HiPure Plasmid Filter Maxiprep kit (Invitrogen) to recover the library of plasmid DNA from the 100 mL culture. Digest the library with *SfiI* for 1 hour at 50°C (shown below) to ensure the insert is the correct size. After digestion use electrophoresis on a 2% agarose TAE gel with EtBr to ensure that a 100-bp band fragment is released. We occasionally see multiple inserts (a 300 bp fragment released) at this step. Please see the troubleshooting guide for solutions to this problem.

Component	Amount
pSCV-1ZFGNN	100 ng
<i>SfiI</i> (20 U/µL)	0.5 µL
10× Buffer (NEB 2)	2 µL
10× BSA	2 µL
Nuclease-free water	to 20 µL
Total	20 µL

?TROUBLESHOOTING

PAUSE POINT Purified plasmid DNA can be stored at -20°C indefinitely.

10| (optional) It may be helpful to pick, miniprep, and sequence individual clones from the 1ZF library. This step ensures that the library is diverse and not contaminated by a single clone. These clones can also be saved and used for cloning designed multi-finger proteins.

Pick 20 colonies from the transformed ligation plates and inoculate 2.5 mL SB media containing 50 µg/mL carbenicillin. Shake (250 rpm) overnight at 37°C.

Prepare small-scale plasmid purifications (PureLink Quick Plasmid Miniprep kit from Invitrogen) from the overnight cultures and sequence with pSCVseqF primer.

PAUSE POINT Purified plasmid DNA can be stored at -20°C indefinitely

11| Create 2ZF GNN library. Digest SuperZifGNN with *XmaI* and *SpeI* (shown below) at 37°C for 4 hours to create a 1ZF insert.

Component	Amount
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SuperZifGNN	10 µg
<i>XmaI</i> (10 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
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Total	50 µL
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Also digest 5 µg pSCV-1ZFGNN with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a 1ZF vector. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-1ZFGNN	5 µg
<i>AgeI</i> (5 U/µL)	1 µL
<i>SpeI</i> (10 U/µL)	1 µL
10× Buffer (NEB 4)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
<hr/>	
Total	25 µL
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12| Repeat step 2 to purify the insert and vector. The insert fragment should be 100 bp.

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

13| Ligate purified 1ZF GNN vector and the 1ZF GNN insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess the library background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
1ZF GNN vector	100 ng	100 ng
1ZF GNN insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL
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Total	20 µL	20 µL
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14| Repeat steps 4 through 7.

15| Count colonies on the transformed ligation and control plates. To ensure 10× coverage of the library 2560 ($16^2 \times 10$) colonies per 1000 µL of SOC culture are required. The transformed ligation plates should have at least 10-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

16| Repeat steps 9 and 10. The fragment released from the library should be 200 bp. We occasionally see multiple inserts (a 400 bp fragment released) at this step. Please see the troubleshooting guide for solutions to this problem.

?TROUBLESHOOTING

17| Create 3ZF GNN library. Digest 5 µg pSCV-2ZFGNN with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a 2ZF vector. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-2ZFGNN	5 µg
<i>AgeI</i> (5 U/µL)	1 µL
<i>SpeI</i> (10 U/µL)	1 µL
10× Buffer (NEB 4)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

18| Purify the pSCV-2ZFGNN vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector can be stored at -20°C indefinitely.

19| Ligate purified 2ZF GNN vector and the 1ZF GNN insert (from step 11) using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess the library background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
2ZF GNN vector	200 ng	200 ng
1ZF GNN insert	100 ng	-----
5× T4 DNA Ligase Buffer	8 µL	8 µL
T4 DNA Ligase	2 µL	2 µL
Nuclease-free water	to 40 µL	to 40 µL
Total	40 µL	40 µL

20| Ethanol precipitate the ligation reactions. Add 4 µL 3 M sodium acetate (NaOAc, pH ~5.2-6.0), 1 µL glycogen (1 mg/mL) (optional), and 100 µL of absolute ethanol. Let stand at -20°C for at least 1 hour. Centrifuge at ≥ 15,000 xg for 30 minutes. Remove supernatant and wash with 500 µL 70% ethanol. Centrifuge at ≥ 15,000 xg for 15 minutes. Remove supernatant and allow pellet to air-dry for 15 minutes. Resuspend DNA pellet in 10 µL nuclease-free water.

PAUSE POINT Ligations can be stored in the NaOAc/ethanol solution or after resuspension in water at -20°C indefinitely.

21| Add 10 µL of the ligation mixture into 200 µL of competent cells and split into two cuvettes; transform via electroporation (2.5 kV, 15.0 µF, 200 Ohms). Transform 5 µL of the control in 100 µL competent cells via electroporation (2.5 kV, 15.0 µF, 200 Ohms). We recommend SS320⁶⁸ cells due to their high competency.

22| After electroporation, add 1 mL SOC media each to the transformed ligation and control and transfer to 15 mL culture tubes combining the two transformed ligation cuvettes. Incubate with agitation (250 rpm) at 37°C for 1 hour.

23| Make a 1:100 dilution of the transformed ligation culture in SOC media. Plate 2 µL and 20 µL of the diluted transformed ligation culture and the transformed control culture onto SB plates containing 100 µg/mL carbenicillin. Allow these to grow overnight at 37°C. Mix the remaining transformed ligation with 100 mL SB with 50 µg/mL carbenicillin. Incubate the culture with agitation (250 rpm) overnight at 37°C.

24| Count colonies on the transformed ligation and control plates. To ensure 10× coverage of the library 40,960 ($16^3 \times 10$) colonies per 2000 µL of SOC culture are required. The transformed ligation plates should have at least 20-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

25| Repeat steps 9 and 10. The fragment released from the library should be 300 bp. We occasionally see multiple inserts (a 500 bp fragment released) at this step. Please see the troubleshooting guide for solutions to this problem.

?TROUBLESHOOTING

26| Create 6ZF GNN library. Digest pSCV-3ZFGNN with *XmaI* and *SpeI* (shown below) at 37°C for 4 hours to create a 3ZF insert.

Component	Amount
pSCV-3ZFGNN	10 µg
<i>XmaI</i> (10 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 10 µg pSCV-3ZFGNN with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a 3ZF vector. Once this digestion is complete, add 2 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-3ZFGNN/ANN	10 µg
<i>AgeI</i> (5 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

27| Repeat step 2 to purify the insert and vector. The insert fragment should be 300 bp.

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

28| Ligate purified 3ZF GNN vector and 3ZF GNN insert using T4 DNA ligase (shown below). This master mix should be divided into three microcentrifuge tubes. Also set up a control ligation with no insert to assess the library background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
3ZF GNN vector	600 ng	200 ng
3ZF GNN insert	900 ng	-----
5× T4 DNA Ligase Buffer	48 µL	16 µL
T4 DNA Ligase	12 µL	4 µL
Nuclease-free water	to 240 µL	to 80 µL
Total	240 µL	80 µL

29| Ethanol precipitate the ligation and control reactions. Add 8 µL 3 M sodium acetate (NaOAc, pH ~5.2-6.0), 1 µL glycogen (1 mg/mL) (optional), and 200 µL of absolute ethanol. Let stand at -20°C for at least 1 hour. Centrifuge at $\geq 15,000 \times g$ for 30 minutes. Remove supernatant and wash with 1 mL 70% ethanol. Centrifuge at $\geq 15,000 \times g$ for 15 minutes. Remove supernatant and allow pellet to air-dry for 15 minutes. Resuspend DNA pellet in 10 µL nuclease-free water.

PAUSE POINT Ligations can be stored in the NaOAc/ethanol solution or after resuspension in water at -20°C indefinitely.

30| Transform all 30 µL of the three ligation reactions (5 µL + 100 µL of competent cells $\times$ 6 cuvettes) and 5 µL of the control in 100 µL competent cells via electroporation (2.5 kV, 15.0 µF, 200 Ohms). We recommend SS320⁶⁸ cells due to their high competency.

31| After electroporation, add 1 mL SOC media each to the transformed ligations and control and transfer to 14-mL culture tubes pooling two transformed ligation cuvettes into one culture tube (three ligation tubes and one control culture tube). Incubate with agitation (250 rpm) at 37°C for 1 hour.

32| Combine the three ligation cultures into one tube. Make a 1:100 dilution of the ligation culture in SOC media. Plate 0.6 and 6 µL of the diluted ligation culture and the control culture onto SB plates containing 100 µg/mL carbenicillin. Allow these to grow overnight at 37°C. Split the remaining transformed ligation culture into two 150-mL flasks of SB with 50 µg/mL carbenicillin. Incubate the culture with agitation (250 rpm) overnight at 37°C.

33| Count colonies on the transformed ligation and control plates. To ensure 10 $\times$ coverage of the library 1.7×10^8 ($16^6 \times 10$) colonies per 6000 µL of SOC culture are required. The transformed ligation plates should have at least 60-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

34| Repeat steps 9 and 10. The fragment released from the library should be 600 bp.

?TROUBLESHOOTING

Construction of a five-finger GNH/ANN library. Timing: 11 days

35| Create a one finger GNH (H= A, T, or C) library. Digest 10 µg of the SuperZifGNN plasmid with *NheI* and *EcoRI* (shown below) at 37°C for 4 hours to remove the GNGs from the vector.

Component	Amount
SuperZifGNN	10 µg
<i>NheI</i> (10 U/µL)	1.5 µL
<i>EcoRI</i> (20 U/µL)	1.5 µL
10× Buffer (NEB 2)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

36| Purify the digested SuperZifGNH DNA fragment by electrophoresis on 1.5% agarose gel (1× TAE with EtBr). Cut out the ~4550-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

PAUSE POINT Purified insert can be stored at -20°C indefinitely.

37| Digest the SuperZifGNH fragment with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours to create a 1ZFGNH insert.

Component	Amount
SuperZifGNN	2.5 µg
<i>XhoI</i> (20 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 2)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

38| Purify the digested GNH DNA fragment by electrophoresis on 2% agarose gel (1× TAE with EtBr). Cut out the 100-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

39| Digest, CIP, and purify the pSCV vector as instructed in steps 1 and 2.

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

40| Ligate the GNH insert and pSCV vector as instructed in step 3.

41| Perform steps 4 through 7 with the pSCV-GNH ligation.

42| Count colonies on the transformed ligation and control plates. To ensure 10× coverage of the library 120 colonies per 1000 µL of SOC culture are required. The transformed ligation

plates should have at least 10-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

43| Perform steps 9 and 10 with pSCV-1ZFGNH. We occasionally see multiple inserts (a 300 bp fragment released) at this step. Please see the troubleshooting guide for solutions to this problem. We highly recommend sequencing several clones from this library to check for possible GNG contamination.

?TROUBLESHOOTING

44| Create a one finger GNH/ANN library. Digest 10 µg of pSCV-1ZFGNH and 10 µg of SuperZiFANN with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours to create 1ZFGNH and 1ZFANN inserts.

Component	Amount
pSCV-1ZFGNH or SuperZifANN	10 µg
<i>XhoI</i> (20 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 2)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

45| Purify the digested GNH and ANN DNA fragments by electrophoresis on 2% agarose gel (1× TAE with EtBr). Cut out the 100-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

PAUSE POINT Purified inserts can be stored at -20°C indefinitely.

46| Ligate purified pSCV vector (from step 38) and the 1ZF GNH and ANN inserts using T4 DNA ligase (shown below). To ensure that each zinc-finger is present in equal amounts, the ratio of GNH to ANN should be 4:5. Also set up a control ligation with no inserts to assess the library background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
pSCV vector	100 ng	100 ng
1ZF GNH insert	22.2 ng	-----
1ZF ANN insert	27.8 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase (1 U/µL)	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL
Total	20 µL	20 µL

47| Perform steps 4 through 7 with the pSCV-GNH/ANN ligation.

48| Count colonies on the transformed ligation and control plates. To ensure 10× coverage of the library 270 colonies per 1000 µL of SOC culture are required. The transformed ligation plates should have at least 10-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

49| Perform steps 9 and 10 with pSCV-1ZFGNH/ANN. We occasionally see multiple inserts (a 400 bp fragment released) at this step. Please see the troubleshooting guide for solutions to this problem.

?TROUBLESHOOTING

50| Proceed as described in steps 11 through 25 to make pSCV-3ZFGNH/ANN, except at step 11 use the pSCV-1ZFGNH/ANN library to make the 1ZFGNH/ANN insert. The 2ZF library should have 7290 ($27^2 \times 10$) colonies per 1000 µL of SOC culture and the 3ZF library should have 1.96×10^5 ($27^3 \times 10$) colonies per 2000 µL of SOC culture.

?TROUBLESHOOTING

51| Create a 5ZF GNH/ANN library. Digest pSCV-2ZFGNHANN with *XmaI* and *SpeI* (shown below) at 37°C for 4 hours to create a 2ZF insert.

Component	Amount
pSCV-2ZFGNH/ANN	10 µg
<i>XmaI</i> (10 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 10 µg pSCV-3ZFGNH/ANN with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a 3ZF vector. Once this digestion is complete, add 2 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-3ZFGNN	10 µg
<i>AgeI</i> (5 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

52| Repeat step 2 to purify the insert and vector. The insert fragment should be 200 bp.

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

53| Ligate purified 3ZF GNH/ANN vector and 2ZF GNH/ANN insert using T4 DNA ligase (shown below). This master mix should be divided into three microcentrifuge tubes. Also, set up a control ligation with no insert to assess the library background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
3ZF GNH/ANN vector	600 ng	200 ng
2ZF GNH/ANN insert	900 ng	-----
5× T4 DNA Ligase Buffer	48 µL	16 µL
T4 DNA Ligase	12 µL	4 µL
Nuclease-free water	to 240 µL	to 80 µL
Total	240 µL	80 µL

54| Perform steps 29 through 34 to complete the construction of pSCV-5ZFGNH/ANN. To ensure 10× coverage of the library 1.4×10^8 ($27^5 \times 10$) colonies per 6000 µL of SOC culture are needed. The transformed ligation plates should have at least 60-fold more colonies than the control plates to ensure less than 10% background. It is not feasible to make a 6ZF-GNH/ANN library due to limitations of transformation efficiency ($27^6 \times 10 = 3.9 \times 10^9$).

?TROUBLESHOOTING

Construction of a designed six-finger clone (for example, recognizing 5'-GAA GAA GAA GAA GAA GAA-3') Timing: 13 days

55| Use Zinc Finger Tools website to design a polydactyl zinc-finger.

56| Finger 1 cloning: Digest 10 µg SuperZifGNN with unique enzymes that release the desire first zinc finger. As an example, use *AccI* and *AflIII* to cut out the GAA-specific helix and digest (shown below) at 37°C for 4 hours to create the ZF1 insert.

Component	Amount
SuperZifGNN	10 µg
<i>AccI</i> (10 U/µL)	1.5 µL
<i>AflIII</i> (20 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 5 µg pSCV with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV	5 µg
<i>XhoI</i> (20 U/µL)	1 µL

<i>SpeI</i> (10 U/μL)	1 μL
10× Buffer (NEB 2)	2.5 μL
10× BSA	2.5 μL
Nuclease-free water	to 25 μL
<hr/>	
Total	25 μL

57| Purify the digested ZF1 DNA fragment by electrophoresis on 2% agarose gel (1× TAE with EtBr). Cut out the 100-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

Purify the pSCV vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

58| Digest the purified ZF1 fragment with *XhoI* and *SpeI* (shown below) at 37°C for 4 hours to create a compatible ZF1 insert.

Component	Amount
Purified ZF1	2.5 μg
<i>XhoI</i> (20 U/μL)	1.5 μL
<i>SpeI</i> (10 U/μL)	1.5 μL
10× Buffer (NEB 2)	5 μL
10× BSA	5 μL
Nuclease-free water	to 50 μL
<hr/>	
Total	50 μL

Purify the *XhoI*/*SpeI* ZF1 insert with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified insert can be stored at -20°C indefinitely.

59| Ligate purified pSCV vector and the ZF1-compatible insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess the ligation background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
pSCV vector	100 ng	100 ng
ZF1 insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 μL	4 μL
T4 DNA Ligase (1 U/μL)	1 μL	1 μL
Nuclease-free water	to 20 μL	to 20 μL
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Total	20 μL	20 μL

60 Ethanol precipitate the ligation reactions. Add 2 μL of 3 M sodium acetate (NaOAc, pH ~5.2-6.0), 1 μL glycogen (1 mg/mL) (optional), and 50 μL of absolute ethanol. Let stand at -20°C for at least 1 hour. Centrifuge at ≥ 15,000 xg for 30 minutes. Remove supernatant and

wash with 500 μ L 70% ethanol. Centrifuge at $\geq 15,000$ xg for 15 minutes. Remove supernatant and allow pellet to air-dry for 15 minutes. Resuspend DNA pellet in 10 μ L nuclease-free water.

PAUSE POINT Ligations can be stored in the NaOAc/ethanol solution or after resuspension in water at -20°C indefinitely.

61| Transform 5 μ L each of the ligation and control in 75 μ L XL1-Blue competent cells via electroporation in a 2-mm gap cuvette (2.5 kV, 15.0 μ F, 200 Ohms).

62| After electroporation, add 1 mL SOC media each to the transformed ligation and control and transfer to 15-mL culture tubes. Incubate with agitation (250 rpm) at 37°C for 1 hour.

63| Plate 1 μ L and 10 μ L of both the transformed ligation and control onto SB plates containing 100 μ g/mL carbenicillin. Allow these to grow overnight at 37°C .

64| Pick several colonies, miniprep, and *Sfi*I digest for 1 hour at 50°C (shown below) to ensure the insert is the correct size. Clones that have ZF1 inserted should release a 100-bp band and will become the ZF1 clone. Clones can be checked by sequencing with pSCVseqF primer.

Component	Amount
pSCV-ZF1	100 ng
<i>Sfi</i> I (20 U/ μ L)	0.5 μ L
10 $\times$ Buffer (NEB 2)	2 μ L
10 $\times$ BSA	2 μ L
Nuclease-free water	13.5 μ L
Total	20 μ L

Optional: Individual clones from a one-finger library can be used as ZF1.

PAUSE POINT Purified plasmid DNA can be stored at -20°C indefinitely.

?TROUBLESHOOTING

65| Create ZF1-2 clone. Digest SuperZifGNN plasmid with unique enzymes that release the desired second zinc finger domain. As an example, use *Acc*I and *Afl*III to cut out GAA specific helix and digest (shown below) at 37°C for 4 hours to create a ZF2 insert.

Component	Amount
SuperZifGNN	10 μ g
<i>Acc</i> I (10 U/ μ L)	1.5 μ L
<i>Afl</i> III (20 U/ μ L)	1.5 μ L
10 $\times$ Buffer (NEB 4)	5 μ L
10 $\times$ BSA	5 μ L
Nuclease-free water	to 50 μ L
Total	50 μ L

Also digest 5 µg pSCV-ZF1 with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a pSCV-ZF1 vector. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-ZF1	5 µg
<i>AgeI</i> (5 U/µL)	1 µL
<i>SpeI</i> (10 U/µL)	1 µL
10× Buffer (NEB 4)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

Purify the pSCV-ZF1 vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

66| Digest the purified ZF2 fragment with *XmaI* and *SpeI* (shown below) at 37°C for 4 hours to create a compatible ZF2 insert.

Component	Amount
Purified ZF2	2.5 µg
<i>XmaI</i> (10 U/µL)	1.5 µL
<i>SpeI</i> (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Purify the *XmaI*/*SpeI* ZF2 insert with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified insert can be stored at -20°C indefinitely.

67| Ligate purified pSCV-ZF1 vector and the ZF2 insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
pSCV-ZF1 vector	100 ng	100 ng
ZF2 insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL
Total	20 µL	20 µL

68| Repeat steps 60 through 64 to obtain a clone that contains ZF1-2. In this case the released band after a *Sfi*I digestion should be 200 bp.

?TROUBLESHOOTING

69| Create ZF1-2-3 clone. Digest SuperZifGNN plasmid with unique enzymes that release the desired zinc finger. As an example, use *AccI* and *AflIII* to cut out GAA specific helix and digest (shown below) at 37°C for 4 hours to create a ZF3 insert.

Component	Amount
SuperZifGNN	10 µg
<i>AccI</i> (10 U/µL)	1.5 µL
<i>AflIII</i> (20 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 5 µg pSCV-ZF1-2 with *AgeI* and *SpeI* (shown below) at 37°C for 4 hours to create a ZF1-2 vector. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-ZF1-2	5 µg
<i>AgeI</i> (5 U/µL)	1 µL
<i>SpeI</i> (10 U/µL)	1 µL
10× Buffer (NEB 4)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

70| Repeat digestion and purification described in step 66 to generate a compatible ZF3 insert.

PAUSE POINT Purified insert can be stored at -20°C indefinitely.

71| Ligate purified pSCV-ZF1-2 vector and the ZF3 insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
pSCV-ZF1-2 vector	100 ng	100 ng
ZF3 insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL

Total	20 µL	20 µL
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72| Repeat steps 60 through 64 to obtain a clone that contains ZF1-2-3. In this case the released band after a *Sfi*I digestion should be 300 bp.

?TROUBLESHOOTING

73| Create pSCV-ZF4-5-6 by repeating steps 56 through 72 using the appropriate zinc fingers for positions 4, 5, and 6.

74| Create pSCV-ZF1-2-3-4-5-6 clone. Digest pSCV-ZF4-5-6 plasmid with *Xma*I and *Spe*I (shown below) at 37°C for 4 hours to create a ZF4-5-6 insert.

Component	Amount
pSCV-ZF4-5-6	10 µg
<i>Xma</i> I (10 U/µL)	1.5 µL
<i>Spe</i> I (10 U/µL)	1.5 µL
10× Buffer (NEB 4)	5 µL
10× BSA	5 µL
Nuclease-free water	to 50 µL
Total	50 µL

Also digest 5 µg pSCV-ZF1-2-3 with *Age*I and *Spe*I (shown below) at 37°C for 4 hours. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
pSCV-ZF1-2-3	5 µg
<i>Age</i> I (5 U/µL)	1 µL
<i>Spe</i> I (10 U/µL)	1 µL
10× Buffer (NEB 4)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

75| Purify the digested ZF4-5-6 DNA fragment by electrophoresis on 2% agarose gel (1× TAE with EtBr). Cut out the 300-bp band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

Purify the pSCV-ZF4-5-6 vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

76| Ligate purified pSCV-ZF1-2-3 vector and the purified ZF4-5-6 insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess background. Allow the ligation reaction to proceed overnight at room temperature.

Component	Ligation	Control
ZF1-2-3 vector	100 ng	100 ng
ZF4-5-6 insert	50 ng	-----
5× T4 DNA Ligase Buffer	4 µL	4 µL
T4 DNA Ligase	1 µL	1 µL
Nuclease-free water	to 20 µL	to 20 µL
Total	20 µL	20 µL

77| Repeat steps 60 though 64 to obtain a 6-finger clone. In this case the released band after a *Sfi*I digestion should be 600 bp.

?TROUBLESHOOTING

Transferring a zinc-finger library or protein to an expression vector. Timing: 3 days

78| Digest pSCV-Library/Clone with *Sfi*I (shown below) at 50°C for 5 hours to create a Library or Clone insert.

Component	Library	Clone
pSCV-Library/Clone	10 µg	5 µg
<i>Sfi</i> I (40 U/µL)	1.5 µL	1 µL
10× Buffer (Roche M)	5 µL	2.5 µL
Nuclease-free water	to 50 µL	to 25 µL
Total	50 µL	25 µL

Also digest the target expression vector (See Table 1) with *Sfi*I (shown below) at 50°C for 5 hours. Once this digestion is complete, add 1 U calf intestinal phosphatase (CIP) and incubate at 37°C for 1 hour.

Component	Amount
Expression Vector	5 µg
<i>Sfi</i> I (20 U/µL)	1 µL
10× Buffer (NEB 2)	2.5 µL
10× BSA	2.5 µL
Nuclease-free water	to 25 µL
Total	25 µL

79| Purify the digested Library or Clone insert fragment by electrophoresis on 1.5% agarose gel (1× TAE with EtBr). Cut out the appropriate band and purify by “Freeze and Squeeze”⁶⁹ or with the QIAquick Gel Extraction kit.

Purify the expression vector with the PureLink PCR Purification kit (Invitrogen).

PAUSE POINT Purified vector and insert can be stored at -20°C indefinitely.

80| Ligate purified expression vector and the Library/Clone insert using T4 DNA ligase (shown below). Also set up a control ligation with no insert to assess the library background. For large libraries two to three ligation reactions may be required to reach the desired library size. Allow the ligation reaction to proceed overnight at room temperature.

Component	Library Ligation	Library Control	Clone Ligation	Clone Control
Expression Vector	1 µg	1 µg	100 ng	100 ng
Library/Clone Insert	750 ng	-----	50 ng	-----
5× T4 DNA Ligase Buffer	20 µL	20 µL	4 µL	4 µL
T4 DNA Ligase (1 U/µL)	10 µL	10 µL	1 µL	1 µL
Nuclease-free water	to 100 µL	to 100 µL	to 20 µL	to 20 µL
Total	100 µL	100 µL	20 µL	20 µL

81| For a clone ligation, follow steps 4 through 6. Next, plate 50 µL each of the transformed ligation and control onto SB plates containing 100 µg/mL carbenicillin. Allow these to grow overnight at 37°C.

Pick five colonies from the transformed ligation plates and inoculate 2.5 mL SB media containing 50 µg/mL carbenicillin. Shake (250 rpm) overnight at 37°C.

Prepare small-scale plasmid purifications (PureLink Quick Plasmid Miniprep kit from Invitrogen) from the overnight cultures and sequence with an appropriate primer.

PAUSE POINT Purified plasmid DNA can be stored at -20°C indefinitely

82| For a library ligation, ethanol precipitate the ligation and control reactions. Add 10 µL of 3 M sodium acetate (NaOAc, pH ~5.2-6.0), 1 µL glycogen (1 mg/mL) (optional), and 250 µL of absolute ethanol. Let stand at -20°C for at least 1 hour. Centrifuge at ≥ 15,000 xg for 30 minutes. Remove supernatant and wash with 1 mL 70% ethanol. Centrifuge at ≥ 15,000 xg for 15 minutes. Remove supernatant and allow pellet to air dry for 15 minutes. Resuspend DNA pellet in 10 µL nuclease-free water.

PAUSE POINT Ligations can be stored in the NaOAc/ethanol solution or after resuspension in water at -20°C indefinitely.

83| Transform entire 10-µL ligation reaction (5 µL + 100 µL of competent cells × 2 cuvettes) and 5 µL of the control in 100 µL competent cells via electroporation (2.5 kV, 15.0 µF, 200 Ohms). We recommend SS320⁶⁸ cells due to their high competency.

84| After electroporation, add 1 mL SOC media each to the transformed ligations and control and transfer to 14-mL culture tubes, pooling the two transformed ligation cuvettes. Incubate with agitation (250 rpm) at 37°C for 1 hour.

85| Make a 1:100 dilution of the transformed ligation culture in SOC media. Plate 0.2 µL and 2 µL of the diluted transformed ligation culture and the transformed control culture onto SB plates containing 100 µg/mL carbenicillin. Allow these to grow overnight at 37°C. Add the remaining transformed ligation culture to 150 mL of SB with 50 µg/mL carbenicillin. Incubate the culture with agitation (250 rpm) overnight at 37°C.

86| Count colonies on the transformed ligation and control plates to ensure 10× coverage of the library. The transformed ligation plates should have at least 20-fold more colonies than the control plates to ensure less than 10% background.

?TROUBLESHOOTING

87| Repeat steps 9 and 10. The fragment released from the library should be 600 bp.

?TROUBLESHOOTING

TROUBLESHOOTING

Steps	Problems	Possible Reason	Solution
9, 16, 25, 34, 43, 49, 64, 72, 77, 87	Band after <i>Sfi</i> I digestions is too large or there is more than one band.	Multiple inserts	A) Repeat ligation with less insert; or B) Digest library with <i>Spe</i> I, clean vector by gel extraction, and religate; or C) use <i>Ahd</i> I instead of <i>Spe</i> I to cut the vector and insert; this will give a larger insert size (see Fig. 2 for approximate <i>Ahd</i> I location).
8, 15, 24, 33, 42, 48, 50, 54, 86	Library size is too small	Low cell competency	A) Use a strain with higher competency potential; or B) make a fresh batch of competent cells; or C) do two (or more) simultaneous ligation reactions.
8, 15, 24, 33, 42, 48, 50, 54, 86	Background is too high	Insufficient digestion of vector	Re-digest vector with higher concentration of enzyme or for a longer period of time.
8, 15, 24, 33, 42, 48, 50, 54, 86	Background is too high	Insufficient dephosphorylation of vector	Clean vector by running on a 1.5% agarose gel and clean via "Freeze and Squeeze" ⁶⁹ or electroelution.

Timing

Construction of a six-finger GNN library: Steps 1 – 34, 9 days

Construction of a five-finger GNN/ANN library: Steps 35 – 54, 11 days

Construction of a designed six-finger clone: Steps 55 – 77, 13 days

Transferring a zinc-finger library or protein to an expression vector: Steps 78 – 87, 3 days

Anticipated Results

In our experience, selection of zinc-fingers from libraries has proven to be a reliable method for discovering proteins that are able to bind specific genomic target sites⁴⁴⁻⁵³. Designed zinc-fingers, with domains designed based on a selected sequence, are not always able to bind their intended genomic target due to secondary structure, chromatin structure, or occlusion of the DNA sequence by other endogenous DNA-binding proteins. Selection for functional zinc finger proteins with activity in the native environment of the cell thus leads to more reliably active zinc-finger proteins. The library construction method presented in this protocol provides libraries that contain fewer mutations and less bias than libraries created using PCR-based methods or by mixing of individual zinc-fingers. These libraries can also be used in bacterial^{55,56}, yeast^{57,58}, and cell-free^{59,60} zinc-finger selection systems.

We have built a variety of libraries and specific clones that have been successfully used in different assays. Libraries constructed using this protocol have a very low background of

nonfunctional proteins and insert-free vector DNA. As shown in Fig. 3, all of the randomly-selected clones analyzed in the process of building the 5ZF GNH/ANN libraries contained the correct size insert for a five-finger protein. For these libraries, the background of insert-free vector was between 1 and 5%.

To demonstrate the success of library construction, the 4ZF and 5ZF GNH/ANN libraries assembled using the described protocol and cloned into pMX-VP64-IRES-GFP and pMX-KRAB-IRES-GFP were used to retrovirally transduce MDA-MB-231 cells. A western blot of the cell extract with detection via the HA tag showed strong expression of all four libraries (Fig. 4). Toxicity problems during the construction of the libraries have been overcome by performing all the cloning in a non-expressing vector (pSCV), thus ensuring a non-biased library.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Plasmid map of the SuperZiF plasmids GNN, ANN, and CNN. Unique restriction sites were placed between each zinc-finger module to enable isolation of individual and subsets of zinc fingers. The zinc-finger sequence was synthesized by Blue Heron and cloned into the pUCminusMCS vector. See Supplementary Figure 1 for complete maps and the Supplementary Sequence Archive for plasmid sequences.

Figure 2.

Library cloning scheme. The SuperZiF vector is digested with *XhoI* and *SpeI* and ligated into pSCV (see Supplementary Figure 2 for a complete map and the Supplementary Sequenced Archive for the plasmid sequence). Next, the 1ZF library is cut with *XmaI* and *SpeI* to create a 1ZF insert and with *AgeI* and *SpeI* to create 1ZF vector (*XmaI* and *AgeI* have compatible overhangs and are both destroyed after ligation). These two pieces are ligated to create a 2ZF library. The 2ZF library is cut with *AgeI* and *SpeI* to create a 2ZF vector and the 1ZF insert from the previous step is ligated into this vector to create a 3ZF vector. Additional fingers may be added in a successive manner until the desired library length is obtained. This scheme can also be used to create designed clones by using individual fingers at each step rather than the libraries.

Figure 3.

Agarose gel electrophoresis (1.5% in 1×TAE with EtBr) of individual clones from the 5ZF GNH/ANN library. Colonies were selected randomly and tested by *Sfi*I enzyme digestion. All clones released a single 500-bp insert after digestion, reflecting the low background (library members with more or less than 5 zinc-fingers) of the library.

Figure 4.

Western blot of 4ZF and 5ZF GNH/ANN library expression with detection via an HA tag with anti-HA-peroxidase antibody (Roche). Libraries were cloned into the pMX-IRES-GFP retroviral vectors as both VP64 and KRAB and transduced into MDA-MB-231 cells.

Table 1

Vectors:					
Genome integrated	Regulator Domains	Expression	Origins of Replication	Markers	References
Retroviral:					
pMX-IRES-GFP	VP64, KRAB, SID, P65	Mammalian	ColE1	Amp, GFP	10, 11, 67, unpublished
pMX-CMV-Luc	VP64, KRAB	Mammalian	ColE1	Amp, Luc	54, 67, unpublished
pMX-CMV-GFP	VP64, KRAB	Mammalian	ColE1	Amp, GFP	54, 67
pMX-sv40-Puro	VP64, KRAB	Mammalian	ColE1	Amp, Puro	67, unpublished
Lentiviral:					
pLVEF1a-IRES-EGFP	VP64, KRAB	Mammalian	ColE1, F1	Amp, EGFP	Unpublished
Expression vectors					
pcDNA3.1	VP64	Mammalian	ColE1, F1	Amp, Neo	10, Invitrogen
pcDNA3.1(+Zeo)	KRAB	Mammalian	ColE1, F1	Amp, Zeo	10, Invitrogen
pMALc		<i>E. Coli</i>	pBR, M13	Amp	10, NEB