Chapter 2

Engineering Single Cys2His2 Zinc Finger Domains Using a Bacterial Cell-Based Two-Hybrid Selection System

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Abstract

Individual synthetic Cys2His2 zinc finger domains with novel DNA-binding specificities can be identified from large randomized libraries using selection methodologies such as phage display. We have previously demonstrated that a bacterial cell-based two-hybrid system is at least as effective as phage display for selecting zinc fingers with desired specificities from such libraries. In this chapter we provide updated, detailed protocols for performing zinc finger selections using the bacterial two-hybrid system.

Key words: Bacterial two-hybrid, zinc finger selection.

1. Introduction

Artificial Cys2His2 zinc finger domains (C2H2 ZFs) with engineered DNA-binding specificities have shown promise for applications in both biological research and gene therapy (1–7). Selection-based methods for altering the specificities of individual C2H2 ZFs typically involve randomization of amino acid residues in the DNA recognition helix to generate large libraries followed by use of a selection method to identify variants with desired DNA-binding specificities. Early studies utilized phage display for the selection method (8–10) but more recent studies have demonstrated that a bacterial cell-based two-hybrid (B2H) system works as well as phage display and may be, in certain cases, more effective (11, 12). In addition, the B2H system is somewhat

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easier to use than phage display because it directly selects for proteins in an in vivo, cellular context whereas phage display requires multiple rounds of in vitro selection.

In this chapter, we describe detailed methods for using the B2H system to identify individual C2H2 ZFs with desired DNA-binding specificities from randomized libraries >10^8 in size. Although we have outlined similar protocols in earlier publications (11, 12), the overall approach has evolved in our laboratory as we have gained experience with the method. The protocol described in detail here is the most up-to-date method currently utilized by our laboratory for selections of individual ZFs.

The B2H system, as used in this protocol, links the occurrence of a protein–DNA interaction to the activation of two reporter genes: the yeast HIS3 and the bacterial aadA genes. These genes code for an enzyme that is essential for histidine synthesis and a gene that confers resistance to streptomycin, respectively. These genes are used to create a “selection strain” harboring a co-cistronic HIS3/aadA reporter on a single copy episome. The reporter also contains a target DNA site of interest positioned just upstream of the weak promoter directing HIS3/aadA expression. If a zinc finger domain capable of binding the target DNA site of interest (and fused to a fragment of the yeast Gal11P protein) is expressed in the selection strain, this leads to recruitment of RNA polymerase to the weak promoter and subsequent activated expression of HIS3/aadA transcription; this occurs because the Gal11P fragment interacts with a yeast Gal4 protein fragment that is fused to a subunit of the E. coli RNA polymerase.

Fig. 2.1. Schematic overview of the bacterial two-hybrid selection system. A selection strain harboring the HIS3/aadA reporter and a kanamycin resistance gene on a single copy recombinant F' is transformed with plasmids encoding a hybrid alphaGal4 protein (harboring chloramphenicol resistance [CAM^R]) and a ZF domain-Gal11P hybrid protein (harboring carbenicillin resistance [AMP^R]). If the ZF domain binds to the target DNA site present on the F' reporter (black box), transcription of the HIS3/aadA reporter gene cistron is activated via recruitment of RNA polymerase to the reporter promoter mediated by interaction of the Gal11P and Gal4 domains. See text for additional details.
polymerase alpha-subunit (a hybrid protein we refer to as the alphaGal4 protein, see Fig. 2.1) (12). Cells harboring such a ZF domain can be identified on a medium lacking histidine and containing the antibiotic streptomycin. The stringency of the HIS3 or aadA selections can be increased by adding higher concentrations of 3-AT (3-aminotriazole, a competitive inhibitor of the HIS3 enzyme) or streptomycin, respectively, to the medium.

We first describe methods for engineering “selection strains” harboring target DNA sequences of interest (Section 3.1). We then describe methods for using these strains to identify ZF variants of interest from large randomized libraries (Section 3.2).

2. Materials

2.1. Molecular Biology Reagents

1. 10× annealing buffer (1 mL): 400 μL 1 M Tris base, pH 8.0, 200 μL 1 M MgCl₂, 100 μL 5 M NaCl, 300 μL H₂O.
2. Restriction enzymes (New England Biolabs): EcoRI, HindIII, SapI; all 10× buffers are included with the enzymes.
3. Cloned Pfu polymerase and associated 10× reaction buffer (Stratagene).
5. Expand High-Fidelity thermostable polymerase and associated 10× Expand buffer (Roche).
6. QIAprep Spin Miniprep kit (Qiagen).
7. QIAquick PCR Purification kit (Qiagen).

2.2. Primer Sequences

1. OK.181 sequencing primer: 5′-CCAGAGCATGTATCATA TGGTCCAGAAACCC-3′
2. His3 2F primer: 5′-CGTATCACGAGGCCCTTTC-3′
3. His3 2R primer: 5′-GCAAATCCTGATCCAAACCT-3′
4. OK.61 sequencing primer: 5′-GGGTAGTACGATGACGGAACCTGTC-3′

2.3. Bacterial Strains and Plasmids

1. CSH100 (genotype: F’lac proA⁺B⁺ (lacIq lacPL8)/ara- Δ(gpt-lac)5)
2. KJ1C (genotype: F- ΔhisB463 Δ(gpt-proAB-arg-lac)XIII zaj::Tn10)
3. Reporter plasmid pKJ1712
4. Expression plasmid pAC-alphaGal4

These strains and plasmids are available by request from the Joung laboratory.
2.4. Bacterial Media

1. Ingredients for M9 minimal medium plates: Bacto Agar, M9 salts, MgSO\textsubscript{4}, glucose, 100 mM CaCl\textsubscript{2}.

2. Ingredients for NM medium and plates: 10× M9 salts (see below), 20% glucose, 20 mM adenine, amino acid mixture (see below), 1 M MgSO\textsubscript{4}, 10 mg/mL thiamine, 10 mM ZnSO\textsubscript{4}, and 100 mM CaCl\textsubscript{2}, isopropyl-beta-D-thiogalactopyranoside (IPTG) and 3-aminotriazole (3-AT), Bacto Agar.

3. 200× amino acid mixture: each of the six solutions below should be made separately with ingredients mixed together in the order listed. The six solutions are then mixed together, filter sterilized, and stored at 4°C (see Note 1). This yields a 200× stock containing all amino acids except histidine, cysteine, and methionine.
   a. Solution I (100 mL): 0.99 g phenylalanine, 1.10 g lysine, and 2.50 g arginine in H\textsubscript{2}O.
   b. Solution II (100 mL): 0.20 g glycine, 0.70 g valine, 0.84 g alanine, and 0.41 g tryptophan in H\textsubscript{2}O.
   c. Solution III (100 mL): 0.71 g threonine, 8.40 g serine, 4.60 g proline, and 0.96 g asparagine in H\textsubscript{2}O.
   d. Solution IV (100 mL): 9.1 mL 36.5% HCl to 80 mL H\textsubscript{2}O, dissolve 1.04 g aspartate, and 14.60 g glutamine. Adjust final volume to 100 mL with H\textsubscript{2}O.
   e. Solution V (100 mL): Dissolve 18.70 g potassium glutamate in 80 mL H\textsubscript{2}O, dissolve 0.36 g tyrosine and 4 g NaOH pellets; adjust final volume to 100 mL with H\textsubscript{2}O to 100 mL.
   f. Solution VI (100 mL): 0.79 g isoleucine and 0.79 g leucine in H\textsubscript{2}O.

4. 10× M9 Salts (for 1 L): 67.8 g disodium phosphate (anhydrous), 30 g sodium chloride, 5 g monopotassium phosphate, 10 g ammonium chloride; fill to 1 L with ddH\textsubscript{2}O and filter sterilize.

5. Antibiotics and other media additives:
   a. Carbenicillin (100 μg/mL in plates); stock is 50 mg/mL in ddH\textsubscript{2}O (see Note 14).
   b. Chloramphenicol (30 μg/mL in plates); stock is 30 mg/mL in EtOH.
   c. Kanamycin (30 μg/mL in plates); stock is 30 mg/mL in ddH\textsubscript{2}O.
   d. Tetracycline (12.5 μg/mL in plates); stock is 12.5 mg/mL in 80% EtOH.
   e. Sucrose (5%); stock is 50% in ddH\textsubscript{2}O.
   f. IPTG (50 μM); stock is 50 mM in ddH\textsubscript{2}O.
g. 3-AT (10–60 mM); stock is 1 M in ddH₂O (see Note 2).
h. Streptomycin (20–80 μg/mL); stock is 100 mg/mL in ddH₂O.

6. LB/TKS plates, which contain tetracycline, kanamycin, and sucrose.
7. LB/TK plates, which contain tetracycline and kanamycin.
8. LB/Kan plates, which contain kanamycin.
9. LB/CCK plates, which contain carbenicillin, chloramphenicol, and kanamycin.
10. LB/CK plates, which contain chloramphenicol and kanamycin.
11. LB/Tet plates, which contain tetracycline.

2.5. Equipment/Consumables

1. 245 mm square plates (Corning)
2. 100 mm × 15 mm round Petri plates (Fisher)
3. 96-well flat-bottom, microtiter plates (Corning-Costar)
4. Glass beads, 3 mm (Fisher)
5. Sterile wooden sticks (Fisher)
6. 25 mm glass culture tubes (Fisher)
7. 18 mm glass culture tubes (Fisher)
8. 100 mm ×100 mm plates (VWRr)

3. Methods

3.1. Selection Strain Construction

Selection strains are constructed in two steps: initially, a target DNA site of interest is synthesized and then cloned into a multi-copy plasmid reporter vector designed in our laboratory (Section 3.1.1). In a second step, a portion of this reporter plasmid is recombined to a single copy F’ episome in bacterial strain CSH100 and the resulting recombinant F’ is then transferred by conjugation to KJ1C, an F-strain in which one can select for HIS3 and aadA expression (12) (Section 3.1.2). We use recombination to place the reporter construct onto the F’ because the F’ episome is too large to be manipulated using standard subcloning techniques. Our method of selection strain construction is similar to one previously described by Whipple (13) but utilizes a counterselection step which simplifies identification of desired double recombinants (see below). Finally, the creation of the selection strain is verified both by prototrophy tests and by direct sequencing (Section 3.1.3).
3.1.1. Reporter Plasmid Construction

1. Digest \( \sim 1 \, \mu g \) of the reporter plasmid pKJ1712 with SapI. pKJ1712 contains two closely positioned SapI sites (see Fig. 2.2) and therefore digestion with this enzyme releases a small 45 bp fragment. Use the following quantities:

- 1 \( \mu g \) Plasmid pKJ1712
- 5 \( \mu L \) 10× NEB Buffer 4
- 5 \( \mu L \) SapI (2 U/\( \mu L \))
- Fill to 50 \( \mu L \) with H\( _2\)O

Incubate at 37°C for 2 h.

2. Isolate the 8,678 bp pKJ1712 vector backbone on either an agarose or polyacrylamide gel using standard methods. Resuspend the final purified digested vector in 20 \( \mu L \) of ddH\( _2\)O.

3. Treat the purified vector with Pfu polymerase to create extended overhangs. Cloned Pfu DNA Polymerase has a 3’ to 5’ exonuclease activity and by providing only one nucleotide (dCTP) to the reaction, the enzyme will

Fig. 2.2. Structure and sequence of the reporter plasmid vector pKJ1712. A schematic of the reporter plasmid pKJ1712 is shown. The region of the plasmid into which target DNA-binding sites are cloned is represented as a black rectangle with corresponding detailed sequence shown. Digestion of pKJ1712 with TypeII SapI restriction enzyme releases a 45 bp fragment. Further treatment of the SapI-digested vector backbone with Pfu DNA polymerase in the presence of dCTP nucleotide results in formation of the illustrated DNA overhangs.
degrade DNA until it reaches a position that can be filled in with dCTP. At this point, the forward synthesis and reverse exonuclease activities will reach equilibrium, thereby leaving extended overhangs as shown in Fig. 2.2. We utilize this approach to create overhangs for historical reasons related to subcloning in plasmid pKJ1712. Reaction conditions for \textit{Pfu} treatment are as follows:

\begin{itemize}
  \item 2 μL 10 mM dCTP
  \item 2 μL 10× Cloned \textit{Pfu} Buffer
  \item 10 μL pKJ1712 \textit{SapI}-digested vector
  \item 1.2 μL Cloned \textit{Pfu} (2.5 U/μL)
  \item 4.8 μL H₂O
\end{itemize}

Incubate at 72°C for 15 min, then at 4°C for >2 min.

4. As illustrated in Fig. 2.3, design a pair of oligonucleotides that when annealed together will form a double-stranded DNA fragment bearing the target DNA-binding site and extended overhangs compatible with the pKJ1712 vector prepared in Step 3 above.

5. Anneal the target DNA-binding site oligonucleotides together as follows:

\begin{itemize}
  \item 1 μL oligonucleotide 1 (10 pmol/μL)
  \item 1 μL oligonucleotide 2 (10 pmol/μL)
  \item 20 μL 10× annealing buffer
  \item 178 μL H₂O
\end{itemize}

Incubate at 95°C for 2 min, then shut off heat block and let tubes slow cool to 35°C. Put on ice. Alternatively, using a thermocycler, incubate at 95°C for 2 min, then cool at a rate of 1°C/min to 25°C. Change temperature to 4°C.

6. Ligate the purified pKJ1712 vector backbone to the annealed oligonucleotide binding site as follows:

\begin{itemize}
  \item 2 μL purified \textit{SapI}-digested, \textit{Pfu}-treated pKJ1712 vector
  \item 8 μL annealed binding site oligonucleotides
  \item 10 μL 2× Quick Ligase Buffer
  \item 1 μL T4 DNA Ligase (400 U/μL)
\end{itemize}

Incubate at room temperature for 5 min then store on ice.

7. Transform ligations into XL-1 Blue \textit{E. coli} competent cells using standard protocols and plate 1/3 of the transformations on LB/Kan plates. Incubate plates at 37°C overnight.

8. Isolate miniprep plasmid DNA from transformants. Typically, if there are at least 20-fold more colonies than
A. Binding site oligonucleotide design template:

\[
\begin{align*}
5' &\text{TGTGAAAGATCTTCTGACACnnnnnnnnATTACATTA}^3' \\
3' &\text{ACACCTTCTAGAACTGTGnnnnnnnnTAPGTAAT}^5'
\end{align*}
\]

B. Example: for the three-finger binding site 5’GCGTGGGC3’

\[
\begin{align*}
5' &\text{TGTGAAAGATCTTCTGACACGCGTGGGCATTACATTA}^3' \\
3' &\text{ACACCTTCTAGAACTGTGACCCCGCGTAAATGTAAT}^5'
\end{align*}
\]

Synthesize these oligos

\[
\begin{align*}
5' &\text{TGTGAAAGATCTTCTGACACGCGTGGGC3'} \\
5' &\text{TAATGTAATGCGCCCACGGTGTCC}^3'
\end{align*}
\]

Anneal oligos

\[
\begin{align*}
5' &\text{TGTGAAAGATCTTCTGACACGCGTGGGC3'} \\
3' &\text{AGCTGTGCGACCCCGTGTAATGTAAT}^5'
\end{align*}
\]

Fig. 2.3. Template for design of oligonucleotides harboring a target DNA site. (A) The target DNA site of interest is substituted for the “nnnnnnnnnn” sequence illustrated in the template (Note that this sequence can be longer than 10 bp if desired). Oligonucleotides corresponding to the highlighted light and dark gray sequences are then synthesized and annealed together to create an insert that can be ligated to reporter plasmid pKJ1712 (compare overhangs of the annealed oligonucleotide complex with the overhangs of SapI-digested, Pfu-treated pKJ1712 shown at the bottom of Fig. 2.2). (B) Design of binding site oligonucleotides for the 10 bp Zif268 target DNA site. As an example, this figure illustrates the design of binding site oligonucleotides for the 10 bp Zif268 binding site 5’GCGTGGGC3’. In this example, the amino-terminal finger of Zif268 binds to the 3’ end of the target sequence while the carboxy-terminal finger binds to the 5’ end.

9. To verify uptake of the annealed oligonucleotides in the pKJ1712 plasmid, we digest 5 μL of each candidate DNA with EcoRI and HindIII. As a control, we also digest plasmid pKJ1712 with EcoRI and HindIII. Recombinants that have taken up the annealed oligonucleotide insert should yield five bands of sizes 6,109, 1,006, 963, 431, and
190 bp. By contrast, pKJ1712 should yield five bands of sizes 6,109, 1,006, 963, 456, and 190 bp (see Note 3).

10. Plasmids that look correct by restriction digest should be sequenced to confirm the target DNA-binding site. We use primer OK.181, a primer which anneals to sense DNA strand just downstream of (and points back toward) the target binding site, to verify the sequence of the insert (see Note 4).

As shown in Fig. 2.4, the reporter plasmid contains portions of the lacIq and lacZ genes that are also present in the F' found in strain CSH100. These regions of matching sequence can serve as point of recombination between the reporter plasmid and the F'. A double crossover event at both regions of sequence identity can lead to transfer of a portion of the reporter plasmid onto the F'.

Fig. 2.4. Homologous recombination between reporter plasmids and the CSH100 strain F' mediated by regions of sequence identity. Schematic of a reporter plasmid showing its structure and its regions of sequence identity with the F' from E. coli strain CSH100. A double homologous recombination event between the two regions of sequence identity (from the lacIq and lacZ genes) leads to insertion of a fragment consisting of the kanamycin resistance (KanR) gene, the target DNA-binding site, and the HIS3/aadA reporter into the F'. Note that a double homologous recombination event does not transfer the counterselectable sacB gene from the reporter plasmid to the F'.
1. Streak out F-strain KJ1C on a LB/Tet plate and grow at 37°C overnight.

2. The next day, transform F+ strain CSH100 with the reporter plasmid and plate enough to obtain a confluent lawn of transformants on a LB/Kan plate. Incubate overnight at 37°C. Also inoculate an overnight culture of KJ1C in LB containing 12.5 μg/mL of tetracycline.

3.1.2.2. Transfer of F’s from CSH100 to KJ1C by Bacterial Mating

The population of transformed CSH100 cells will contain a small number of cells in which a single recombination event has led to integration of the entire reporter plasmid to the F’ (see Fig. 2.5). In an even smaller number of cells, a double recombination event will have exchanged only the target DNA-binding site and promoter present on the reporter plasmid with the promoter on the F’ (see Fig. 2.5). As we describe in this step, all F’s (recombinant and non-recombinant) in the CSH100 transformants are transferred to the F-strain KJ1C by mating. In a subsequent step, KJ1C cells harboring the desired double recombinant F’ can be identified by plating on appropriate selective medium (see Fig. 2.5 and below).

Fig. 2.5. Construction and identification of selection strains by recombination, mating, and selection. Reporter plasmids transformed into CSH100 either undergo no recombination (top left), single recombination (middle left), or double recombination (bottom left) with the F’ present in this strain. Mating of these CSH100 cells with the tetracycline-resistant, F-strain KJ1C results in transfer of the three different F’s into KJ1C cells (top, middle, and bottom right). When these cells are plated on medium containing tetracycline, kanamycin, and sucrose, only KJ1C cells that have received the double recombinant F’ (the desired selection strain, bottom right) will survive. All of CSH100 cells (left side) will fail to grow due to sensitivity to tetracycline. KJ1C cells that do not receive an F’ or that receive a non-recombinant F’ (top right) will fail to grow due to sensitivity to kanamycin. KJ1C cells that receive a single recombinant F’ (middle right) will fail to grow due to their sensitivity to sucrose owing to the presence of the sacB gene. KJ1C cells harboring a double recombinant F’ will be resistant to kanamycin, tetracycline, and sucrose and thus will be the only surviving cells on the plate.
1. Scrape the confluent plate of CSH100 transformants with a sterile wooden stick and transfer cell paste to a sterile 25-mm glass tube containing 10 mL of LB (see Note 5).

2. Vortex to resuspend the CSH100 transformants (see Note 6) and transfer ~200 μL of this cell resuspension to a fresh 25-mm tube with 5 mL of LB (see Note 7).

3. Transfer ~200 μL of an overnight culture of KJ1C cells (inoculated the night before, grown in LB containing 12.5 μg/mL of tetracycline) to a sterile 25 mm tube containing 10 mL of LB.

4. Prepare a control 25 mm tube containing 10 mL of LB.

5. Incubate all tubes from Steps 2, 3, and 4 for 2 h at 37°C without agitation, thereby allowing cells to grow to log phase and for CSH100 cells to form F pili.

6. To perform matings, mix together the following combinations of the cultures from Steps 2, 3, and 4 in sterile 18 mm glass tubes. Use 1 mL of each liquid culture (i.e., each mating will consist of a total of 2 mL).
   a. CSH100 transformants + KJ1C (actual mating)
   b. CSH100 transformants + LB (negative control)
   c. KJ1C + LB (negative control)
   d. LB + LB (negative control)

   Allow matings to proceed at 37°C for 1 h without agitation.

7. Put tubes on a rotating wheel at 37°C for 90 min.

8. Plate 300 μL of the actual mating from Step 6a above (CSH100 transformants + KJ1C) on a LB/TK plate and on a LB/TKS plate. Spot 5 μL of each of the negative controls (Steps 6b, 6c, and 6d above) on a LB/TK plate and on a LB/TKS plate. Incubate all plates overnight at 37°C.

   As shown in Fig. 2.5, only KJ1C cells harboring the desired double recombinant F’ should grow on LB/TKS plates. We plate all the matings on LB/TK plates as well to check that the counterselectable marker (sacB) is working to eliminate KJ1C cells harboring single recombinant and non-recombinant F’s (see Fig. 2.5). (Note that expression of the sacB gene is lethal in E. coli cells when they are plated on medium containing sucrose.) We typically observe at least a 10-fold reduction in the number of colonies when comparing the number of colonies on LB/TK to the number of colonies on LB/TKS plates.

9. After KJ1C cells harboring the desired double recombinant F’ have been identified on the basis of growth on LB/TKS plates (see Note 8), we purify clonal isolates by re-streaking candidates two times to LB/TKS plates. We typically choose two candidates (which we designate “A” and “B”) for each
desired clone because a small percentage of cells that survive on LB/TKS plates will fail additional subsequent verification tests (as described below).

3.1.3. Selection Strain Verification

3.1.3.1. Confirmation of F' Transfer by Prototrophy Test (See Note 9)

1. Prepare M9 plates: For 500 mL, autoclave 439 mL H$_2$O with 7.5 g Bacto Agar. After agar has cooled to $\sim$65°C, add 50 mL 10× M9 salts, 1 mL 1 M MgSO$_4$, 10 mL 20% glucose, and 0.5 mL 100 mM CaCl$_2$, and then pour plates.

2. Resuspend each colony (A & B candidates) in 100 μL of 1× M9 salts (we typically do this in wells of a sterile 96-well plate). Spot 5 μL of each cell suspension on an M9 minimal medium plate. Do not discard the resuspended colonies because they will be required for Step 1 of Section 3.1.3.2 below.

3. After overnight growth at 37°C, verify growth of cells within the spots. Candidates that fail to grow should be discarded.

3.1.3.2. Sequencing of the Recombinant F' Reporter

For candidates that successfully demonstrate growth on M9 minimal medium plates, we amplify the portion of the F' harboring the target DNA site and perform DNA sequencing to verify the reporter.

1. Use 20 μL of the cell suspension from Step 1 of Section 3.1.3.1 above, to inoculate a 4 mL LB/kanamycin (30 μg/mL) culture and grow overnight on a roller wheel or shaker at 37°C.

2. Transfer $\sim$100 μL of the saturated overnight culture to a 1.5 mL microcentrifuge tube. Spin at maximum speed for 1 min in a microfuge. Remove as much of the media as possible using a pipet tip and then resuspend cells in 100 μL PCR-grade ddH$_2$O. Heat the cell resuspension at 95°C for 10 min and then spin at maximum speed for 1 min in a microfuge. Remove 50 μL of the supernatant to a fresh tube and use this crude preparation of F' DNA as template for a PCR reaction as follows:

<table>
<thead>
<tr>
<th>PCR conditions</th>
<th>Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μL crude F' DNA</td>
<td>95°C, 5 min</td>
</tr>
<tr>
<td>5 μL 10× Expand Buffer (Roche)</td>
<td>94°C, 30 s$^a$</td>
</tr>
<tr>
<td>4 μL 10 mM dNTPs</td>
<td>55°C, 30 s$^a$</td>
</tr>
<tr>
<td>1 μL His3 2R primer (10 pmol/μL)</td>
<td>72°C, 1 min$^a$</td>
</tr>
<tr>
<td>1 μL His3 2F primer (10 pmol/μL)</td>
<td>72°C, 7 min</td>
</tr>
<tr>
<td>0.375 μL Expand enzyme (Roche)</td>
<td>33.625 μL H$_2$O</td>
</tr>
</tbody>
</table>

$^a$Indicates repeated steps in 35 cycles.
3. Purify PCR product using a Qiagen QIAquick PCR purification kit and elute in 50 μL of 0.1× EB. Sequencing of the target DNA-binding site can be performed using primer OK.181 (we typically send 10 μL of purified PCR for sequencing).

4. We typically also make prepare glycerol stocks and competent cells of the selection strain using the overnight culture inoculated in Step 1 of Section 3.1.3.2 above.

The final step in preparation of the selection strain is to transform the KJ1C strain harboring a sequence verified F’ reporter episome with the pAC-alphaGal4 plasmid (12) (see Note 10).

1. KJ1C cells bearing a sequence-verified recombinant F’ reporter episome are transformed with plasmid pAC-alphaGal4 using standard chemical transformation protocols. Transformations are plated on LB/CK plates since the recombinant F’ episome in the KJ1C cells confers resistance to kanamycin while the pAC-alphaGal4 plasmid encodes a chloramphenicol resistance gene.

2. Transformants are inoculated into overnight LB cultures containing chloramphenicol (30 μg/mL) and kanamycin (30 μg/mL) and grown overnight at 37°C.

3. Glycerol stocks of the final selection strains are prepared using the overnight culture.

To perform selections, phagemid libraries encoding randomized zinc fingers are introduced into a selection strain and then plated on selective media. These zinc finger variants are expressed as fusions to a fragment of the yeast Gal11P protein that interacts with the fragment of the yeast Gal4 protein present in the alphaGal4 protein expressed in selection strains. Binding of a zinc finger domain to the target DNA sequence leads to recruitment of RNA polymerase complexes that have incorporated the alphaGal4 hybrid protein. This recruitment in turn leads to activation of reporter gene expression and survival on selective medium.

We typically perform selections in two stages. In Stage A selections, large numbers of transformants (typically ~10^9) are plated on a low stringency selection plate. Zinc finger-encoding phagemids are rescued from surviving cells (Section 3.2.1). In Stage B selections, this enriched population of phagemids are then re-introduced into the selection strain cells and plated on a series of higher stringency selection plates (Section 3.2.2). Phagemid DNA is then isolated from cells that grow on the highest stringency plate and sequenced to determine the identity of the fingers.
3.2.1. Stage A
Selections

3.2.1.1. Streak Plates

Streak out the selection strain on an LB/CK plate and incubate overnight at 37°C.

3.2.1.2. Prepare NM Medium and Plates

1. Autoclave 836 mL H₂O, 15 g Bacto Agar and a magnetic stir bar together.

2. While agar is cooling, mix together the following components in a sterile flask: 100 mL of 10× M9 salts, 20 mL of 20% glucose, 10 mL of 20 mM adenine, 30 mL of 200× amino acid mixture (see below), 1 mL of 1 M MgSO₄, 1 mL of 10 mg/mL thiamine, 1 mL of 10 mM ZnSO₄, 1 mL of 100 mM CaCl₂, and antibiotics, IPTG and 3-AT as desired.

3. When agar has reached a temperature of ∼65°C, add the above mixture to the agar, stir well and pour plates.

4. NM/CCKI plates are NM agar plates supplemented with carbenicillin (100 μg/mL), chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and IPTG (50 μM).

3.2.1.3. Culture Cells

Use a fresh, well-isolated colony to start an overnight culture of your strain in 20 mL of NM media supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and 50 μM IPTG. Because selection strain cells are sensitive to detergents and rapid agitation, this culture should be grown in a sterile 125 mL glass flask that was rinsed thoroughly with MilliQ water before autoclaving and with shaking at 110 rpm at 37°C for ∼16–24 h.

3.2.1.4. Introduction of Zinc Finger Phagemid Libraries into Selection Strain Cells

The construction of the randomized zinc finger phagemid libraries used in this step has been previously described (11, 12).

1. Thaw phagemid phage library on ice. Extreme care is required to prevent phage contamination in the laboratory as it may persist. We change our bench paper and gloves frequently and we use racks designated for phage work only. In addition, we use barrier pipet tips and expel used tips into a bucket of soapy water to inactivate the phage.

2. Transfer 5 mL of the saturated overnight culture of selection strain cells to a sterile 125 mL flask.

3. Add ∼6 × 10⁸ ATU (ampicillin transducing units; see Note 11) of phagemid library to the selection strain cells and gently swirl immediately. Allow the cell/phagemid mixture to sit at room temperature for 30 min without agitation.

4. Add 20 mL of pre-warmed NM medium supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and 50 μM IPTG to the infected cells. Shake at 110 rpm, 37°C for 90 min.
5. Transfer culture to a sterile 50 mL conical tube and pellet cells by spinning at 2,500 rpm in a table top centrifuge for 25 min at room temperature.

6. Pour off medium and resuspend the cell pellet in 2.5 mL of pre-warmed NM medium supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and 50 μM IPTG.

7. Serially dilute 100 μL aliquots of the cell resuspension in a sterile 96-well microtiter plate. Perform three independent dilution sets using NM medium supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and 50 μM IPTG. Perform dilutions from 10^{-1} to 10^{-8} (Note that you will only plate dilutions 10^{-3}–10^{-8}).

8. Spot 5 μL of the 10^{-3}–10^{-8} dilutions each in triplicate on LB/CK, LB/CCK, and NM/CCK/50 μM IPTG plates. Incubate LB/CK and LB/CCK plates for 16–18 h at 37°C and NM/CCK/50 μM IPTG plates for 24 h at 37°C. Titers from these plates can be calculated the next day after colonies have formed (see below).

9. Pour some sterile glass beads (3 mm) onto a large (245 mm × 245 mm) NM/CCK/50 μM IPTG/10 mM 3-AT plate.

10. Measure the remaining suspension volume, record the value (this volume will be used when calculating titers), and then add the cells to the NM/CCK/50 μM IPTG/10 mM 3-AT plate.

11. Spread the cells with circular motions using the beads to distribute the cells evenly.

12. When plates have dried, turn plates over, and tap beads from the agar onto the inverted plate cover. Incubate at 37°C for 24 h and then at room temperature for 18 h.

13. The next day, count colonies on the serial dilution plates to calculate the total number of cells and total number of transformed cells plated on the selection plate. LB/CK plates are used to determine the total number of cells plated and the LB/CCK and NM/CCK/150 plates are used to determine the total number of transformed cells plated. The following formula is used to perform these calculations:

\[
\text{Titers} = \left( \frac{\text{# colonies}}{\text{volume of spots (μL)}} \right) \times \text{dilution factor} \times \text{volume (μL) plated on large dish}
\]

Example: 65 total colonies in nine 5 μL spots of a 10^{-6} dilution together with 2,650 μL plated on the large plate would give the following equation:
3.2.1.5. Recovery of Zinc Finger-Encoding Plasmids from Cells Surviving the Selection

In this step, ZF-encoding plasmids from surviving cells are rescued as phagemids by infecting these cells with M13K07 helper phage.

1. Turn the large selection plate over and tap the glass beads back onto the agar and add 15 mL of pre-warmed NM media to the plate. Agitate the plates in a circular motion using the glass beads to resuspend the cells in the media.

2. Transfer the suspension to a sterile 25 mm glass tube.

3. Remove 3 mL of cell resuspension to make glycerol stocks in case this recovery step needs to be redone.

4. Add enough of the cell suspension to 90 mL of 2×YT supplemented with carbenicillin (50 μg/mL) and kanamycin (30 μg/mL) to give it a pre-log appearance (i.e., an OD_{600} of ~0.1). Shake this culture at 120 rpm, 37°C for 1 h.

5. Infect the log phase culture with 10^{12} kanamycin-transducing units (KTU) of M13K07 helper phage. Allow the phage to adsorb to the cells, without shaking, at room temperature for 30 min.

6. Add kanamycin to a final concentration of 100 μg/mL (including the original 30 μg/mL present in the culture). Shake the culture at 125 rpm, 37°C for 6 h. During this incubation, ZF-encoding phagemids from the cells will be packaged as infectious phage particles harboring single-stranded DNA molecules and extruded into the culture medium.

7. Harvest the ZF-encoding phagemid phage by filtering the culture through a 0.2 μm PES filter membrane (no need to centrifuge the cells away first). This enriched phagemid phage library can be stored at 4°C for several weeks (for long-term storage, we freeze the phage at –80°C).

3.2.2. Stage B Selections

3.2.2.1. Introduction of Enriched Library of Zinc Finger-Encoding Phagemid Phage into the Selection Strain

1. Start a 20 mL overnight culture of the selection strain in NM medium supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and 50 μM IPTG in a sterile 125 mL flask. Shake for 16–24 h at 110 rpm, 37°C.

2. In a 96-well plate, aliquot 50 μL of the selection strain overnight culture into five wells.

3. In another column of a 96-well plate, add 100 μL of the enriched phagemid phage library to one well. Perform serial 10-fold dilutions of the enriched library by removing 10 μL of phage and adding it to a well containing 90 μL of NM
medium supplemented with chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and IPTG (50 μM). Repeat to create $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions.

4. Infect each of the wells containing 50 μL of selection strain overnight culture with 10 μL of each of the following: undiluted enriched phagemid library and $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions of the phagemid library. Allow phage to adsorb by leaving them (without shaking) at room temperature for 30 min.

5. Add 190 μL of pre-warmed NM medium containing chloramphenicol (30 μg/mL), kanamycin (30 μg/mL), and IPTG (50 μM) to each well. Incubate for 2 h at 37°C (no shaking).

6. Spot 5 μL aliquots of the phagemid-infected selection strain cells on the following plates:
   a. NM/CCKI plates (six 5 μL spots on standard Petri dishes)
   b. NM/CCKI/20 mM 3-AT/20 μg/mL streptomycin (ten 5-μL spots on small square 100 mm × 100 mm plates)
   c. NM/CCKI/25 mM 3-AT/40 μg/mL streptomycin (ten 5-μL spots on small square 100 mm × 100 mm plates)
   d. NM/CCKI/40 mM 3-AT/60 μg/mL streptomycin (ten 5-μL spots on small square 100 mm × 100 mm plates)

7. Incubate plates 37°C for 48 h and inspect for colonies. Colonies may not form on NM/CCKI/40 mM 3-AT/60 μg/mL streptomycin plates until ∼72–96 h of incubation at 37°C.

3.2.2.2. Isolation and Sequencing of Plasmid DNA from Selected Colonies

1. Pick 8–12 well isolated colonies from the highest stringency selection plate on which colonies appear and inoculate them into 4 mL of LB supplemented with carbenicillin (50 μg/mL). Incubate overnight at 37°C with agitation.

2. Prepare miniprep plasmid DNA from the saturated 4 mL overnight cultures using QIagen’s QIAprep Spin Miniprep Kit and their protocol but with the following differences:
   a. Perform triple washes with both PB and PE buffers (see Note 12).
   b. Elute the DNA with 60 μL of pre-warmed (60°C) 0.1× EB (see Note 13).

3. Send the plasmids for sequencing with sequencing primer OK.61, a sense strand primer which anneals just upstream of the region encoding the zinc finger domains.
4. Notes

1. It is important to dissolve the amino acids in each of the six solutions in precisely the order listed as this avoids potential solubility issues. We typically keep the amino acid mixture for no more than 2–3 months.

2. 3-AT should be prepared using gloves. In addition, we have found that the solubility and purity of 3-AT vary from lot to lot. Some preparations have the appearance of a white powder, whereas others look like brown flakes. For certain lots, we have found that heating the solution to 50°C can aid with solubility.

3. We run these digests on 5% polyacrylamide gels made with 0.5× TBE buffer to visualize the relatively small change in fragment size in clones that have taken up the annealed oligonucleotide insert.

4. We sequence verify the entire sequence between the unique EcoRI site (positioned just upstream of the ZF binding site) and the unique SalI site (positioned at the start site of transcription). Verifying this entire span of sequence ensures that both the ZF binding site and the promoter do not have undesired mutations.

5. We have found that using a resuspension of multiple transformed CSH100 colonies rather than an overnight culture grown from a single transformed colony helps ensure that a relatively consistent percent of transformed CSH100 cells contain the desired double recombinant F'.

6. Set vortex to half-maximum speed to ensure that resuspension does not spill over the top of the glass tube.

7. The initial density of this subculture should correspond to OD_{600} of ~0.1 (i.e., pre-logarithmic phase). Depending upon the density of the resuspension culture, we add more or less culture as needed to achieve this target OD_{600}.

8. We occasionally see some small colonies on the LB/TKS plates. We avoid picking these colonies because we have found that these colonies do not yield the desired recombinants.

9. KJ1C cells will not grow unless histidine and proline are provided in their medium due to deletion of the proAB gene cluster and a deletion within the hisB gene, respectively. A double recombinant F' transferred from CSH100 cells harbors an intact proAB gene cluster and expresses a basal level of the yeast HIS3 gene which is sufficient to complement the hisB deletion of strain KJ1C. Thus, KJ1C
cells that receive a double recombinant F’ should be able to grow on M9 minimal medium lacking proline and histidine.


11. We typically aim for a threefold oversampling of the size of the randomized library being interrogated and for a fivefold ratio of total cells to transformed cells. For example, for a randomized library with a complexity of \(\sim 2 \times 10^8\), we would aim to plate a total of \(\sim 6 \times 10^8\) transformed cells and of \(>3 \times 10^9\) total cells on the selection plate.

12. We have found that these triple washes are critical for obtaining good quality sequencing reads. We believe these washes help reduce contaminating endonuclease activity from the *endA* + selection strains.

13. 0.1× EB is Buffer EB from the Qiagen miniprep kit diluted 10-fold with ddH2O.

14. We use carbenicillin at a final concentration of 50 μg/mL in liquid media.

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**References**


