**INDEX OF EXPERIMENTAL PROTOCOLS FOR**

**"UTAH-BIG LOVE" APPROACH TO CONSTRUCTION OF ZINC FINGER NUCLEASES**

**(February 2011)**

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**1. Overview and Experimental Flow**

**Contents**

**1.1. Background**

**1.2. Brief description of ZFNs constructed here**

**1.3. Experimental flow of the protocol**

**1.4. Implementing the Utah-Big Love approach to ZFNs**

**1.1. Background**

The “Utah-Big Love” approach to Zinc Finger Nuclease (ZFN) production is a composite of two published approaches that have been used successfully to generate ZFNs. We use concepts, reagents, and methods described in the publications of Joung and colleagues, who developed the OPEN pools, and Wolfe and colleagues, who developed the Bacterial One-Hybrid selection scheme employed here. Our protocol “marries” efficient elements of the two approaches. Many of the steps described in this protocol are adapted directly from their published methods and placed here to make this protocol self-contained. We do not reference the original sources at each point but you may wish to consult the original publications that are most closely relevant to the methods described here:

Foley, J. E., J. R. Yeh, et al. (2009). "Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN)." PLoS One **4**(2): e4348.

Maeder, M. L., S. Thibodeau-Beganny, et al. (2008). "Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification." Mol Cell **31**(2): 294-301.

Maeder, M. L., S. Thibodeau-Beganny, et al. (2009). "Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays." Nat Protoc **4**(10): 1471-501.

Meng, X., M. B. Noyes, et al. (2008). "Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases." Nat Biotechnol **26**(6): 695-701.

Meng, X. and S. A. Wolfe (2006). "Identifying DNA sequences recognized by a transcription factor using a bacterial one-hybrid system." Nat Protoc **1**(1): 30-45.

Noyes, M. B., X. Meng, et al. (2008). "A systematic characterization of factors that regulate Drosophila segmentation via a bacterial one-hybrid system." Nucleic Acids Res **36**(8): 2547-60.

Sander, J. D., E. J. Dahlborg, et al. (2011). "Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA)." Nat Methods **8**(1): 67-9.

Assembling libraries of ZFNs and selecting the ZFNs specific for your target is a multi-step process. In the following sections we aim to describe every experimental method you need to perform. We have tried to make each Section readable, so that you understand what part of the entire process is being accomplished in that Section. Thus the protocol may seem long but in fact once you have a set of starting materials, the entire process can be completed routinely in a month, while you still have your hands in other experiments.

**1.2. Brief description of ZFNs constructed here**

Zinc Finger Nucleases function as dimers. Each ZFN monomer built here is composed of two domains: a three-finger zinc-finger protein (ZFP) fused with the cleavage domain of the Fok I restriction enzyme. The Fok I cleavage domains used here are derived from Meng et al. (2008) and function as an obligate heterodimer. Successful target cutting therefore requires appropriate target sequence binding and interaction of two ZFN monomer partners. Spacer bases between each 3-finger target are required for dimerization of ZFN monomer partners. Each individual Finger Module recognizes a **core triplet** (3 bp) target on one strand and a base on the opposing strand that is adjacent to the 3'-end of the triplet. (Many investigators concern themselves only with binding to the **core triplet**, which for many purposes is sufficient for the design of functional ZFPs.) Interaction between the kind of ZFN constructed here and a host target sequence is depicted in Figure 1, which depicts the arrangement of Finger Modules and their primary interactions with target bases.

Figure 1: Binding of a ZFN to a target sequence. The ZFN functions as a dimer. In the system described here, each monomer component consists of a three-finger ZFP fused to the cleavage domain of the FokI endonuclease. Each three-finger ZFP recognizes a 9bp **core target** and one additional base neighboring the core. Finger Modules and the bases they contact are color coordinated. A 5-7bp spacer between the core target sequences is required for effective dimerization of the endonuclease domains.

**1.3. Experimental flow of the protocol**

1. ZFN target sites that are: 1) within your gene of interest, and 2) suitable for ZFNs constructed with reagents from the OPEN pool system are identified using available Web-based search programs in **Section 2**.
2. We build each three-finger monomer component independently. Three-finger ZFPs that bind 9+1 are created in two steps: 1) a library encoding three-finger ZFPs is created and cloned into an expression vector; and 2) the library is subjected to selection by co-expressing the ZFPs with a selectable reporter plasmid that carries the 9+1 target sequence.
3. The library encoding the three-finger ZFPs is constructed from sequences that pre-exist in the OPEN pools. Each OPEN pool contains an archive of plasmids that encode three-finger proteins, of which **only one Finger Module** had been subjected to low stringency selection against a target triplet. Therefore: each OPEN pool contains a library of sequences that vary only at a single Finger Module; all variants of the Finger Module in a single OPEN pool have been selected because they likely recognize a known triplet at a specific position within **a 9bp core target**; but not all the Finger Modules present in an OPEN pool may be optimal for binding in the context of **your 9+1 target sequence.** To generate a library encoding three-finger proteins that are varied in all three Finger Module positions and that likely can bind your desired 9bp core target sequence, it is necessary to join sequences encoding Finger Module 1 (F1) from one OPEN pool, sequences encoding F2 from a different OPEN pool, and sequences encoding F3 from a third OPEN pool.
4. Sequences encoding three-finger ZFPs are fused by overlapping PCR (**Section 3)** and then cloned into expression vectors (**Section 4**) that will be used in the B1H selection scheme. In **Section 5** the selectable reporter plasmid containing **your 9+1 target sequence** is built. The B1H selection is performed in and analyzed in **Section 6**. Finally, a CS2-based expression plasmid containing the sequence encoding the selected ZFP fused to the sequence encoding a cleavage domain of the FokI endouclease is built in **Section 7**. This plasmid is appropriate as a template for generating ZFN mRNAs in vitro.

**1.4. Implementing the Utah-Big Love approach to ZFNs**

* The key to efficiency in the long run, and the only daunting aspect when you start out, is generating all the necessary starting materials. We suggest spending your tedious first week or two preparing the following reagents:
	+ Ordering and aliquoting all oligonucleotides for primers, reporter sequences, etc
	+ Generating cleaved and dephosphorylated backbone vectors for 1) the B1H expression library; 2) the reporter library; 3) the two CS2-FokI expression plasmids.
	+ Creating all the amino acid stock solutions and antibiotics etc. needed for making selective media and plates
	+ Preparing electrocompetent cells (you will need to make your own at least for B1H selection)
	+ Amplifying and storing sequences encoding individual Finger Modules (explained in **Section 3**).

**2. TARGET SITE SELECTION**

**Contents**

**2.1. Considerations and goals for selecting target sequences**

**2.2. Identifying potential target sequences**

**2.3. Analysis and prioritization of candidate target sequences**

**2.4. Identifying OPEN Pools that encode zinc Finger Modules that recognize the triplets of your candidate target sequence**

**2.5. Assembly of a gene encoding a ZFP using previously identified ZFPs**

**2.1. Considerations and goals for selecting target sequences**

* ZFNs function as dimers. Each OPEN pool-based ZFN monomer is composed of two domains: a three-finger zinc-finger protein (ZFP) fused with the cleavage domain of the Fok I restriction enzyme. The Fok I cleavage domains used here are derived from Meng et al. (2008), and function as an obligate heterodimer. Successful target cutting therefore requires appropriate target sequence binding and interaction of two ZFN monomer partners.
* The three-finger ZFP domain is responsible for sequence-specific DNA binding:

- Individual single-Finger Modules recognize a **core triplet** (3 bp) target

- The base adjacent to the 3'-end of the triplet affects binding of a zinc-Finger Module to the core triplet. We call this the context base.

- Each three-finger ZFP recognizes 10bp (3 core triplets +1 context base adjacent to the 3'-end)

* The OPEN pools encode only a subset of possible zinc Finger Modules, and thus can be used to target only a subset of core triplets at each finger position.
* The full genomic target sequence of a ZFN consists of 25 - 27 bp:

- 10 bp for left monomer ZFN recognition/binding

- 10 bp for right monomer ZFN recognition/binding

- Spacer bases between each 3-finger target are required for dimerization of ZFN monomer partners: 6bp spacer is preferred, 5bp spacer can be used, 7bp may be acceptable but there is not much data on this.

* Selected target sequences should have the following criteria:
	+ ZFN-initiated mutations at the target site should destroy the function of a target gene. Target sites should be within or upstream of critical domains necessary for gene function.
	+ The actual genomic sequence to be mutagenized must be 100% identical to the designed target sequence. The target site cannot be interrupted by an intron. **Beware of strain polymorphisms** that may differ from a reference sequence.
	+ The target site must be unique in the zebrafish genome. The target site should not reside in repetitive sequences.
	+ It may be desirable to include a restriction enzyme site in the middle of the target sequence. This requirement depends on the method to be used for mutation identification. It is absolutely required if using a Restriction Enzyme-PCR Assay to identify induced mutations.
* To maximize the chance of obtaining mutations, you may choose to generate ZFNs against multiple target sites in a single target gene.

**2.2. Identifying potential target sequences**

* A web-based program, ZiFit, is used to identify target sequences that can be recognized using the OPEN pools: http://bindr.gdcb.iastate.edu/ZiFiT/. To use this, you must prepare query sequences first.
* As intron sequences vary more than exon sequences, we perform two rounds of queries for potential target sites: first we analyze exon sequences alone for potential target sequences - if targets are found in suitable exons we select those targets. If necessary we analyze sequences that include an exon plus 15 bp of intron sequences on both sides of the exon.
* Analyze your gene structure and prepare query sequences.
* To identify the regions of your gene that are optimal for mutation induction, you may wish to identify critical coding domains of your target gene, for example at <http://smart.embl-heidelberg.de/>. This allows you to prioritize use of potential target sites.

**Analyzing Query Sequences on ZiFit**

* Access ZiFit web site: http://bindr.gdcb.iastate.edu/ZiFiT/
* Click ZiFit on Menu
* Choose "Design Zinc Finger Nucleases" with OPEN
* Paste query sequence into sequence box
* Choose spacer size: start with 6 bp; then try 5 bp; 7 bp would be a last option
* Click "submit" to start search
* Select the best candidate sites from the search results:

Candidate site should not be centered in the 5'UTR or 3'UTR

Mutations at the candidate site are likely to disrupt function

Choose candidate sites that include the most GNN triplets **(6 GNN triplets, if possible)**

**2.3. Analysis and prioritization of candidate target sequences**

**2.3.1. Identifying potential off-target sites in the zebrafish genome**

From the ZiFit search results page:

* Click (+) sign next to candidate sites reveals a link to a Blast search engine
* Choose the Danio rerio database
* Analyze the Blast results with the following considerations:

The fewer potential off-target sites the more desirable is a candidate target

Best not to use candidate targets with many potential off-target sites

Avoid candidate targets with off-target sites in other genes

**2.3.2. Examining candidate target sites for the existence of Restriction Enzyme sites**

* Access NEBcutter V2.0: http://tools.neb.com/NEBcutter2/index.php
* Paste candidate target sequence (25~27bp; sense strand) into query box
* Click submit
* Choose the enzyme that recognizes the sequence in the spacer region

**2.3.3 Checking candidate target sites for their presence within repetitive sequences**

Maeder et al. (2009) suggested screening to determine if your target sequence lies within a repeated sequence. We find that if a target is within a repetitive sequence, the Blast search will indicate many off-target sites. However, you can directly search to determine if your target is within a repeated sequence:

* Retrieve the candidate target site along with ~ 300bp of genomic sequence flanking each side (~600bp total)
* Access RepeatMasker: http://www.repeatmasker.org/
* Click "RepeatMasking"
* Paste the query into the Sequence box
* Choose "Danio (zebrafish)" from DNA source
* Click "Submit Sequence"
* Try not to use target sequences that are located within repetitive sequence.

**2.4. Identifying OPEN Pools that encode zinc Finger Modules that recognize the triplets of your candidate target sequence**

* Access ZiFit, click (+) sign next to candidate sequence to reveal more information, and note the **Reference Number** of the OPEN pool corresponding to each triplet of the selected target sequence. The **Reference Number** refers to a particular pool, which can be found in a particular well in the OPEN pool array. The code for connecting **Reference Numbers**, their targets, and the wells in which they reside upon distribution is listed in **Section 3**.

**2.5. Assembly of a gene encoding a ZFP using previously identified ZFPs**

It is possible that ZFPs have been developed already against one of your target sequences. Check whether your candidate target sequences have been analyzed previously and are listed on a database.

* Access ZiFDB: http://bindr.gdcb.iastate.edu:8080/ZiFDB/
* Click "Search ZiFDB"
* Click "Search for a zinc finger array"
* Input candidate sequence, Note: 5' to 3' of a target sequence is recognized by F3 to Fl fingers
* Click "Search"
* Interpret the results

**3. OPEN POOL AMPLIFICATION AND ASSEMBLY OF THREE FINGER LIBRARIES**

**Contents**

**3.1. Considerations and goals: What are the OPEN Pools?**

**3.2. Preparation of Stored and Working Stocks of OPEN Pools**

**3.3. Preparing amplicon libraries representing individual Finger Modules**

**3.4. Assembly of amplicons encoding three-finger ZFPs from libraries of individual Finger Modules**

**3.1. Considerations and goals: What are the OPEN Pools?**

* OPEN Pools are provided as ~1ug of lyophilized plasmid DNA in each well of a 96-well plate.
* Each well/pool contains a series of plasmids that encode three-finger ZFPs. To generate the OPEN pools (Maeder et al., 2009), sequences encoding one zinc Finger Module of a three-finger protein were varied (the other two fingers were held constant), plasmids encoding proteins that could bind a specific 9 bp target sequence were selected under low stringency conditions, and plasmids from up to 95 independent selected clones were pooled. Thus each pool contains a library of sequences encoding three-finger proteins where only one position-specific zinc Finger Module (either F1, F2, or F3) varies and all the variations of this module in the pool should be able to bind one triplet at a specific position within a three triplet target sequence. Each pool is designated by 1) F1, F2, or F3 (indicating the Finger Module that was allowed to vary) and 2) the triplet target sequence the Finger Module was selected to bind.
* The goal of the "Utah - Big Love" approach is 1) to generate from the OPEN pools a library of plasmids that encode three-finger proteins **likely** to bind a desired three-triplet target sequence, and then 2) to select specific three-finger proteins that bind well to the target sequence using a Bacterial One-Hybrid (B1H) selection scheme developed by (Noyes et al., 2008). The OPEN pools contain plasmids that encode three-finger proteins, but we are interested **only** in the Finger Module that has been subjected to selection against the triplet of interest. To generate a library encoding three-finger proteins that can bind a desired 9 bp core target sequence, it will be necessary to join sequences encoding F1 from one OPEN pool, sequences encoding F2 from a different OPEN pool, and sequences encoding F3 from a third OPEN pool. To join specific finger sequences from different OPEN pools, it is useful first to create pools of DNA fragments each of which contains only sequences encoding a single Finger Module of interest. To accomplish this, here we amplify only F1 from the F1 OPEN pools, only F2 from the F2 pools, and only F3 from the F3 pools. Amplification of each finger requires finger-specific primers.
* From the pools of individual Finger Modules we will assemble three-finger ZFP using “overlapping PCR” to join sequences encoding each module.
* The three-finger ZFP amplicons are subsequently cloned (**in Section 4)** into an expression vector that fuses the ZFPs with an RNA Polymerase co-factor, thus driving expression of fusion proteins suitable for the Bacterial 1-Hybrid selection scheme.
* As of 2010, the OPEN systems contained 74 pools: 31 pools for F1 finger, 21 pools for F2 finger, and 22 pools for F3 finger. Each pool has a “Reference Number” and the pools have been arrayed in a 96-well plate. The Table on the following page explains the correspondence between **Reference Number**, **Well Number/Location**, **Finger Module that was under selection,** and **Target Triplet that the Finger Module was selected against.** It is possible either to order individual wells of interest or to order the entire set at once. Here we explain working with an entire set of the OPEN Pools.

**3.2. Preparation of Stored and Working Stocks of OPEN Pools**

* OPEN Pools are obtained as lyophilized DNA arrayed in a 96-well plate. We have found it useful to re-suspend the DNA and generate 1) an original stock plate; 2) two replica stock plates; and 3) diluted "working plates".



**Materials**

10mM Tris-HCI (pH7.4)

96-well plates [ISC BioExpress, #T-3082-I]

Adhesive PCR film [Thermo Scientific, #AB-0558]

**Procedure for making Stock and Working Plates of OPEN pools**

- OPEN Pools are obtained in a 96-well plate: ~1ug lyophilized plasmid DNA/well

- cfg. (centrifuge) 1,000rpm, 1 min @ 4°C. (to spin down any water in the wells)

- Add 100ul 10mM Tris-HCI (pH7.4) into each well.

- Incubate 30 min. @ RT to dissolve DNA.

- Dissolve DNA completely by pipetting with 50ul multi-channel pipettor

 **Label plate**: "OPEN pools, original" [~10ng/ul in each well]

- Transfer 30ul to each of two new 96-well plates

 **Label Plate**: "OPEN pools, replica #1" (or #2) [~10ng/ul in each well]

- Seal all plates with adhesive PCR film

- Wrap the plates with parafilm to completely seal them

- Store replica plates @-80°C

In a new 96-well plate:

- Add 45ul/well of 10mM Tris-HCI (pH7.4)

- Add 5ul/wellof "OPEN pools, original"

- Mix well by pipetting

 **Label Plate**: "OPEN pools, working plate (1/10)" [~lng/ul in each well]

- Seal the plates (original and dilution) with adhesive PCR film

- Wrap plates with parafilm to completely seal

- Store @ -80°C

**3.3. Preparing amplicon libraries representing individual Finger Modules**

* We amplify all the single Finger Modules from all the OPEN pools at once, and then store the entire set of single Finger Module amplicons. However, one can amplify single Finger Modules on an as-needed basis.

**3.3.1 PCR Amplification of selected individual zinc Finger Modules from the OPEN Pools**

* Total pools per Finger Module: 31 F1 pools; 21 F2 pools; 22 F3 pools.
* Individual Finger Modules are amplified in 8-tube strips of PCR tubes. Then each amplicon is gel-purified.

**Materials**

TempAssure PCR 8-tube strip, natural [USA Scientific, #1402-2700]

Expand High Fidelity PCR System [Roche, #11732641001]

Template DNA: "OPEN pools, working (1/10)"

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| --- |
| **OVERVIEW OF INDIVIDUAL FINGER MODULE AMPLIFICATION** |
| **Finger to be amplified: Primers to use** | **OPEN pools** | **Amplicon size** | **PCR Program** |
| F1: OK1424/OK1425 | A1-A11; C7-C12; D2-D5; D10-D12; E1-E4; F11; G1, G2 | 116bp | OPEN-FIB |
| F2: OK1426/OK1427 | A12; B1-B7; E11, E12; F1- F10; F12 | 91 bp | OPEN-F2B |
| F3: OK1428/OK1429 | B8-B12; C1-C6; D1, D6-D9; E5-E10 | 80 bp | OPEN-F3B |

|  |
| --- |
| **Primer Information** |
| **Name** | **Sequence** | **Tm** | **MW** | **nmol/OD** |
| OK1424 | GAGCGCCCCTTCCAGTGTCGC | 72.62°C | 6,359 | 5.41  |
| OK1425 | CGCATACAGATCCGACACTGAAACGG | 71.24°C  | 7,958 | 3.89  |
| OK1426 | GTGTCGGATCTGTATGCGAAATTTCTCC | 69.23°C  | 8,586 | 3.81  |
| OK1427 | TCGGCATTGGAATGGCTTCTCG | 70.46°C  | 6,757 | 4.96  |
| OK1428 | GCCATTCCAATGCCGAATATGCA | 69.68°C  | 6,993 | 4.51 |
| OK1429 | CCCTCAGGTGGGTTTTTAGGTG | 43.26°C → 63.93°C | 6,788 | 4.91 |

|  |
| --- |
| **PCR Protocol** |
|  |  | **Master Mixes**  | **Final Conc.** |
|  |  | 16x | 24x |  |
| 10x Expand Buffer [+15mM MgCI2]  | 5ul | 80ul  | 120ul  | lx, 1.5mM MgCl2 |
| 2mM each dNTPs  | 7.5ul | 120ul  | 180ul  | 300uM each  |
| 10uM forward primers  | 3ul | 48ul  | 72ul | 600nM  |
| 10uM reverse primers  | 3ul | 48ul  | 72ul | 600nM |
| Template DNA | 1ul | - | - | 20pg/ul |
| H2O  | 30.125ul | 482ul  | 723ul |  |
| Expand Enzyme [3.5U/ul] | 0.375ul | 6ul | 9ul | 0.026U/ul |
| **Total volumes** | **50 ul** | **9ul x 16** | **49ul x 24** |  |

|  |
| --- |
| **Finger Module PCR Amplification Programs** |
| OPEN-FIB 94°C, 2min; 35 cycles: {94°C, 15sec; 67°C, 20sec; 72°C*,* 30sec}; 72°C, 2min; 4°C | OPEN-F2B 94°C, 2min; 35 cycles: {94°C, 15sec; 67°C, 20sec; 72°C,30sec}; 72°C, 2min; 4°C |
| OPEN-F3B 94°C, 2min; 10 cycles: {94°C, 15sec; 43°C, 20sec; 72°C*,* 30sec}; 25 cycles: {94°C, 15sec; 62°C, 20sec; 72°C*,* 30sec}; 72°C, 2min; 4°C |

**Procedure:**

* Prepare 100uM Primer Stock Solutions for storage
* Dilute Stock Solutions to generate 10uM Primer ‘Working’ Solutions
* Note the Finger Modules that are to be amplified
* Prepare PCR Reactions according to the PCR Protocol using the Finger-specific Amplification Programs
* Finger Module amplicons can be stored @ -80°C.

**3.3.2. Gel Purification of Finger Module amplicons**

Overview: Amplicons representing individual Finger Modules are gel-purified and then stored.

**Materials**

QIAquick Gel Extraction Kit [QIAGEN, #28706]

**Procedure**

- Run 53ul sample (50ul PCR product + 3ul 10X loading dye (Xylene Cyanol only)) through 1.5% agarose gels

- Purify each DNA by QIAquick Gel Extraction Kit

- Elute in 50ul of 1/10X diluted Elution Buffer (incubate 3min. @ RT prior to centrifugation)

- Measure OD (1ul on NanoDrop ND-1000 spectrophotometer)

- Store @ -20°C

• Amplicons should be: Fl = 116 bp; F2 = 91 bp; F3 = 80 bp. However, two bands (~80 bp and ~170 bp) appear in some F3 pools. In these cases, purify only the smaller ~80 bp fragment

**3.4. Assembly of amplicons encoding three-finger ZFPs from libraries of individual Finger Modules**

Overview: Ready-to-clone DNA fragments encoding three-finger ZFPs are generated from the libraries of individual Finger Modules in two steps. First, because each Finger Module amplicon has been constructed so that one end has sequences identical to those at the end of the adjoining Finger Module, "Overlapping PCR amplification" is used to join Finger 1 Module sequences to Finger 2 Module sequences and Finger 2 Module sequences to Finger 3 Module sequences. As a result, amplicons encoding three-finger ZFPs are generated. In a second step, new ends with restriction enzyme sites that allow directional cloning are added to the termini of the three-finger amplicons.

**3.4.1. Assembly of sequences encoding three-finger ZFPs**

Considerations

* Some F1 modules, but not others, have been screened with consideration of the base adjoining the target triplet.

ex.: GCTg and GGAg vs. GAG and GTA

* Context-considered F1 modules were not prepared for all triplet + 1bp units.

ex.: There are modules specific for GCTg and GCTc, but not GCTt nor GCTa.

* If the F1 target sequence is "GCTa", use a mixture of F1 fragments from the "GCTg" and "GCTc" pools (2ul each) as templates to build the three-finger fragment.

|  |
| --- |
| **Protocol for Overlapping PCR**  |
|  |  | **Master Mix** | **Final Conc.** |
| **Reaction #1** |  | 5x |  |
| 10x Expand Buffer [+15mM MgCI2]  | 5ul | 25ul | lx, 1.5mM MgCl2 |
| 2mM each dNTPs  | 7.5ul | 37.5ul | 300uM each  |
| Purified F1 DNA fragment [~10 ng/ul] | 4ul  | - | ~ 40 ng  |
| Purified F2 DNA fragment [~10 ng/ul] | 4ul  | - | ~ 40 ng  |
| Purified F3 DNA fragment [~10 ng/ul] | 4ul  | - | ~ 40 ng  |
| H2O  | 25.125ul | 125.625ul |  |
| Expand Enzyme [3.5U/ul] | 0.375ul | 1.875ul | 0.026U/ul |
| **Total volumes** | **50 ul** | **38 ul x 5** |  |

**PCR Program: OPEN-AS1**

94°C, 2min;

10 cycles: {94°C, 30sec; 50°C, 30sec; 72°C*,* 1min};

72°C, 7min;

4°C

**Purification using QIAquick PCR Purification Kit**

50ul reaction mixture per column

Elute in 50ul of 1/10X Elution Buffer (incubate 3min. @ RT prior to centrifugation)

**3.4.2. Amplification of the Assembled Three-Finger Fragments with new ends for asymmetric cloning**

**Materials**

Expand High Fidelity PCR System [Roche, #11732641001]

QIAquick PCR Purification Kit [QIAGEN, #28106]

|  |
| --- |
| **Primer Information** |
| Name | Sequence | Tm | MW | nmol/OD |
| B2TO1HF | GTAGGTACCCGCCCCTTCCAGTGTCGC | 67.59°C → 75.56°C | 8,188 | 4.14 |
| B2TO1HR | GTGGGATCCCCTCAGGTGGGTTTTTAGGTG | 63.93°C → 75.35°C | 9,316 | 3.54 |

**Procedure**

* Prepare 100uM Primer Stock Solutions for storage.
* Dilute Stock Solutions to generate 10uM Primer ‘Working’ Solutions.
* For each sample, perform two replicate 50ul PCR reactions according to the Protocol for Overlapping PCR, Reaction #2, utilizing the **OPEN-AS2 PCR Program.** The amplicon size is 267bp

|  |
| --- |
| **Protocol for Overlapping PCR**  |
|  |  | **Master Mix** | **Final Conc.** |
| **Reaction #2** |  | 5x  |  |
| 10x Expand Buffer [+15mM MgCI2]  | 5ul | 25ul | lx, 1.5mM MgCl2 |
| 2mM each dNTPs  | 7.5ul | 37.5ul | 300uM each  |
| B2TO1HF | 3ul  | 15ul | 600nM  |
| B2TO1HR | 3ul  | 15ul | 600nM |
| Overlapping PCR product | 24ul  | - |  |
| H2O  | 7.125ul | 35.625ul |  |
| Expand Enzyme [3.5U/ul] | 0.375ul | 1.875ul | 0.026U/ul |
| **Total volumes** | **50 ul** | **26 ul x 5** |  |

**PCR Program: OPEN-AS2**

94°C, 2min;

10 cycles: {94°C, 10sec; 60°C, 15sec; 72°C*,* 1min};

20 cycles: {94°C, 15sec; 68°C, 15sec; 72°C*,* 1min};

72°C, 7min;

4°C

**Purification using QIAguick PCR Purification Kit**

* 100ul (50ul x2) reaction mixture per column
* Elute in 50ul of 1/10X Elution Buffer (incubate 3min. @ RT prior to centrifugation)
* Measure OD (1ul on NanoDrop ND-1000 spectrophotometer)
* Run 1ul on 1.5% Agarose gel to check size - **Amplicon Size:** 267bp
* Store @ - 20°C

• Typically ~100ng/ul DNA is obtained in the eluted ~50ul solution.

**4. CONSTRUCTION OF THREE-FINGER ZFP LIBRARIES**

**Contents**

**4.1. Required starting materials**

**4.2. KpnI/BamHI Digestion of Assembled Three-Finger PCR Fragments**

**4.3. Preparation of Library Vector**

**4.4. Library Construction**

**4.5. Trouble Shooting**

**4.1. Required Starting Materials**

* 267bp amplicons encoding three-finger ZFPs (generated in **Section 3**) [~100-150ng/ul in ~50ul]
* pB1H2w2-Kp/Bm (5'-OH) [100ng/ul] (vector plasmid for libraries)
* Gene Pulser Cuvettes [BIO-RAD, #165-2086] (2mm cuvette for electroporation)
* XL-I Blue electrocompetent cells [Stratagene, #200228]
* 3mm glass beads [VWR, #26396-508] (autoclaved and stored @ RT)
* 2uM B1H2w2Fl (For PCR and sequencing, including ZFP library screening procedure)
* 2uM H2-R (For PCR, including ZFP library screening procedure)
* 2xYT plates and broth

**4.2. KpnI/BamHI Digestion of Assembled Three-Finger PCR Fragments**

**Materials**

KpnI [NEB, #R0142S]

BamHI [NEB, #R0136S] or [Fermentas, #FD0054]

QIAquick Gel Extraction Kit [QlAGEN, #28706]

Sample DNA: Amplicons encoding three-finger ZFPs (generated in **Section 3**) [100 ~ 150ng/ul]

**Procedure**

**KpnI/BamHI digestion**

 Sample DNA [~100ng/ul] 20ul (2ug)

 10X buffer [NEB, #2] 5ul

 KpnI [NEB] 3ul

 BamHI [NEB] 1.5ul

 H2O up to 50ul

 - Incubate 37°C, ON

 - Add 3ul 0.5M EDTA

 - on ice

**Gel Purification**

* Run 59ul (53ul reaction mixture + 6ul 6x Orange G dye) on 1.5% agarose in 1X TAE
* Purify using QIAquick Gel Extraction Kit: one column/sample
* Elute in <50ul 1/10 diluted EB (incubate 3min. @ RT prior to centrifugation)
* Measure OD [1ul on NanoDrop ND-1000 spectrophotometer]
* Run ~2ul (~40-100ng) on 1.5% agarose in 1X TAE to check size and recovery
* Store @ -20°C

• Size of the pre-digested ZFP fragment is **267bp.**

• Size of the KpnI/BamHI digested ZFP fragment is **255bp.**

**4.3. Preparation of Library Vector**

* Overview: The three-finger ZFPs will be selected in a B1H system. The vector backbone used for the three-finger ZFP library is the pB1H2w2-mcs plasmid cut with KpnI and BamHI and dephosphorylated. The ready-to-use vector is called pB1H2w2-Kp/Bm (5’-OH) [3.7kb]
* pB1H2w2-Kp/Bm (5’-OH) should be gel-purified. Because we produce this plasmid in large quantities for many projects, we perform 20ug digestions and gel purify with a phenol/chloroform (ΦOH/CHCL3) extraction procedure. However, for smaller projects, any simpler method is sufficient.
* The quality of the three-finger ZFP library in pB1H2w2-Kp/Bm (5’-OH) is very important. We measure number of independent transformants (complexity) and cell concentration before proceeding to selection.

**Materials**

KpnI [NEB, #R0142S]

BamHI [NEB, #R0l36S]

SAP (Shrimp Alkaline Phosphatase, 1U/ul) [Fermentas, #EF0511]

Pellet Pestle [Kimble Chase Kontes, #749521-15090 (provided by Fisher Scientific, #K749521-1590)

ΦOH (saturated with TE)

CHCl3 (CHC13: isoamyl-alcohol = 24:1)

**4.3.1. KpnI/BamHI Digestion: Set up 2 digestions, each with 50ul and 10ug DNA**

 pB1H2w2-mcs 10ug

 10X buffer [NEB, #2] 5ul

 KpnI [NEB] 3ul

 BamHI [NEB] 1.5ul

 H20 up to 50ul

 - Incubate 37°C, O/N

 - Incubate 70°C, 20min.

 - on ice

**4.3.2. Dephosphorylation: 130ul x 2 tubes**

 digested DNA 50ul

 10X SAP buffer 13ul

 SAP (1U/ul) 7ul

 H2O 60ul

 - Incubate 37°C, 2h

 - Add 6.5ul 0.5M EDTA

 - Incubate 70°C, 20min.

 - on ice

**4.3.3. Gel Purification**

**Sample Preparation for gel electrophoresis (550ul)**

 digested and dephosphorylated DNA 273ul (130ul x2 + 6.5ul x2)

 TE 222ul

 6x loading dye (Orange G) 55ul

**Electrophoresis**

 0.8% agarose in 1X TAE (13.8cm x 12cm x 0.9cm)

 combs: 8mm x 12

 load: 55ul sample/lane x 10 wells

 run: 80V/20cm, 45-60min.

**Gel Purification by ΦOH Extraction**

* cut out gel slices containing vector DNA (3.7kb)
* divide and place into 5 tubes of 1.5ml microcentrifuge tubes: ~2 wells/tube
* Add TE up to ~400ul/tube)
* crush gel slices with pestle
* Add 600ul/tube ΦOH
* vortex well
* Incubate -80°C, (>30min – ON)
* thaw and mix well by vortexing
* cfg.: 14,000rpm, 5min. @ RT
* Transfer supernatant to new tube (~400ul/tube)
* ΦOH extract
* transfer the upper aqueous phase (~400ul/tube) to new tubes
* ΦOH/CHC13 extract
* transfer the upper aqueous phase (~400ul/tube) to new tubes
* Add 40ul/tube (1/10X vol.) 3M NaOAc
* Add 1ml/tube (2.5X vol.) EtOH @-20°C
* mix well by inverting; incubate 20min on ice
* cfg.: l4,000rpm, 20min. @4°C
* remove supernatant
* Wash pellet with 700ul 70% EtOH
* cfg.: l4,000rpm, 20min. @4°C
* Remove supernatant
* vacuum dry, ~5min.
* dissolve pellet in 20ul/tube TE
* Pool the DNAs into a single tube: ~100ul total
* measure OD
* dilute to 100ng/ul with TE
* load 1ul on 1% agarose gel to check quality
* store @-20°C

**4.3.4. Quality Check of Prepared Vector Plasmid: Transformation Efficiency**

* Check the transformation efficiency in XL-l Blue electrocompetent cells of the prepared vector plasmid without (or with) a control insert, count the colony numbers and record the efficiency.
* Typical results: vector only: <200 colonies/ng vector DNA; vector plus control insert: >2x104 colonies/ng vector DNA

**Example**

 **Vector Insert Plate #1 Plate #2 Plate #3**

pB1H2w2-Kp/Bm (5'-OH) - 1X 1/10X 1/100X

pB1H2w2-Kp/Bm (5'-OH) some Kp/Bm control insert 1/100X 1/1,000X 1/10,000X

**Materials**

Gene Pulser Cuvette [BIO-RAD, #165-2086] (2mm cuvette for electroporation)

Gene Pulser Xcell [BIO-RAD]

T4 DNA Ligase [NEB, 10X buffer including ATP]

XL-l Blue electrocompetent cells [Stratagene, #200228]

SOC

2xYT (or LB)

LB/carb (100ug/ml) plates

**Ligation**

10X T4 DNA ligase buffer [NEB, ATP+] 1ul

pB1H2w2-Kp/Bm (5'-OH) [100ng/ul] 1ul

some Kp/Bm control insert -/+

T4 DNA ligase [NEB] 0.2ul

H2O up to 10ul

* + Mix well and spin down briefly
	+ Incubate 16°C, ON
	+ Store @4°C

**Transformation** (one cuvette/sample)

* Combine on ice: 1ul Ligation mixture + 50ul XL-l Blue electrocompetent cells
* Transfer to electroporation cuvette (on ice)
* Carry out electroporation with GenePulserXcell [condition: 2.5kV, 25uF, 200g]
* add 750ul SOC into the cuvette as quickly as possible (prepare 750ul in pipetman prior to pulse)
* transfer to 1.5ml microcentrifuge tube
* 37°C, 1h rotating @-250rpm
* plate dilutions of cells, incubate 37°C, ON, calculate transformation efficiency

**4.4. Library Construction**

Note: The vector plasmid pB1H2w2-mcs used for cloning three-finger ZFP fragments was created by replacing the Asp7l8-XbaI fragment encoding the zif268 domain of pB1H2w2zif268 0 with an Asp7l8-XbaI linker consisting of: 5'-GTACCCTCGAGAAGCTTGGATCCTAAT-3' (sense); and 5'-CTAGATTAGGATCCAAGCTTCTCGAGG-3 ' (antisense). The entire sequence of pB1H2w2-mcs is in **Section 9**.

**Materials**

Ligation Ready Vector Plasmid: pB1H2w2-Kp/Bm (5'-OH) [100ng/ul]

Insert DNA: ZFP-Kp/Bm (three-finger fragments cut with KpnI and BamHI and gel-purified)

Gene Pulser Cuvette [BIO-RAD, #165-2086] (2mm cuvette for electroporation)

Gene Pulser Xcell [BIO-RAD]

T4 DNA Ligase [NEB, 10X buffer including ATP]

Glycogen [Ambion, #AM95l0]

XL-l Blue electrocompetent cell [Stratagene, #200228]

SOC

2xYTbroth

2xYT/carb (100ug/ml) plate (or LB/carb (100ug/ul) plate):

 150mm plates 4 plates/2 libraries (two plates for right ZFP and two plates for left ZFP)

 100mm plates 6 ~ 8 plates/2 libraries

**4.4.1. Library Ligation**

NOTE: Perform EtOH precipitation very carefully to avoid disturbing DNA pellet and losing it.

10X T4 DNA ligase buffer [NEB, ATP+] 2ul

Vector: pB1H2w2-Kp/Bm (5'-OH) [100ng/ul] 4ul (400ng)

Insert: ZFP-Kp/Bm ~100ng

T4 DNA ligase [NEB] 1ul

dH20 up to 20ul

* Incubate 16°C, ON
* Add 72ul TE
* Add 8ul 1ug/ul glycogen
* Add 10ul (1/10 vol.) 3M NaOAc (pH7.0)
* Mix well (vortex and spin down)
* Add 250ul (2.5 vol.) -20°C EtOH
* Mix well by inverting
* Incubate on ice, 20min
* cfg: 14,000rpm, 20min @ 4°C
* Carefully remove the supernatant with P1000 or P200 pipetman)
* Wash the pellet with 500ul -20°C 70% EtOH by:

 Slowly adding the 70% EtOH to the pellet (don't disturb the pellet)

 Closing the lid and rotating the tube to wash inner wall with the 70% EtOH

* cfg: 14,000rpm, 5min. @ 4°C
* Carefully remove the supernatant with P1000 or P200 pipetman)
* Vacuum dry, ~5-8min
* Dissolve DNA in 6ul dH20

**4.4.2. Library Transformation**

**Transformation**

* Perform 2 transformations per sample (2 cuvettes per sample)
* Plate dilutions onto 100mm plates for transformation efficiency analysis and plate onto 150mm plates for library DNA preparation. Both types of plating will be performed simultaneously.
* Combine in microfuge tube on ice:

 6ul DNA ligation mixture + 130ul XL-I Blue electrocompetent cells

* Mix **gently** by flicking
* Transfer half the cell suspension to each of two electroporation cuvettes on ice
* Carry out electroporation with GenePulserXcell [condition: 2.5kV, 25uF, 200Q]
* Add 750ul SOC into the cuvette as quickly as possible (prepare 750ul in pipetman prior to pulse)
* Combine both electroporation solutions into a l5ml tube: total volume ~1.6ml per sample
* Rotate 1hr at 37°C, 250rpm (stand tubes on an angle)
* Centrifuge tubes at 3,500xg, 10min. @ RT
* Re-suspend pellet in 1ml sterile dH20 and transfer to 1.5ml microcentrifuge tube
* Cfg.: 14,000rpm, 2min. @ RT
* Wash pellet 1X more with dH20 [suspend 1ml sterile dH20 and cfg. 14,000rpm, 2min @ RT]
* Re-suspend pellet in 800ul dH20
* Transfer 5ul to a 1.5ml microcentrifuge tube for **Transformation check (See below)**
* Plate ~400ul of the suspension onto each of two 150mm 2xYT/carb plates
* Incubate at 37°C, ON
* Prepare plasmid DNA (**See 4.3 Midi-Prep of ZFP plasmid library DNA**) from transformed cells on 150mm plates if the titer is >3 x 106.

**Transformation Check**

Note: Keep titer plates made here to pick colonies for Colony-PCR and Sequencing (**see 4.4 Assessing ZFP library quality/diversity**)

* Prepare four 10-fold serial dilutions:

 1/10X: 5ul of 1X + 45ul 2xYT

 1/100X: 5ul of 1/10X + 45ul 2xYT

 1/1000X: 5ul of 1/100X + 45ul 2xYT

 1/10000X: 5ul of 1/1000X + 45ul 2xYT

* Plate 20ul cell suspension of lower dilutions onto 2xYT/carb or LB/carb (100ug/ml)
* Incubate at 37°C, ON
* Count the number of colonies on each plate and determine titer of cell suspension

**Example**: if there are 200 colonies on the 1/1,000X dilution plate,

200 x 1,000 = 2 X 105 in 20ul cell suspension

2 x 105 x 800/20 = 8 x 106 colonies in 800ul cell suspension (acceptable, as we need >3 x 106 independent transformants).

**4.4.3. Midi-Prep of ZFP plasmid library DNA**

* ZFP plasmid library DNAs are prepared directly from just-plated (150mm plates) transformed cells. 100ng of each library is required for screening in the B1H system. 100ng/ul or higher final concentration is recommended for the next screening step.
* The pB1H2w2 plasmid is a low copy number plasmid that always gives low yields. Because of this, the plasmid DNA fraction is easily contaminated with bacterial genomic DNA. Contaminating bacterial genomic DNA reduces the net amount of library DNA and will affect the total titer of library screening on next step. Using any plasmid purification procedure/kit that can reduce the contamination of the genomic DNA is appropriate. One good approach is to use a commercial midi-prep kit using larger volumes of lysis buffers, as described in the example below.

**Materials**

2xYTbroth

3mm glass beads [VWR, #26396-508] (autoclaved and stored @RT)

PureLink HiPure Plasmid Filter Purification Kits [Invitrogen, #K2l00] (or other plasmid purification kit)

60ml hand-made pre-filter (Stuff 6 pieces of Kimwipe into 60ml syringe and push down to the bottom with plunger)

**Procedure: Midi-Prep using PureLink HiPure Plasmid Filter Purification Kit**

* Start with two 150mm plates (confluent with colonies) of just-plated ZFP library-transformed cells

To wash off all the colonies on the plates:

* Add 10ml 2xYT broth/plate
* Add ~20 3mm glass beads per plate
* Rotate @ 100rpm for ~10min (try to make sure the beads are distributed over the plate)
* Collect cell suspensions from the two plates and combine into a single 50ml tube
* Rinse each plate with 5ml 2xYT broth and add to the 50ml tube: total volume is ~30ml cell suspension from 2 plates
* Cfg. 4,000xg, 10min. @ RT (or 4°C)
* Remove supernatant
* Purify cell pellet through a column according to Invitrogen's manual with brief modifications:
* Use 15ml each ofR3, L7 and N3 (cell lysis with larger volumes is best for avoiding genomic DNA contamination)
* Put cell lysis solution through 60ml hand-made pre-filter before loading onto column
* After the isopropanol precipitation, wash the pellet with 1ml 70% EtOH and transfer to a new l.5ml tube
* Cfg at 14,000rpm, 5min. @ 4°C
* Wash pellet with 700ul 70% EtOH
* Cfg at 14,000rpm, 5min. @ 4°C
* Remove EtOH and vacuum-dry for ~5-8min
* Dissolve the DNA pellet in 100ul H20
* Measure OD
* Run ~100ng on 0.8% Agarose gel (to check for contamination with genomic DNA)
* Store @ -20°C

**4.4.4. Assessing ZFP library quality/diversity: Sequencing plasmids from the titer plates**

* The goal is to obtain a rough estimate of whether the ZFN library has enough complexity to give good enrichment/selection in the B1H screen.
* Sequence and analyze 8 clones from the titration plate (representing the library prior to selection). If 3 or more of the 8 clones have an identical sequence in a particular finger position (this does happen), you should be concerned that there may not have been a lot of variability in the starting OPEN pool used for this experiment.
* Nevertheless, we recommend trying ZFPs that can be recovered following stringent selection.

**Materials**

LB/carb (100ug/ml) broth

**Procedure for recovering and storing colonies**

* Dispense 20ul LB/carb (100ug/ml) broth into each well of 96-well cell culture plate
* Pick 8 colonies from titration plates and suspend in the broth
* Mix well by pipetting
* Use 2ul of cell suspension for **colony-PCR (see below)**

As a backup for failed colony-PCR we grow an overnight culture of each colony and store for a few days:

* After removing 2ul for colony-PCR, add 82ul LB/car (100ug/ml) broth
* Cover and parafilm
* Incubate at 37°C, ON
* Add 25ul 60% glycerol to each well
* Mix well by pipetting
* Store @ -80°C

**Colony-PCR**

**Materials**

Taq DNA polymerase [NEB, #M0273S or M0273L (with Standard Taq Reaction Buffer, Mg+)]

|  |
| --- |
| **Primer Information** |
| Name | Sequence | Tm |
| B1H2w2F1 | CAAGAGCAGGAAGCCGCTG | 64.6°C |
| H2-R | TTGTCGGCCTTTTTCTAGTCTCTAGA | 62.9°C  |

|  |
| --- |
| **Colony-PCR Protocol**  |
|  |  | **Master Mixes**  |
|  |  | **17x** | **34x** |
| 10x PCR Buffer [NEB Mg+]  | 2ul | 34ul  | 68ul  |
| 2mM each dNTPs  | 2ul | 34ul  | 68ul  |
| 2uM B1H2w2F1 | 0.5ul | 8.5ul  | 17ul |
| 2uM H2-R | 0.5ul | 8.5ul  | 17ul |
| Cell suspension | 2ul | - | - |
| 1X DNA polymerase mix | 0.15ul | 2.55ul | 5.1ul |
| H2O  | 12.85ul | 218.45ul  | 436.9ul |
|  |  |  |  |
| **Total volumes** | **20 ul** | **18ul x 17** | **18ul x 34** |

* Dispense 18ul Master Mix into 200ul PCR tube or wells of a 96-well plate
* Transfer 2ul from each cell suspension/tube

**Colony-PCR Program**

94°C, 1min;

35 cycles: {94°C, 10sec; 59°C, 15sec; 68°C*,* 1min};

72°C, 1min;

4°C

**Electrophoresis**

Run 5ul on 1% agarose gel, expecting 407bp amplicon

**Sequence Sample**

2uM BIH2w2Fl 4ul

PCR fragment 2ul

H20 4ul

Total: 10ul

**Sequence Analysis**

* Extract (copy) the KpnI-XbaI sequences (should be about 270bp)
* Paste into a file of any DNA analysis application
* Build an excel file.
* Translate retrieved DNA sequences into amino acids
* Identify core residues in each finger (a Template for how to analyze positions in the ZFP sequence is presented in the figure on the following page)
* Create an Excel file and input the core residues:

Column #1: clone#

Column #2: selected 7 residues in Finger 1

Column #3: selected 7 residues in Finger 2

Column #4: selected 7 residues in Finger 3

Column #5: length of KpnI - XbaI stretch

Column #6: comments (i.e., ambiguity, base alterations in the fixed sequences, etc)

**4.5. Trouble Shooting**

• If the titer of the prepared library is lower than 3 x 106, you should look at:

 i) Transformation efficiency (especially if the cells were prepared manually);

 ii) Loss of ligated DNA during EtOH ppt;

 iii) Ratio of insert DNA to vector plasmid DNA.



**5. REPORTER PLASMID CONSTRUCTION**

**Contents**

**5.1. Considerations and Goals**

**5.2. Required Starting Materials**

**5.3. Oligonucleotide Synthesis to Construct Reporter Plasmids**

**5.4. Preparation of Reporter Vector Plasmid**

**5.5. Ligation and Transformation**

**5.6. Cell Storage, Colony-PCR and Sequencing**

**5.7. Midi-Prep of Reporter Plasmid and Preparation of the Cell Stock**

**5.1. Considerations and Goals**

Here the reporter plasmid for the Bacterial One-Hybrid selection is constructed. Candidate Target Sequences, including the two nucleotides that neighbor the 9bp Core Target Sequence in the zebrafish genome, will be inserted just upstream from the promoter of a plasmid gene that supports Histidine auxotrophy. Note that the reporter plasmid, like the ZFP expression plasmid, is a low copy number plasmid and will produce low yields of DNA. Also note the reporter plasmid carries the kanamycin resistance gene.

**5.2. Required Starting Materials**

Oligonucleotide sequences to be synthesized: 2 oligos per reporter plasmid

pH3U-EI/RI (5'-OH) [20ng/ul] (a digested and dephosphorylated vector plasmid that is ready to use)

2uM HU100 (PCR and sequencing primer)

2uM H3U3R (PCR primer)

**5.3. Oligonucleotide Synthesis to Construct Reporter Plasmids**

**Design**

* Generate two complementary oligonucleotides that contain an 11bp recognition sequence along with single strand end sequences that allow simple cloning into the Reporter Plasmid. The two oligonucleotides should have 5' phosphates in preparation for ligation.
* 11bp recognition sequence = 9bp core target recognized by three fingers + 1bp 5' and 1bp 3' that are present in the zebrafish genome adjoining the 9bp core target sequence.
* After ordering oligos, calculate MW and coefficient *(i.e.:* nmol/OD260) at the Ambion site: http://www.ambion.com/techlib/misc/oligo\_calculator.html
* Prepare oligonucleotides at 100uM (= 0.1 nmol/ul) in dH20

**Name Template sequence**

H3U3**gene ZFP**-F 5’-pCCGGA**xX3X3X3X2X2X2X1X1X1x**TGTG-3'

H3U3**geneZFP**-R 5’-pAATTCACA**yY1Y1Y1Y2Y2Y2Y3Y3Y3y**T-3'

**x:** left and right context sequence

**X1X1X1**: 3bp recognized by F1 finger

**X2X2X2**: 3bp recognized by F2 finger

**X3X3X3**: 3bp recognized by F3 finger

**yYIYIYIY2Y2Y2Y3Y3Y3y**: complementary sequence of **xX3X3X3X2X2X2XIXIXIx**

underlined sequences indicate the BspEI or EcoRI annealing sites

**Example: The genomic target for tbx6ZFP2 is:**

5' -gCGCTTCACCTTTCCTGAAGCTGCTt-3' sense strand

3' -cGCGAAGTGGAAAGGACTTCGACGAa-5'

Therefore, for the left and right ZFPs (ZFP2L and ZFP2R):

 **Core target triplets**

**ZFP name F3 F2 Fl Reporter Sequences**

tbx6ZFP2L GGT GAA GCGc 5'-aGGTGAAGCGc-3'

tbx6ZFP2R GAA GCT GCTt 5'-tGAAGCTGCTt-3'

You would need to assemble the following pairs of complementary oligonucleotides:

**Name Sequence**

H3U3tbx6ZFP2L-F 5'-pCCGGA**AGGTGAAGCGC**TGTG-3'

H3U3tbx6ZFP2L-R 5'-pAATTCACA**GCGCTTCACCT**T-3'

H3U3tbx6ZFP2R-F 5'-pCCGGA**TGAAGCTGCTT**TGTG-3'

H3U3tbx6ZFP2R-R 5'-pAATTCACA**AAGCAGCTTCA**T-3'

**Generating double stranded oligonucleotides**

**Annealing Procedure (in Thermal Cycler)**

(31ul total reaction volume)

15ul 100 uM oligo-F

15ul 100 uM oligo-R

1ul 3M NaCl (final ~100mM)

Incubate at 70°C, 5min

Gradually cool down to 25°C (0.1° C/sec)

Hold at 4°C

Store@-20°C [as 50uM stock (= 610ng/ul)]

**Prepare Dilution of Annealed Reporter Oligonucleotides (with 5’ phosphates)**

Prepare l/5000X dilution of 50uM ds-oligos containing reporter target sequence in STE [TE + 100mM NaCl].

* 1/50X dilution: 1ul of 50uM (~610ng/ul) + 49ul STE = 1uM (~12.2ng/ul)
* 1/5000X dilution: 1ul of 1/50X (1uM) + 99ul STE = 10nM (~122pg/ul)

**5.4. Preparation of Reporter Vector Plasmid**

Overview: The reporter plasmid used in the Bacterial 1-Hybrid (B1H) Selection is pH3U3-mcs [5,834bp]. It is a **kanamycin-resistant, low copy number of plasmid.** Here pH3U3-mcs is prepared to readily accept the oligonucleotide target sites. The plasmid is digested with BspEI and EcoRI, dephosphorylated at the 5'-ends, and gel purified.

**Materials**

BspEI [NEB, #R0540S]

EcoRI [NEB, #R0101S]

SAP (Shrimp Alkaline Phosphatase, 1U/ul) [Fermentas, #EF0511]

Pellet Pestle [Kimble Chase Kontes, #749521-15090 (provided by Fisher Scientific, #K749521-1590)

QIAquick Gel Extraction Kit [QIAGEN, #28704 or #280706]

Starting plasmid: pH3U3-mcs

**5.4.1. BspEI/EcoRI Digestion:**

pH3U3-mcs [1ug/ul] 10ul (10ug)

10X buffer [NEB, #2] 5ul

BspEI [NEB] 2ul

EcoRI [NEB] 2ul

H2O 31ul

**Total 50ul**

* Incubate 37°C, ON
* Incubate 70°C, 20 min
* on ice

**5.4.2. Dephosphorylation: 130ul x 1 tube**

digested DNA 50ul

10X SAP buffer 13ul

SAP (1U/ul) 7ul

H2O 60ul

**Total 130ul**

* Incubate 37°C, 2h
* Add 6.5ul 0.5M EDTA
* Incubate 70°C, 20 min
* on ice

**5.4.3. Gel Purification**

**Preparing the Sample for gel loading** (330ul)

digested and dephosphorylated DNA 136.5ul (130ul + 6.5ul)

TE 160.5ul

6x loading dye (Orange G) 33ul

**Electrophoresis**

0.8% agarose in 1x TAE (13.8cm x 12cm x 0.9cm)

combs: 8mm x 12

load 55ul sample/lane x 6 wells

load 15ul ladder marker in a lane

running: 80V/20cm, c.v., 45-60min.

~14bp of BspEI/EcoRI-digested short fragment may be seen on this preparative gel.

**Gel Purification by QIAquick Gel Extraction Kit**

* cut out gel slices containing vector DNA (~3.7kb)
* divide the fragments from the 6 lanes among 6 tubes of 1.5ml microcentrifuge tubes
* Follow manufacturer’s directions for extraction and applying to column
* To elute, add 50ul of 1/10X EB [preincubated @37°C] and incubate 3 min @ RT prior to centrifugation
* cfg: 13,000 rpm, 1 min @ RT
* combine eluants into a single tube: ~300ul
* measure OD
* dilute to 20ng/ul in l/10X EB
* dispense into 20ul aliquots
* store @-20°C

**5.4.4. Quality Check: Transformation Efficiency**

Carry out transformation with or without control insert and count the colony numbers. If the dephosphorylation was successful, there should be considerable stimulation in the number of recovered colonies following ligation with phosphorylated insert.

**5.5. Ligation and Transformation**

**Materials**

DNA Ligation Kit ver. 2.1 [TAKARA, #6022] (or any other ligation kit)

DH5α chemical competent cell

SOC

LB/kan (25ug/ml) plate: one plate/plasmid

**Ligation: with DNA Ligation Kit ver. 2.1 [TAKARA, #6022]**

 **Sample control**

20 ng/ul vector plasmid 0.5ul (10ng) 0.5ul

122 pg/ul insert ds-oligo 1ul (122pg) -

STE - 1ul

Solution I 1.5ul 1.5ul

* Incubate at 16°C, 30min
* Add 0.3ul Solution III
* Transform into cells

**Transformation**

* Combine

 3.3ul Ligation mixture

 30ul DH5α chemical competent cell

* Incubate on ice, 30 min
* Heat shock at 42°C, 45 sec
* Immediately add 125ul SOC
* Incubate at 37°C, 1h, shaking at 200rpm
* Plate 20ul to an LB/kan plate (one plate per reporter ligation)
* Incubate at 37°C, ON

**Interpretations & Trouble Shooting**

Compare the numbers of colonies on sample plates with the numbers on control (no insert) plates:

If >10-fold increase in the numbers of colonies on sample plates compared to control plates, proceed immediately to **5.6. Cell Storage, Colony-PCR, and Sequencing**

If 2-10-fold stimulation, you may need to check >10 colonies per reporter

If <2-fold stimulation, prepare again the vector plasmid and/or reporter insert oligos

**5.6. Cell Storage, Colony-PCR, and Sequencing**

**Materials**

LB/kan (25ug/ml) broth: ~300ul/construct

60% Glycerol [autoclaved]

Taq DNA polymerase [NEB, #M0273S or M0273L (with Standard Taq Reaction Buffer, Mg+)]

**Preparation of Cell Stock**

* Dispense 20ul each of LB/kan broth into 8-strip PCR tubes (or 1.5ml microcentrifuge tubes)
* Pick 2-4 colonies from sample plates and suspend into different wells
* Use 2ul of cell suspension for **colony-PCR** (**see** **below)**
* After removing the 2ul for Colony PCR, Add 82ul LB/kan broth
* Close lid
* Incubate at 37°C, ON
* Add 25ul 60% glycerol
* Mix well by pipetting or by vortexing
* Store @-80°C

**Colony-PCR**

|  |
| --- |
| **Primer Information** |
| **Name** | **Sequence** | **Tm** |
| HU100 | CAAATATGTATCCGCTCATGAC | 57.16°C |
| H3U3R | CCAGAGCATGTATCATATGGTC | 57.06°C |

|  |
| --- |
| **Colony-PCR Protocol**  |
|  |  | **Master Mixes**  |
|  |  | **9x** | **34x** |
| 10x PCR Buffer [NEB Mg+]  | 2ul | 18ul  | 68ul  |
| 2mM each dNTPs  | 2ul | 18ul  | 68ul  |
| 2uM HU100 | 0.5ul | 4.5ul  | 17ul |
| 2uM H H3U3R | 0.5ul | 4.5ul  | 17ul |
| Cell suspension | 2ul | - | - |
| 1X DNA polymerase mix | 0.15ul | 1.35ul | 5.1ul |
| H2O  | 12.85ul | 115.65ul | 436.9ul |
|  |  |  |  |
| **Total volumes** | **20 ul** | **18ul x 9** | **18ul x 34** |

* Dispense 18ul each in PCR tubes
* Dispense 2ul cell suspension individually

**PCR program**

94°C, l min

40 cycles: {94°C, 10 sec; 54°C, 15 sec; 68°C,1 min}

72°C, l min

4-10°C

**Electrophoresis**

Run 10ul on 1.5% Agarose gel, amplicon should be 404bp

**Expected Results**

**Detection interpretation**

~400bp fragment correct insert (or no insert), go to sequencing

Longer fragments double or multiple inserts, incorrect and discard

No amplification incorrect for whatever reason, discard

**Sequencing**

**Sample Preparation**

 PCR product 2ul

 2uM H3U3R 4ul

 H2O 4ul

 Total: 10ul

**5.7. Midi-Prep of Reporter Plasmid and Preparation of the Cell Stock**

**Comments**

* 1ug of reporter plasmid is required for screening of a ZFP library in B1H system.
* >500ng/ul reporter plasmid concentration is recommended for ZFP library screening in the next step.
* Midi-preps from 100mls should yield enough DNA, but remember the plasmid is a low copy number plasmid that always gives low yields.
* Because of this, the plasmid DNA fraction is easily contaminated with bacterial genomic DNA.
* Contaminating bacterial genomic DNA reduces the net amount of library DNA and will affect the total titer of library screening on the next step.
* Using any plasmid purification procedure/kit that can reduce the contamination of the genomic DNA is appropriate. One good approach is to use a commercial midi-prep kit using larger volumes of lysis buffers, as described in the example below.

**Materials**

LB/kan or 2xYT/kan (25ug/ml)broth

60% glycerol [autoclaved]

PureLink HiPure Plasmid Filter Purification Kits [Invitrogen, #K2100] (or other plasmid purification kit)

60ml hand-made pre-filter:

Stuff 6 pieces of Kim paper into 60ml syringe [BD, #309653] and push down to the bottom with plunger

**Midi-Culture**

* Inoculate from a colony or glycerol stock into a small ‘starter culture’. Inoculate from a fresh ‘starter culture’ into100ml 2xYT/kan (25ug/ml)in 500ml flask (or 300ml in 2L flask).
* Incubate ON @37°C

**Cell Stock**

* Dispense 750ul ON culture into cryostat tubes
* Add 250ul 60% glycerol (sterile)
* Mix well by vortex
* Store @-80°C

**Midi-Prep using PureLink HiPure Plasmid Filter Purification Kits**

Purify the plasmids according to the manufacture's manual with some modifications:

* Load cell lysate from 100ml ONculture onto one column
* Use 15ml each of R3, L7 and N3 buffer
* Very gently mix after adding L7 and N3 to avoid breaking up genomic DNA
* Use 60ml hand-made prefilter
* Dissolve the precipitated DNA in small volume of H2O (25-50ul) to obtain higher concentration

**Procedure**

* ~100ml ON cell culture
* Add to two 50ml tubes
* Centrifuge at 4,000xg, 10 min @ RT (or 4°C)
* Purify cell pellet through a column according to Invitrogen's manual with brief modification:

Use 15ml each of R3, L7 and N3 (cell lysis with larger volumes are best for avoiding genomic DNA contamination)

Put cell lysis solution through 60ml hand-made pre-filter before loading onto column

Suspend DNA pellet after isopropanol precipitation with 1ml 70% EtOH and transfer to a new 1.5ml tube

Centrifuge at 14,000 rpm, 5 min @ 4°C

Wash pellet with 700ul 70% EtOH

Centrifuge at 14,000 rpm, 5 min @ 4°C

Remove EtOH and vacuum-dry for ~5-8min

Dissolve the DNA pellet in 25-50ul H2O

* Measure OD
* Run ~100ng on gel
* Store @-20°C

**Sequencing**

Confirm insert sequence of the purified reporter plasmids using the Primer: H3U3R (HU100)

**6. SCREENING OF THREE-FINGER ZFP LIBRARIES BY B1H SELECTION**

**Contents**

**6.1. Required Starting Materials**

**6.2. Preparation of the Selective Media and Plates**

**6.3. Transformation for BIH selection**

**6.4. Storage and analysis of selected colonies**

**6.5. Isolation of the Candidate ZFP Plasmids**

**6.6. Trouble Shooting**

**6.1. Required Starting Materials**

NM medium (His+)

NM medium (His-)

His selective plates: 8 different stringencies per library

Electrocompetent *US0ΔhisBΔpyrFΔrpoZ* cells [stored @-80°C] (~60ul / library)

Gene Pulser Cuvettes (2mm) [BIO-RAD, #165-2086] (1 cuvette / library)

2uM B1H2w2F1 primer

2uM H2-R primer

**6.2. Preparation of the Selective Media and Plates**

* Media and plates are prepared according to **Section 8. Bacterial Media and Plates**
* Generate 8 different stringencies of His selective plates (150mm plates) per library screening. IPTG induction raises the level of expression of the ZFP. 3-AT (3-amino-1, 2, 4-triazole) is a competitive inhibitor of the product of the HIS3 gene on the reporter, and thus places higher demand for reporter gene expression.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Plate** | **IPTG** | **3-AT** | **Carbenicillin** | **Kanamycin** | **Stringency** |
| 10-05 | 10uM | 5mM | 100ug/ml | 25ug/ml | Lowest |
| 00-05 | 0 | 5mM | 100ug/ml | 25ug/ml | Low |
| 10-10 | 10uM | 10mM | 100ug/ml | 25ug/ml | Low |
| 00-10 | 0 | 10mM | 100ug/ml | 25ug/ml | Middle |
| 10-20 | 10uM | 20mM | 100ug/ml | 25ug/ml | Middle |
| 00-20 | 0 | 20mM | 100ug/ml | 25ug/ml | High |
| 00-30 | 10uM | 30mM | 100ug/ml | 25ug/ml | Higher |
| 00-40 | 0 | 40mM | 100ug/ml | 25ug/ml | Higher |

**6.3. Transformation for B1H selection**

**Host Bacteria Strain**

*US0ΔhisBΔpyrFΔrpoZ* (electrocompetent cells). Protocol for preparing these electrocompetent cells is found in **Section 10**.

**Materials**

Gene Pulser Cuvette [BIO-RAD, #165-2086] (2mm cuvette for electroporation)

Gene Pulser Xcell [BIO-RAD]

SOC broth

2xYT broth

2xYT/carb (100ug/ml) & kan (25ug/ml) plates (or LB/carb & kan plates) [100mm plates, 6 plates/library]

NM His selective plates [150mm plates, use 8 different stringencies/library]

NM medium (His+) with carb (100ug/ml) & kan (25ug/ml) [“NM His+/carb&kan”]

NM medium (His-) with carb (100ug/ml) & kan (25ug/ml)

**6.3.1. Co-Transformation of ZFP library and reporter plasmids**

Use one cuvette per a library.

* Combine:

 100ng/ul ZFP library 1ul (100ng)

 1ug/ul reporter plasmid 1ul (1ug)

 *USOL1hisBL1pyrFL1rpoZ* electrocompetent cells 60ul

* Mix well on ice
* Transfer to chilled electroporation cuvette on ice
* Carry out electroporation with GenePulserXcell [condition: 2.5kV, 25uF, 200Q]

 Following electroporation, add 1ml SOC into the cuvette as quickly as possible

 (Prepare 1ml prior to starting pulse)

* Transfer to 15ml tube
* Incubate at 37°C, 1h shaking @ 250rpm
* Cfg. at 3,500xg, 10min. @ RT
* Remove supernatant
* Re-suspend pellet with 1ml NM **His+**/carb & kan
* Incubate at 37°C, 1h shaking @250rpm
* Transfer to 1.5ml microcentrifuge tube
* Cfg. at 14,000xg, 30-60sec. @ RT
* Remove supernatant
* Re-suspend pellet well in 1ml sterile dH20
* Centrifuge at 14,000xg, 2min. @RT
* Remove supernatant
* Repeat wash step three times with 1ml H2O (total of 4 washes)

 Dead cells will lyse and form a separate non-compact layer above cells.

 Remove this layer from the solution.

* Resuspend cells thoroughly in 650ul NM **His-**/carb & kan
* Transfer 5ul to a new microcentrifuge tube to check titer (**see 6.3.2 Checking Titer #1**)
* Store re-suspended cells in NM His-/carb & kan @ 4°C ON(until calculating the titer the next day)

**6.3.2. Checking the Titer #1: Prior to Plating on Screening Plates**

For B1H screening, aim to plate ~1x107 transformed cells/150mm plate. To do this, first determine the titer of the original cell transformation suspension and then calculate a dilution scheme.

* Prepare six 10-fold serial dilutions:

 1X = cell suspension (in NM His-)

 1/10X: 5ul of 1X + 45ul 2xYT

 1/100X: 5ul of 1/10X + 45ul 2xYT

 1/1,000X: 5ul of 1/100X + 45ul 2xYT

 1/10,000X: 5ul of 1/1,000X + 45ul 2xYT

 1/100,000X: 5ul of 1/10,000X + 45ul 2xYT

* Plate 20ul of the lowest three dilutions onto 2xYT/carb & kan plates
* Incubate at 37°C, ON
* Count the number of colonies on each plate and calculate the concentration of transformed cells in the 650ul cell suspension. We require about 8x107 transformed cells!

 **Example**: if there are 250 colonies on the 1/10,000X dilution plate, then

 250 x 10,000 = 2.5x106 transformed cells in 20ul cell suspension

 2.5x106 x 650/20 = 8.1x107 transformed cells in 650ul cell suspension

**6.3.3. Plating on NM His- Selection Plates**

* Aim to plate 8 different selective plates with 200ul each, so need at least 1.6ml of cell suspension
* Dilute original 650ul suspension to 1x107cells/200ul (= 5x104/ul) with NM His-/carb & kan
* Plate 200ul each of diluted cell suspension on NM His- selective plates
* After drying on lab bench, seal the plates with parafilm to avoid evaporation during the long incubation
* Incubate at 37°C, 2 days (~48h but longer if necessary)
* Store @4°C

**Comments**

* Depending on the titer of transformed cells there may not be enough cell suspension to plate all 8 different selective plates. Two options are: 1) to get a higher titer by repeating the transformation, or 2) to use less than 8 plates:

 6 plates: eliminate the highest and lowest stringency plates (00-40 and 10-05)

 4 plates: eliminate the two highest and lowest stringency plates

* Check colony growth first at 40h, and then check repeatedly every 2 or 4h.
* Incubation time depends on library and plate stringency: stop incubation when well-visible colonies are seen
* If no colonies have grown by 48h at 37°C, then incubate further (up to 60h) at 37°C or RT, but **be careful to avoid overgrowth**.

**6.3.4. Checking the Titer #2: at the time of Plating on Selective Plates**

Cells will be lost during overnight storage at 4°C. Therefore it is necessary to determine the actual titer the day of plating onto selective plates.

* Starting with stored cell suspension diluted to 1x107cells/200ul, prepare the following dilutions

 1X 1x107cells/200ul = 1x106cells/20ul

 1/10X 1x105cells/20ul

 1/100X 1x104cells/20ul

 1/1,000X 1x103cells/20ul

 1/10,000X 1x102cells/20ul

* Plate 20ul diluted suspension onto 2xYT/carb&kan plates
* Incubate at 37°C, ON
* Count the number of colonies on each plate and determine titer

**6.4. Storage and analysis of selected colonies**

Overview: Pick >24 colonies that grew under the most stringent conditions. Cell stocks will be generated and prepared for longterm storage. Selected ZFP plasmids will be sequenced.

**Materials**

LB/carb (100ug/ml) broth

60% Glycerol (autoclaved)

Toothpicks (sterile)

**6.4.1. Generating cell stocks**

* Dispense 20ul LB/carb (100ug/ml) broth into each well ofa 96-well cell culture plate
* Pick colonies from sample plates and suspend in the broth
* Mix well by pipetting
* Use 2ul of cell suspension for **colony-PCR** (see **6.4.2 Colony-PCR and Sequencing)**
* After removing 2ul for colony-PCR:
* Add 82ul LB/carb (100ug/ml) broth
* Cover and parafilm
* Incubate at 37°C, ON
* Add 25ul 60% glycerol
* Mix well by pipetting
* Store @-80°C

**6.4.2. Colony-PCR and Sequencing**

Overview: You want to determine if a subset of ZFP sequences were selected following B1H selection. Sequence analysis of ~20 PCR products should be enough to choose candidate ZFPs. Here we use colony-PCR to amplify selected ZFP sequences of each selected plasmid.

**Protocol for Colony-PCR**

**Materials**

Taq DNA polymerase [NEB, #M0273S or M0273L (with Standard Taq Reaction Buffer, Mg+)]

|  |
| --- |
|  **Primer Information** |
| Name | Sequence | Tm |
| B1H2w2F1 | CAAGAGCAGGAAGCCGCTG | 64.6°C |
| H2-R | TTGTCGGCCTTTTTCTAGTCTCTAGA | 62.9°C  |

|  |
| --- |
| **Colony-PCR Protocol**  |
|  |  | **Master Mixes**  |
|  |  | **22x** | **50x** |
| 10x PCR Buffer [NEB Mg+]  | 2ul | 44ul  | 100ul  |
| 2mM each dNTPs  | 2ul | 44ul  | 100ul  |
| 2uM B1H2w2F1 | 0.5ul | 11ul  | 25ul |
| 2uM H2-R | 0.5ul | 11ul  | 25ul |
| Cell suspension | 2ul | - | - |
| 1X DNA polymerase mix | 0.15ul | 3.3ul | 7.5ul |
| H2O  | 12.85ul | 282.7ul  | 642.5ul |
|  |  |  |  |
| **Total volumes** | **20 ul** | **18ul x 22** | **18ul x 50** |

* Dispense 18ul Master Mix into 200ul PCR tube or wells of a 96-well plate
* Transfer 2ul from each cell suspension/tube

**Colony-PCR Program**

94°C, 1min;

40 cycles: {94°C, 10sec; 59°C, 15sec; 68°C*,* 1min};

72°C, 1min;

4-10°C

**Electrophoresis**

Run 5ul on 1% agarose gel, expecting 407bp amplicon

**Sequence Sample**

2uM B1H2w2Fl 4ul

PCR fragment 2ul

H2O 4ul

Total: 10ul

**Sequence Analysis**

* Extract (copy) the KpnI-XbaI sequences (should be about 270bp)
* Paste into a file of any DNA analysis application
* Build an excel file.
* Translate retrieved DNA sequences into amino acids
* Identify core residues in each finger (a Template for how to analyze positions in the ZFP sequence is presented in the figure on the following page)
* Create an Excel file and input the core residues:

Column #1: clone#

Column #2: selected 7 residues in Finger 1

Column #3: selected 7 residues in Finger 2

Column #4: selected 7 residues in Finger 3

Column #5: length of KpnI - XbaI stretch

Column #6: comments (i.e., ambiguity, base alterations in the fixed sequences, etc)

**6.4.3 Choosing candidate ZFP clones**

If selection has worked properly, only a few sets of residue sequences will be recovered at any one Finger Module.

* Color the highly selected residue sets for each finger (eg. red as most common, blue as second most common)
* Identify the most common selected three-finger set (and the second and the third)

****

**6.4.4 Create logo (optional)**

Creating a logo for each finger makes it easy to visualize the residues that are selected frequently.

* Open web site, http://weblogo.berkeley.edu/logo.cgi
* Paste single column of 7 residues on individual fingers into the query box
* Click "Create Logo"
* Copy the logo and paste it into some presentation software application.

**6.5. Isolation of the Candidate ZFP Plasmids**

Prepare DNA corresponding to candidate ZFP plasmids (pB1H2w2geneZFP#) from cell stocks for sub-cloning

into the pCS2-Fokl expression vector.

* Inoculate bacteria from cell stock into 3ml 2xYT/carb (100ug/ml) or LB/carb (100ug/ml) broth.
* Incubate at 37°C, ON*.*
* Purify the plasmid according to manufacturer's manual: one column/sample.
* Measure OD.
* Run an aliquot on 0.8% Agarose gel.
* Store @-20°C.

**6.6. Trouble Shooting**

**6.6.1 Isolation of Single Clones**

If more than one base peak can be seen at the positions of the randomized finger sequences (encoding the core 7 residues) but not at other scaffold sequence regions, it indicates 2 or more clones are in a cell stock. You can isolate single clones by streaking the cell stock on His selective plates:

* Use plates of the same stringency as the one from which the cell stock was prepared.
* ~8 cell stocks can be streaked onto a single 150mm plate.
* Incubate 40 - 60h at 37°C.

**6.6.2 Re-Screening Candidate Clones**

It is possible that several different ZFPs are recovered following selection and that no single ZFP appears predominant. It is possible that all of these bind well and any one of them might function well to build a ZFN. Alternatively you may choose to re-select a subset of the candidates under higher stringency conditions:

* Choose individual clones that you want to re-assess in the subsequent screening
* Beginning with a DNA prep that contains both the reporter plasmid and an individual ZFP expression plasmid, destroy the reporter plasmid by digestion with XmaI, which will cut only the reporter plasmid.
* Retransform XL-1B cells, selecting only for carb (amp) resistance.
* Prepare miniprep DNA corresponding to each plasmid.
* Mix equal amounts of the different ZFP expression plasmids and retransform under high stringency conditions.

**7. SUBCLONING CANDIDATE ZFPS INTO pCS2-FOKI EXPRESSION VECTORS**

**Contents**

**7.1. Considerations and Goals**

**7.2. PCR Amplification of the ZFP Fragments**

**7.3. Asp718/BamHI Digestion**

**7.4 Preparation of ZFN Fusion Vector Plasmids**

**7.5. Ligation and Transformation**

**7.6. Colony Storage, Colony-PCR and Sequencing**

**7.7. Synthesizing sense strand mRNA for injections**

**7.1. Considerations and Goals**

* There are two different pCS2-FokI expression vectors, DD and RR. Each encodes a different version of the FokI cleavage domain. Expression of both a ZFP-DD and ZFP-RR fusion protein is required to generate the obligate heterodimer ZFN. Therefore, you need to clone the “left” and “right” ZFPs into different vectors. Pay attention!
* For subcloning into the pCS2-FokI expression vectors, you will need to purify a KpnI (Asp718) to BamHI fragment containing the three finger ZFP domain. We sometimes find that ZFP expression plasmids purified from the selection host bacterium cannot be digested well with Asp718 and/or BamHI, perhaps due to methylation interference. For this reason, we routinely amplify DNA fragments encoding ZFP regions by PCR from the mini-prepped plasmid DNA, and then digest this DNA for sub-cloning.

**7.2. PCR Amplification of the ZFP Fragments**

**Materials**

Expand High Fidelity PCR System [Roche, #11732641001]

QIAquick PCR Purification Kit [QIAGEN, #28104 or #28106]

|  |
| --- |
|  **Primer Information** |
| Name | Sequence | Tm |
| B1H2w2F1 | CAAGAGCAGGAAGCCGCTG | 64.6°C |
| H2-R | TTGTCGGCCTTTTTCTAGTCTCTAGA | 62.9°C  |

|  |
| --- |
| **PCR Protocol for amplifying selected ZFPs**  |
|  |  | **Master Mix** |
|  |  | **9x** |
| 10x Expand Buffer [+ 15mM MgCl2] | 5ul | 45ul |
| 2mM each dNTPs  | 5ul | 45ul |
| 2uM B1H2w2F1 | 7.5ul | 67.5ul |
| 2uM H2-R | 7.5ul | 67.5ul |
| Template plasmid | 2ul | - |
| Expand Enzyme [3.5U/ul] | 0.375ul | 3.375ul |
| H2O  | 22.625ul | 203.625ul |
|  |  |  |
| **Total volumes** | **50 ul** | **48ul x 9** |

* Dispense 48ul Master Mix into PCR tube or wells of a 96-well plate
* Add 2ul template DNA/tube

**PCR program: 59-72X30**

94°C, l min

30 cycles: {94°C, 10sec; 59°C, 15 sec; 72°C,1min}

72°C, 7 min

4 - 10°C

**Purification using QIAquick PCR Purification Kit [QIAGEN]**

Purify PCR products according to the manufacturer's manual:

* Use one column for 50ul PCR reaction
* Elute in 50ul 1/10X EB (incubate @ RT 3 min prior to centrifugation)
* Measure OD (1ul by NanoDrop ND-1000 spectrophotometer)
* Run 1ul on 1.5% Agarose gel (amplicon should be 407bp)
* Store @ -20°C

**7.3. Asp718/BamHI Digestion of ZFP amplicons**

Digestion of the 407bp PCR fragment with Asp718 and BamHI produces 151bp and 256bp fragments. The 256bp fragment should be purified following gel electrophoresis.

**Materials**

Asp718 [Roche, #10814245001]

BamHI [NEB, #R0136S] or [Fermentas, #FD0054]

QIAquick Gel Extraction Kit [QIAGEN, #28704 or #28706]

**Asp718/BamHI Digestion**

10x buffer [Roche, B] 5ul

PCR amplicon DNA 25ul

Asp718 1.5ul

BamHI 1.5ul

H2O 17ul

**Total volume 50ul**

Incubate at 37°C, 2h-ON (ON is preferred)

**Gel Purification Using QIAquick Gel Extraction Kit**

* Run 50ul digested products on 1.5% agarose in 1x TAE
* Slice out gels containing 256bp fragments
* Purify PCR products according to the manufacturer's manual
* Elute in 50ul 1/10X EB (incubate @ RT 3 min prior to centrifugation)
* Measure OD (1ul by NanoDrop ND-1000 spectrophotometer)
* Run 1ul on 1.5% Agarose gel to check product
* Store @ -20°C

**7.4. Preparation of ZFN Fusion Vector Plasmids**

Overview: Here the two forms of the “ready-to-use” CS2-FokI vector are prepared, following digestion of starting plasmids with Asp718 and BamHI, backbone purification, and dephosphorylation. “Ready-to-use” vectors are to be ligated with three-finger ZFP purified fragments.

**Ready-to-Use Vectors**

pCS2-DD-Asp/Bm (5’-OH) [4.8kb, 20ng/ul]

pCS2-RR-Asp/Bm (5’-OH) [4.8kb, 20ng/ul]

**Starting Plasmid Vectors**

pCS2-Flag-TTGZFP-FokI-DD [1ug/ul]

 pCS2-HA-GAAZFP-FokI-RR [1ug/ul]

**Materials**

Asp718 [Roche, #10814245001]

BamHI [NEB, #R0136S]

SAP (Shrimp Alkaline Phosphatase, 1U/ul) [Fermentas, #EF0511]

Pellet Pestle [Kimble Chase Kontes, #749521-15090 (provided by Fisher Scientific, #K749521-1590)

QIAquick Gel Extraction Kit [QIAGEN, #28704 or #280706]

**7.4.1. Asp718/BamHI Digestion CS2-FokI starting plasmid vectors:**

Digestion of the plasmids with Asp718 and BamHI produces a ~3700bp vector backbone and a 261bp ZFP-encoding insert. The ~3.7kbp fragment should be purified following gel electrophoresis.

 **DD vector RR vector**

pCS2-Flag-TTGZFP-FokI-DD [1ug/ul] 10ul [10ug] -

pCS2-HA-GAAZFP-FokI-RR [1ug/ul] - 10ul [10ug]

10x buffer [Roche, B] 5ul 5ul

Asp718 2ul 2ul

BamHI 2ul 2ul

H2O 31ul 31ul

**Total volume 50ul 50ul**

* Incubate @ 37°C, ON
* Incubate @ 70°C, 20min
* On ice

**7.4.2. Dephosphorylation (the vectors are treated independently):**

 digested DNA 50ul

 10X SAP buffer 13ul

 SAP (1U/ul) 7ul

 H2O 60ul

* Incubate 37°C, 2h
* Add 6.5ul 0.5M EDTA
* Incubate 70°C, 20min
* On ice

**7.4.3. Gel Purification**

**Preparing the Sample for gel loading (330ul)**

digested and dephosphorylated DNA 136.5ul (130ul + 6.5ul)

TE 160.5ul

6x loading dye (Orange G) 33ul

**Electrophoresis**

 0.8% agarose in 1x TAE (13.8cm x 12cm x 0.9cm)

 combs: 8mm x 12

 load: 55ul sample/lane x 6 wells

 load: 15ul ladder marker in a lane

 run: 80V/20cm, 45-60min.

* cut out gel slices containing vector DNA (~3.7kb)
* divide the fragments from the 6 lanes among 6 tubes of 1.5ml microcentrifuge tubes
* measure gel weight (= weight of a tube containing a gel slice – weight of a empty tube)
* Follow manufacturer’s directions for extraction and applying to column
* To elute, add 50ul of 1/10X EB [preincubated @37°C] and incubate 3 min @ RT prior to centrifugation
* cfg: 13,000 rpm, 1 min @ RT
* combine eluants into a single tube: ~300ul
* measure OD
* dilute to 20ng/ul in l/10X EB
* dispense into 20ul aliquots
* store @-20°C

**Comment**

• ~261bp of Asp718I/BamHI-digested short fragment can be seen on the preparative gel.

**7.4.4. Quality Check: Transformation Efficiency**

Carry out transformation with or without control insert and count the colony numbers. If the dephosphorylation was successful, there should be considerable stimulation in the number of recovered colonies following ligation with phosphorylated insert.

**7.5. Ligation and Transformation**

Choose pCS2-DD and pCS2-RR vectors for leftZFP and rightZFP, respectively.

**Materials**

DNA Ligation Kit ver. 2.1 [TAKARA, #6022]

DH5α chemical competent cell

SOC

LB/carb (100ug/ml) plate

**Vector Plasmids**

pCS2-DD-Asp/Bm (5'-OH) [20ng/ul]

pCS2-RR-Asp/Bm (5'-OH) [20ng/ul]

**Diluted Insert DNA**

Dilute insert DNA to 6ng/ul with 1/10X EB

**Ligation: with DNA Ligation Kit ver. 2.1 [TAKARA, #6022]**

 **Sample** **Control**

20ng/ul vector plasmid 0.5ul 0.5ul

6ng/ul insert DNA 1ul -

H2O - 1ul

Solution I 1.5ul 1.5ul

Incubate at 16°C, 30min

Add 0.3ul Solution III

Go to transformation

**Transformation**

* Combine on ice:

 Ligation mixture 3.3ul

 DH5α chemical competent cell 30ul

* Incubate on ice, 30min
* Heat Shock at 42°C, 45sec
* Immediately add 125ul SOC
* Incubate at 37°C, 1h, shaking at 200rpm
* Plate 20ul to LB/carb (100ug/ml) plate
* Incubate at 37°C ON

**7.6. Colony Storage, Colony-PCR and Sequencing**

Analyzing a couple of colonies from ON plates should be enough to identify the correct clone.

**Materials**

LB/carb (100ug/ml)broth

60% Glycerol (autoclaved)

Toothpicks (sterile)

**7.6.1. Generating cell stocks**

* Dispense 20ul LB/carb (100ug/ml) broth into each well of 96-well cell culture plate (or 8-well PCR strip)
* Pick colonies from sample plates and suspend in broth
* Mix well by pipetting
* Use 2ul of cell suspension for **colony-PCR (see below)**
* After removing cells for Colony PCR
* Add 82ul LB/carb (100ug/ml) broth
* Cover and parafilm
* Incubate at 37°C, ON
* Use these cultures to initiate a fresh ON for long-term storage, as described below

**7.6.2. Colony-PCR**

**Primer Information**

**Name Sequence Tm Amplicon**

CS2ZFNF1 CCACCATGGCTCCAAAGAAG 62.87°C 566bp (in DD) or 596bp (in RR)

CS2ZFNR1 AAATTGCTCCGTCCGGTTTC 63.45°C

**Materials**

Taq DNA polymerase [NEB, #M0273S or M0273L (with Standard Taq Reaction Buffer, Mg+)]

|  |
| --- |
| **PCR Protocol for Colony PCR from CS2-ZFN plasmids**  |
|  |  | **Master Mix** |
|  |  | **6x** |
| 10x PCR buffer [NEB, Mg+] | 2ul | 12ul |
| 2mM each dNTPs  | 2ul | 12ul |
| 2uM CS2ZFNF1 | 0.5ul | 3ul |
| 2uM CS2ZFNR1 | 0.5ul | 3ul |
| Cell suspension | 2ul | - |
| 1x DNA polymerase mix. | 0.15ul | 0.9ul |
| H2O  | 12.85ul | 77.1ul |
|  |  |  |
| **Total volumes** | **20 ul** | **18ul x6** |

* Dispense l8ul each into 200ul PCR tube or 96-well plate
* Transfer 2ul from each cell suspension well

**PCR program: 59-68X40**

94°C, 1min

40 cycles: {94°C, 10sec; 59°C, 15sec; 68°C,1min}

72°C, 7 min

4 - 10°C

**Electrophoresis**

Run 5ul on 1.5% Agarose gel

**7.6.3. Sequencing**

**Prepare Sequence Samples**

PCR fragment 2ul

2uM CS2ZFNF1 4ul

H2O 4ul

 **Total 10ul**

**7.6.4. Mini-Prep and Preparation of the Cell Stock**

**Comments**

The pCS2 vector plasmid is a high copy number plasmid, so 2 mini-preps should yield sufficient plasmid.

**Materials**

2xYT/carb (100ug/ml) or LB/carb (100ug/ml) broth

E.Z.N.A. Plasmid Miniprep Kit [Omega, #D6942-02] (or other plasmid mini-prep kit)

60% Glycerol (autoclaved)

**Mini-Prep**

* Inoculate bacteria from the stored master plate into fresh 5ml 2xYT/carb (or LB/carb) culture
* Incubate at 37°C, ON
* Transfer 750ul to cryostat tube for cell stock (below)
* Purify the plasmid according to manufacturer's manual: **2 columns/sample**
* Elute in 50ul of 1/10X EB (incubate @ RT 3 min prior to centrifugation
* Measure OD
* Run 1ul (~100ng) on 0.8% Agarose gel
* Store @ -20°C

**Cell Stock**

750ul cell suspension in cryostat tube

250ul 60% glycerol

Mix well

Store @ -80°C

**Sequence Sample**

2uM CS2ZFNF1 4ul

Plasmid DNA 600ng-1ug

H2O up to 10ul

**7.7. Synthesizing sense strand mRNA for injections**

Generally we digest pCS2-ZFP-FokI plasmids with NotI and transcribe with SP6 using a commercial kit that generates 5'capped mRNA.

**8. BacteriaL Media and Plates**

**Contents**

**8.1. Reagents & Materials Obtained from Providers**

**8.2. Stock Solutions**

**8.3. Amino Acid Mix (Histidine–)**

**8.4. NM Medium**

**8.5. NM Plates**

**8.1. Reagents & Materials Purchased from Providers**

**Reagent MW Provider, Catalog# size storage**

Carbenicillin•Na2 422.4 Invitrogen, #10177-012 5g 4°C

Kanamycin (sulfate salt) 582.58 Sigma, #K-4000 5g 4°C

Tetracycline•HCl 480.90 Sigma, #T-3383 25g -20°C

2xYT Becton Dickinson, #244020 500g RT

(VWR, #90003-330)

SOB Becton Dickinson, #244310 500g RT

(VWR #90003-336)

Bacto Agar Becton Dickinson, #214010 454g RT

(VWR #90000-760, 454g)

D-glucose (= dextrose) 180.16 J.T.Baker, #1916-07 12kg RT

L-phenylalanine 165.2 Sigma, #P-5482 25g RT

L-lysine•HCl 182.6 Sigma, #L-8662 100g RT

L-arginine 210.7 Sigma, #A-8094 25g RT

Glycine 75.07 Sigma, #G-8898 1kg RT

L-valine 117.1 Sigma, #V-0513 25g RT

L-alanine 89.09 Sigma, #A-7469 25g RT

L-tryptophan 204.2 Sigma, #T-0254 5g RT

L-threonine 119.12 Sigma, #T-8441 25g RT

L-serine 105.1 Sigma, #S-4311 25g RT

L-proline 115.1 Sigma, #P-5607 25g RT

L-asparagines 132.1 Sigma, #A-4159 25g RT

L-asparatic acid (free acid)133.1 Sigma, #A-4534 100g RT

L-glutamic acid•K•H2O 203.23 Sigma, #G-1501 100g RT

L-glutamine 146.1 Sigma, #G-8540 100g RT

L-tyrosine 181.2 Sigma, #T-8566 25g RT

L-isoleucine 131.2 Sigma, #I-7403 25g RT

L-leucine 131.2 Sigma, #L-8912 25g RT

L-histidine•HCl•H2O 209.63 Sigma, #H-5659 25g RT

Adenine-HCl 171.59 Sigma, #A-9795 1g RT

Thiamine•HCl 337.3 Sigma, #T-4625 5g RT

3-AT 84.08 Sigma, #A-8056 100g -20°C

(3-amino-1, 2, 4-triazole)

Uracil 112.1 Sigma, #U-1128 25g RT

IPTG 238.3 Sigma, #I-6758 10g 4°C

(isopropyl b-D-1-thiogalactopyranoside)

CaCl2 110.98 Sigma, #C-5670 500g RT

ZnSO4•7H2O 287.56 J.T.Baker, #4382-01 500g RT

MgSO4 120.37 J.T.Baker, #2506-01 500g RT

Na2HPO4•7H2O 268.07 Fisher Scientific, #S373-500 500g RT

KH2PO4 136.09 Fisher Scientific, #BP362-500 500g RT

NaCl 58.44 J.T.Baker, #3624-05 2.5kg RT

NH4Cl 53.49 Fisher Scientific, #A661-500 500g RT

0.22um filter (with 500ml reservoir) Nalgene, #566-0020

3mm glass beads VWR, #26396-508 RT (autoclaved)

**8.2. Stock Reagents**

100mg/ml Carbenicillin (in sterile H2O) [store @-20°C]

 Carbenicillin (disodium salt) 1g

 sterile H2O 10ml

 filter sterilize through 0.22um filter

 dispense 1ml per 1.5ml tube

 (working concentration: 100ug/ml)

50mg/ml Kanamycin (in sterile H2O) [store @-20°C]

 Kanamycin (sulfate salt) 0.5g

 sterile H2O 10ml

 filter sterilize through 0.22um filter

 dispense 1ml per 1.5ml tube

 (working concentration: 25ug/ml or 30ug/ml)

10mg/ml Tetracycline (in 70% EtOH) [store @-20°C]

 Tetracycline (hydrochloride) 100mg

 70% EtOH 10ml

 filter sterilize through 0.22um filter

 dispense 1ml per 1.5ml tube

 (working concentration: 10ug/ml)

60% glycerol [RT] (200ml)

 glycerol 120ml

 H2O 80ml

 autoclave

10% glycerol [4°C] (1L) (4L)

 glycerol 100ml 400ml

 H2O 900ml 3.6L

 autoclave

2xYT medium (use as a rich medium) [r.t] (100ml) (300ml) (1L)

 2xYT [Becton Dickinson, #244020] 3.1g 9.3g 31g

 H2O up to 100ml 300ml 1L

 autoclave

2xYT/carb plate [4°C, ~3months] (500ml)

 2xYT [Becton Dickinson, #244020] 15.5g

 H2O 500ml

 mix well to dissolve

 Add 7.5g Bacto agar (final 1.5%)

 autoclave

 cool down to ~55°C in water bath

 Add antibiotic [500ul 100mg/ml carbenicilin and/or 250ul 50mg/ml kanamycin]

 Pour into 10cm (~25 dishes) or 15cm (~10 dishes) Petri dishes

1M Glucose [RT] (100ml)

 glucose 18g

 H2O 90ml

 Dissolve in a measuring cylinder by inverting

 Bring final volume to 100ml with H2O

 filter sterilize through 0.22um filter

SOB medium (prepared with individual reagents) [RT]

 tryptone 20g

 yeast extract 5g

 NaCl 0.5g

 dissolve in 950ml H2O

 add 10ml 250mM KCl

 adjust pH to 7.0 with ~10N NaOH

 bring final volume to 1L

 autoclave

 add 5ml 2M MgCl2 (autoclaved)

SOB medium (pre-mix) [RT] (1L)

SOB [Becton Dickinson, #244310] 28g

 H2O up to 1L

 autoclave

SOC medium [store @-20°C] (50ml) (1L)

 SOB medium 50ml 1L

 1M glucose (filtered) 1ml 20ml

 dispense 1ml aliquots into 1.5ml tubes or 10ml aliquots into 15ml tubes

SOC medium (prepared from Mg-free SOB) [@-20°C]

 SOB (Mg-) 100ml

 1M MgCl2 1ml

 1M Glucose 2ml

 Filter sterilize through 0.22um filter

 dispense 10ml each into 15ml tubes

10x M9 salt solution [RT] (1L)

 Na2HPO4•7H2O 128g

 KH2PO4 30g

 NaCl 5g

 NH4Cl 10g

 H2O up to 1L

 autoclave

100mg/ml (10%) Histidine [store @-20°C]

histidine•HCl•H2O 2g

 sterile H2O up to 20ml

 filter sterilize through 0.22um filter

 dispense 10ml per 15ml tube

400mg/ml glucose [store @-20°C]

 D-glucose 16g

 sterile H2O up to 40ml

10mg/ml thiamine (= 1%) [store @-20°C]

 thiamine 50mg

 sterile H2O 5ml

20mM adenine-HCl [store @-20°C]

 adenine-HCl 171.59mg

 sterile H2O 50ml

 dispense 10ml per 15ml tube

20mM uracil [store @-20°C]

 uracil 112.1mg

 sterile H2O 40ml

Add ~1ml (200ul x5) 2N NaOH slowly until all uracil goes into solution

 Add ~1ml 2N HCl to neutralize: check the pH with pH paper

 Bring final volume to 50ml with H2O

 dispense 10ml per 15ml tube

1M 3-AT (3-amino-1, 2, 4-triazole) [store @-20°C]

 (20ml) (40ml)

 3-AT 1.68g 3.36g

 sterile H2O up to 20ml up to 40ml

 Filter sterilize through 0.22um filter

 dispense 10ml per 15ml tube

0.1M CaCl2 [RT]

 CaCl2 1.11g

 H2O up to 100ml

 autoclave

20mM ZnSO4 [RT] (50ml)

 ZnSO4•7H2O 287.6mg

 H2O up to 50ml

 Autoclave

1M MgSO4 [RT] (50ml)

 MgSO4 6.02g

 H2O up to 50ml

 autoclave

100mM IPTG [prepare just before use]

 IPTG 23.8mg

 H2O 1ml

 filter sterilize through 0.22um filter

10mg/ml (1%) yeast extract [store @RT]

 yeast extract 1g

 H2O 100ml

 autoclave

**8.3. Amino Acid Mix (Histidine–)**

Subsets of the Amino Acids will be prepared as Solutions I – VI. The exact volumes of these premix Solutions is not important because the entirety of these Solutions will be combined and then the volume will be adjusted for the final stock Amino Acid Mix [33.3x “Amino Acid Mix 17/20”, lacking His, Met, and Cys].

amino acid solution I (~90ml)

 phenylalanine 0.99g

 lysine 1.1g

 arginine 2.5g

 sterile H2O ~90ml (not necessary to be accurate)

amino acid solution II (~90ml)

 glycine 0.2g

 valine 0.7g

 alanine 0.84g

 tryptophan 0.41g

 sterile H2O ~90ml (not necessary to be accurate)

amino acid solution III (~90ml)

 threonine 0.71g

 serine 8.4g

 proline 4.6g

 asparagine 0.96g

 sterile H2O ~90ml (not necessary to be accurate)

amino acid solution IV (~90ml)

 asparatic acid 1.04g

 glutamate•K 18.7g

 sterile H2O ~90ml (not necessary to be accurate)

amino acid solution V (~**180ml**)

 glutamine 14.6g

 tyrosine 0.36g

 sterile H2O ~70ml

 **add entire solution IV ~90ml**

 add ~15ml 10N NaOH (add slowly and stir until all amino acids dissolve completely)

amino acid solution VI (200x, ~90ml)

 isoleucine 0.79g

 leucine 0.36g

 sterile H2O ~90ml (not necessary to be accurate)

33.3x amino acid mix (17/20 = His–, Met–, Cys–, 600ml) [@4°C, **~6 months**]

 amino acid solution I ~90ml

 amino acid solution II ~90ml

 amino acid solution III ~90ml

 amino acid solution V ~180ml

 amino acid solution VI ~90ml

 adjust pH to 7.5 with conc. HCl

 Add H2O to 600ml final volume

 filter sterilize through 0.22um filter

**8.4. NM Media**

• includes uracil in the media

NM medium (50ml) [4°C] **His+ His- (final conc.)**

 10x M9 salt 5ml 5ml 1x

 33.3x amino acid mix (17/20) 1.5ml 1.5ml 1x

 400mg/ml glucose 500ul 500ul 4mg/ml

 10mg/ml thiamine 50ul 50ul 10ug/ml

 20mM adenine-HCl 500ul 500ul 200uM

 20mM ZnSO4 25ul 25ul 10uM

 0.1M CaCl2 50ul 50ul 100uM

 1M MgSO4 50ul 50ul 1mM

 20mM uracil 500ul 500ul 0.2mM

 100mg/ml (=10% histidine) 500ul - 0.1%

 H2O 41.3ml 41.8ml

 filter sterilize through 0.22um filter (0.45um filter)

**8.5. NM Plates for ZFP Library Screening**

* B1H screening requires NM plates prepared for a variety of Histidine selection conditions (different stringencies). We prepare the NM Media+Agar for each condition independently, because the selective agents and other additives are added **after** autoclaving, while the Media+Agar is hot. We generally prepare batches of 250ml Media+Agar; each batch is sufficient to pour about 5 – 150mm petri plates. **[250ml = ~ 5 150mm plates]**.
* To make batches of NM Media+Agar, we prepare **concentrated NM+ medium**, and use 40.9ml of concentrated NM+ medium per 250ml NM Media+Agar.

**Concentrated NM+ medium**

**To prepare: (40.9ml) (x6.6) (x8.4) (final conc.)**

 10x M9 salt 25ml 165ml 210ml 1x

 400mg/ml glucose 2.5ml 16.5ml 21ml 4mg/ml

 20mM adenine-HCl 2.5ml 16.5ml 21ml 200uM

 33.3x amino acid mix (17/20) 7.5ml 49.5ml 63ml 1x

 20mM uracil 2.5ml 16.5ml 21ml 200uM

 0.1M CaCl2 250ul 1.65ml 2.1ml 100uM

 1M MgSO4 250ul 1.65ml 2.1ml 1mM

 10mg/ml thiamine 250ul 1.65ml 2.1ml 10ug/ml

 20mM ZnSO4 125ul 825ul 1.05ml 10uM

 filter sterilize through 0.22um (or 0.45um) filter

**NM plates** [store @ 4°C, ~3weeks]

Recipe is for 250ml batches of NM Media+Agar. For each Selection Condition: the Plate code, and the amounts of H2O, IPTG, and 3-AT are noted in the following Table:

 **Plate code[IPTG, 3-AT] H2O 100mM IPTG 1M 3-AT**

 00-05 [0, 5mM] 207ml - 1.25ml

 10-05 [10uM, 5mM] 207ml 25ul 1.25ml

00-10 [0, 10mM] 206ml - 2.5ml

 10-10 [10uM, 10mM] 206ml 25ul 2.5ml

 00-20 [0, 20mM] 204ml - 5ml

 10-20 [10uM, 20mM] 204ml 25ul 5ml

 00-30 [0, 30mM] 201ml - 7.5ml

 00-40 [0, 40mM] 199ml - 10ml

* Add 199~207ml H2O (depending on selection condition) to a flask
* Add 3.75g Bacto Agar
* Add a stirring bar
* Autoclave
* Cool to ~60°C in water bath
* Add 40.9ml conc. NM+ medium
* Add appropriate 100mM IPTG
* Add appropriate 1M 3-AT
* Add 250ul of 100ug/ml carbenicillin
* Add 125ul of 50mg/ml kanamycin
* Swirl or mix with stir bar
* Pour into 150mm Petri dishes: ~50ml/plate

**9. Sources of Plasmids & Bacteria**

**Contents**

**9.1. Bacterial Strains obtained from Addgene**

**9.2. Additional Bacterial Strains**

**9.3. Plasmids obtained from Addgene**

**9.4. Plasmids generated in the Grunwald Laboratory**

**9.5. Entire sequence of pB1H2w2-mcs**

**9.1. Bacterial Strains obtained from Addgene**

**strain name catalog# resistance growth medium**

*US0DhisBDpyrFDrpoZ* 18049 Tet, (Zeo) 2xYT, 10ug/ml tetracycline

**Important features**

• bacteria strain for B1H system

• US0 selection strain has deletions in the *hisB*, *pyrF* and *rpoZ* genes (bacterial homologs of HIS3, URA3 and omega subunit, respectively)

**9.2. Additional Bacterial Strains**

**strain name provider, catalog# Features**

XL1-blue Stratagene, #200228 for library construction

 plating and cultivation with 10ug/ml tetracycline

good for generating lab electrocompetent cells (**see Section 10**)

**9.3. Plasmids Obtained from Addgene**

**Plasmid Name catalog# size resistance copy# provided in**

pB1H2w2zif268 18045 3,966bp carb (high) DH5a

pCS2-Flag-TTGZFP-FokI-DD 18755 5,026bp carb high DH5a

pCS2-HA-GAAZFP-FokI-RR 18754 5,056bp carb high DH5a

pH3U3-mcs 12609 5,834bp kan low DH5a

pH3U3-zif268 (omega) 18046 5,828bp kan low DH5a

copy# (high) means the yield is lower than typical high copy number plasmids.

**Important features**

pB1H2w2zif268

• Expresses w-fused Zif268 ZFP and thus can serve as a positive control in the B1H system.

pCS2-Flag-TTGZFP-FokI-DD [labeled as pCS2-DD]

• Plasmid in which sequences encoding a selected ZFP can be fused with sequences encoding the DD version of the FokI cleavage domain and a Flag epitope. ZFPs are cloned into *Kpn*I (=*Asp*718) and *Bam*HI sites.

• RNA expression vector for expressing ZFNs.

pCS2-HA-GAAZFP-FokI-RR [labeled as pCS2-RR]

• Plasmid in which sequences encoding a selected ZFP can be fused with sequences encoding the RR version of the FokI cleavage domain and an HA epitope. ZFPs are cloned into *Kpn*I (=*Asp*718) and *Bam*HI sites.

• RNA expression vector for expressing ZFNs.

pH3U3-mcs

 • reporter plasmid vector for B1H system

• HIS3 URA3 chaperon is located downstream of multiple cloning site.

pH3U3-zif268 (omega)

 • positive control reporter for B1H system: use with pB1H2w2-zif268.

 • preferred binding site for the Zif268 ZFP is inserted 10bp upstream of -35 box.

**9.4. Plasmids Obtained from Grunwald Laboratory**

**Plasmid Name size resistance copy# host strain**

pB1H2w2-mcs 3,722bp amp (high) DH5a

copy# (high) means the yield is lower than typical high copy number plasmids.

**Important features**

 • pB1H2w2 backbone plasmid into which three-finger ZFP libraries are cloned. Plasmids with a three-finger ZFP can be screened in the B1H selection system.

• Prepared by replacing Asp718-XbaI fragment encoding zif268 domain in pB1H2w2zif268 with Asp718-XbaI linker:



**9.5. Entire sequence of pB1H2w2-mcs**

>pB1H2w2-mcs (3722bp)

GAATTCCGGGCTTTACACTTTATGCTTCCGGCTCGCATTATGTGTCGACTTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGCCTCAACAGCAGCAAATGCAACCTCCCAATTCAAGTGCGGACAACAACCCTTTGCAACAGCAATCATCACAAAATACCGTACCAAACGTCCTCAACCAAATTAACCAAATCTTTTCTCCAGAGGAGCAACGCAGCTTATTACAAGAAGCCATCGAAACCTGCAAGAATTTTGAAAAAACACAATTGTAAGTCTCGGCTCCAAGAAGGAGATATACCCATGGCACGCGTAACTGTTCAGGACGCTGTAGAGAAAATTGGTAACCGTTTTGACCTGGTACTGGTCGCCGCGCGTCGCGCTCGTCAGATGCAGGTAGGCGGAAAGGACCCGCTCGTACCGGAAGAAAACGATAAAACCACTGTAATCGCGCTGCGCGAAATCGAAGAAGGTCTGATCAACAACCAGATCCTCGACGTTCGCGAACGCCAGGAACAGCAAGAGCAGGAAGCCGCTGAATTACAAGCCGTTACCGCTATTGCTGAAGGTCGTGCGGCCGCGGACTACAAGGATGACGACGACAAGTTCCGGACCGGTTCCAAGACACCCCCCCATGGTACCCTCGAGAAGCTTGGATCCTAATCTAGAGACTAGAAAAAGGCCGACAAGTCCCGCTCCGCTGAAGATCCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGG

**10. Making Electrocompetent Bacteria**

**Contents**

**10.1. Bacterial Strains and Growth Media**

**10.2. Protocol #1**

**10.3. Checking the transformation efficiency**

**10.1. Bacterial Strains and Growth Media**

**bacterial strain medium/antibiotic**

*US0DhisBDpyrFDrpoZ* 2xYT/tet (10ug/ml tetracycline)

XL-1 blue 2xYT/tet (10ug/ml tetracycline) or LB/tet

**10.2. Protocol**

**Materials**

2xYT/tet (10ug/ml) or LB/tet (10ug/ml) plates

2xYT/tet (10ug/ml) broth: 300ml in 2L flask for large culture

~3L 10% glycerol [ice-cold, 4°C]

500ml centrifuge bottles [autoclaved]

**Procedure [from four 300ml cultures (1.2L cells)]**

Streak frozen bacterial stock on 2xYT/tet (or LB/tet) plates

Grow @37°C, ON

Inoculate a colony into 5ml liquid 2xYT/tet broth

Grow @37°C, ON, shaking @250rpm

Transfer 600ul of the ON culture to each 300ml 2xYT/tet in 2L flask (x4 flasks = 1.2L)

Incubate @37°C, shaking @250rpm

Grow until OD595 reaches to ~0.8 (check the OD occasionally, 3.5 ~ 8h incubation)

Chill cell culture in an ice-water bath **[from this step forward, keep cells chilled]**

Transfer cultures to 4 chilled 500ml centrifuge bottles

cfg. 4,500rpm, 8min. @4°C

Remove supernatant

Rinse inner walls of bottles very gently with ~10ml ice-cold 10% glycerol (don’t disturb cell pellet) and discard liquid

Resuspend in 300ml (1 vol.) 10% glycerol per bottle (total 1.2L)

cfg. 4,500rpm, 8min. @4°C

Remove supernatant

Repeat the cell wash step with 300ml 10% glycerol per bottle (total 1.2L)

cfg. 4,500rpm, 8min. @4°C

Remove supernatant

Wipe inner wall of centrifuge bottles with a Kimwipe

Resuspend the pellet with residual (~500ul) 10% glycerol (by gently rotating bottles but not pipetting)

Dispense 60 ~ 240ul into microcentrifuge tubes on ice

Place tubes on powdered dry-ice

Store @-80°C

**Comments:**

• *US0DhisBDpyrFDrpoZ* grows slowly: it takes ~8h for the OD595 to reach to 0.8.

• After growing the cells to OD595 = 0.8, all steps should be performed cold.

• Typical yield is 1.2 ~ 2.4ml competent cells from 1.2L culture

**10.3. Assaying Transformation Efficiency**

**Materials**

Gene Pulser Xcell [Bio-Rad]

Gene Pulser Cuvette [BIO-RAD, #165-2086] (2mm wide)

LB/carb (50ug/ml) plates

SOC

**Control Intact Plasmid**

10ng/ul pCS2HA-GAAZFP-FokI-RR (carbr) or any carbr = ampr plasmid

**Transformation**

* Combine and mix on ice:

10ng/ul control plasmid 1ul

electrocompetent cells (thaw on ice) 60ul

* Transfer bacteria+plasmid to electroporation cuvette (on ice)
* Carry out electroporation with GenePulserXcell
* Add 750ul SOC as quickly as possible (prepare in 1ml pipettman prior to electroporation)
* Transfer to 1.5ml microcentrifuge tube
* Incubate 37°C, 1h, rotating @~250rpm
* Plate dilutions

**Titration Plating**

* Prepare 10-fold serial dilutions:

 1X: = cell suspension in ~1ml SOC

 1/10X: 5ul of 1X + 45ul 2xYT

 1/100X: 5ul of 1/10X + 45ul 2xYT

 1/1,000X: 5ul of 1/100X + 45ul 2xYT

 1/10,000X: 5ul of 1/1,000X + 45ul 2xYT

* Plate 20ul each of cell suspension on LB/carb (50ug/ml)
* Incubate 37°C, ON
* Count the number of colonies on each plate and calculate transformation efficiency