INDEX OF EXPERIMENTAL PROTOCOLS FOR
"UTAH-BIG LOVE" APPROACH TO CONSTRUCTION OF ZINC FINGER NUCLEASES
(February 2011)

by Kazuyuki Hoshijima, Timothy Dahlem, and David Jonah Grunwald
(please contact DJG at: grunwald@genetics.utah.edu regarding corrections/improvements)

1. OVERVIEW AND EXPERIMENTAL FLOW

Contents

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.

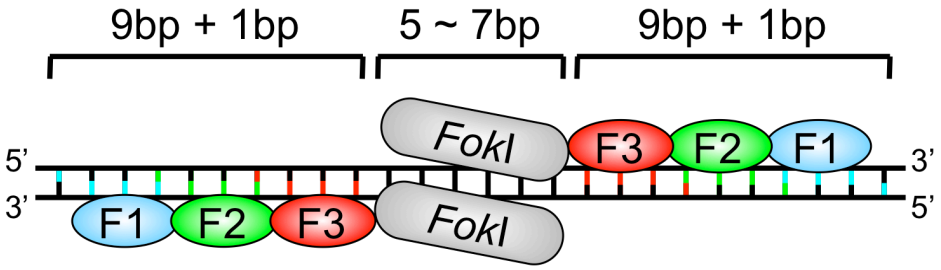


Figure 1: Binding of a ZFN to a target sequence. The ZFN functions as a dimer. In the system described here, each monomer component consists of a three-finger ZFP fused to the cleavage domain of the FokI endonuclease. Each three-finger ZFP recognizes a 9bp **core target** and one additional base neighboring 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

- 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?

- 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.

Reference number	Well number	Selected Finger	Target triplet		Reference number	Well number	Selected Finger	Target triplet		Reference number	Well number	Selected Finger	Target triplet
1	A1	F1	GAG		30	C1	F3	GTC		64A	E1	F1	TAC
2	A2	F1	GCTg		31	C2	F3	GTT		65	E2	F1	TAA
3	A3	F1	GGAg		32	C3	F3	GCC		68	E3	F1	TTC
4	A4	F1	GGCa		33	C4	F3	GAC		70	E4	F1	TTT
5	A5	F1	GGTg		34	C5	F3	GCA		87	E5	F3	TGG
6A	A6	F1	GTA		35	C6	F3	GCT		88	E6	F3	TGC
7	A7	F1	GTC		36	C7	F1	GCTc		94B	E7	F3	TCT
8A	A8	F1	GTG		37	C8	F1	GGCc		97C	E8	F3	TAA
9	A9	F1	GTT		38	C9	F1	GGTc		101A	E9	F3	TTA
10	A10	F1	GCCg		39A	C10	F1	GCAc		102B	E10	F3	TTT
11	A11	F1	GCAg		40	C11	F1	GCCc		106	E11	F2	GAC
13	A12	F2	GGA		41	C12	F1	GGAc		107	E12	F2	GAG
14	B1	F2	GGG		43A	D1	F3	GAT		108	F1	F2	GGC
15	B2	F2	GTA		46	D2	F1	GAC		109	F2	F2	GCG
16	B3	F2	GTC		48	D3	F1	GAA		110	F3	F2	GGT
17	B4	F2	GTG		49A	D4	F1	GCG		111	F4	F2	GAA
19	B5	F2	GCC		50	D5	F1	GGG		112	F5	F2	GCT
20	B6	F2	GAT		51A	D6	F3	GGA		113	F6	F2	TTG
22	B7	F2	GCA		52	D7	F3	GGT		114	F7	F2	TTT
25	B8	F3	GAA		53A	D8	F3	GGC		116	F8	F2	TGA
26	B9	F3	GAG		54	D9	F3	GTG		117	F9	F2	TGG
27	B10	F3	GCG		58	D10	F1	TGT		118	F10	F2	TAG
28	B11	F3	GGG		62	D11	F1	TCT		123	F11	F1	GATt
29	B12	F3	GTA		63	D12	F1	TAG		126	F12	F2	GTT
										127	G1	F1	GATg
F1	F2	F3								128	G2	F1	GATa

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/well of "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)"

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 5x	**Final Conc.**
Reaction #1			
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 5x	**Final Conc.**
Reaction #2			
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

- 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: >$2x10^4$ 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 Asp718-XbaI fragment encoding the zif268 domain of pB1H2w2zif268 0 with an Asp718-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, #AM9510]
XL-1 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 10^6.

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 10^5 in 20ul cell suspension
2 x 10^5 x 800/20 = 8 x 10^6 colonies in 800ul cell suspension (acceptable, as we need >3 x 10^6 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 1.5ml 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 10^6, 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.

Template for analyzing a three-finger ZFP sequence in the B1H2 vector

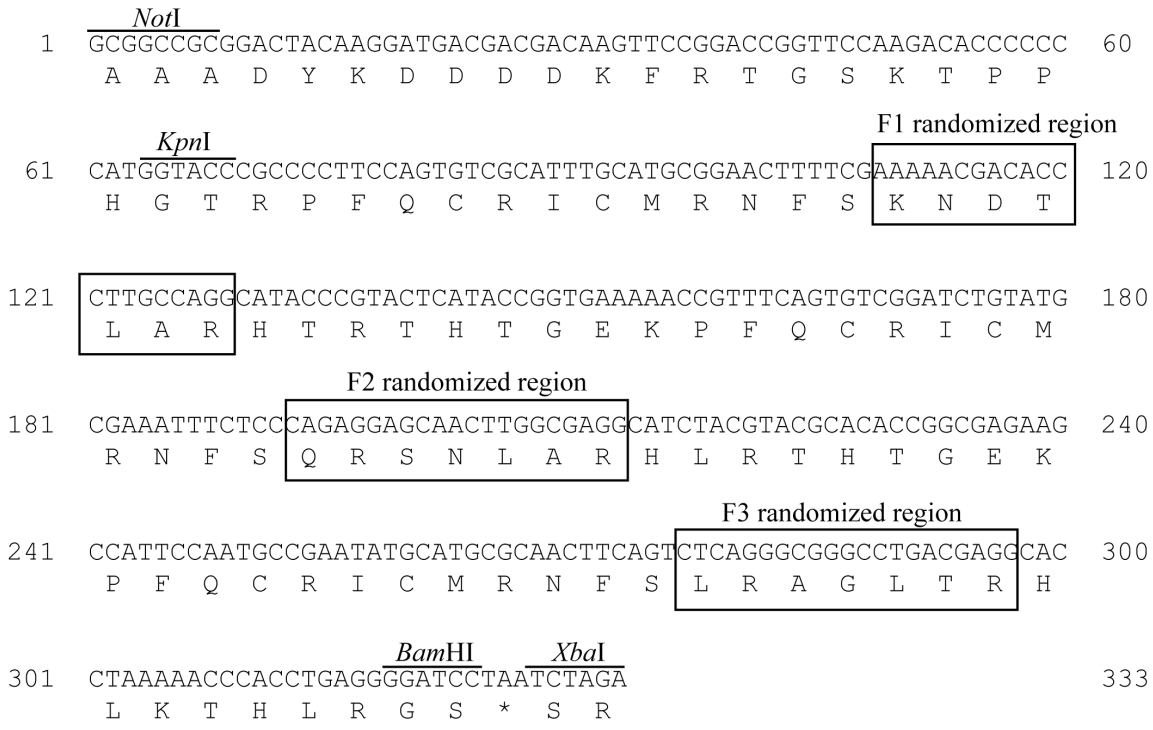
library vector: pB1H2w2-mcs [3,722bp]

ZFP PCR fragment was inserted at KpnI/BamHI sites

insert length: 261bp (KpnI-BamHI)

analyzed sequence: 333bp (NotI-XbaI)

randomized regions: originally XXXXLXX



5. REPORTER PLASMID CONSTRUCTION

Contents

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/OD_{260}) 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**x**$\mathbf{X_3X_3X_3X_2X_2X_2X_1X_1X_1}$**x**TGTG-3'
H3U3**geneZFP**-R	5′-pAATTCACA**y**$\mathbf{Y_1Y_1Y_1Y_2Y_2Y_2Y_3Y_3Y_3}$**y**T-3'

x: left and right context sequence
$\mathbf{X_1X_1X_1}$: 3bp recognized by F1 finger
$\mathbf{X_2X_2X_2}$: 3bp recognized by F2 finger
$\mathbf{X_3X_3X_3}$: 3bp recognized by F3 finger
y$\mathbf{Y_IY_IY_IY_2Y_2Y_2Y_3Y_3Y_3}$**y**: complementary sequence of **x**$\mathbf{X_3X_3X_3X_2X_2X_2X_IX_IX_I}$**x**
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, 1 min
40 cycles: {94°C, 10 sec; 54°C, 15 sec; 68°C, 1 min}
72°C, 1 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 ON culture 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

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 ~1x10^7 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 8x10^7 transformed cells!
  Example: if there are 250 colonies on the 1/10,000X dilution plate, then
  250 x 10,000 = 2.5x10^6 transformed cells in 20ul cell suspension
  2.5x10^6 x 650/20 = 8.1x10^7 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 1x10^7cells/200ul (= 5x10^4/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 $1x10^7$cells/200ul, prepare the following dilutions
  - 1X $1x10^7$cells/200ul = $1x10^6$cells/20ul
  - 1/10X $1x10^5$cells/20ul
  - 1/100X $1x10^4$cells/20ul
  - 1/1,000X $1x10^3$cells/20ul
  - 1/10,000X $1x10^2$cells/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)

Template for analyzing a three-finger ZFP sequence in the B1H2 vector

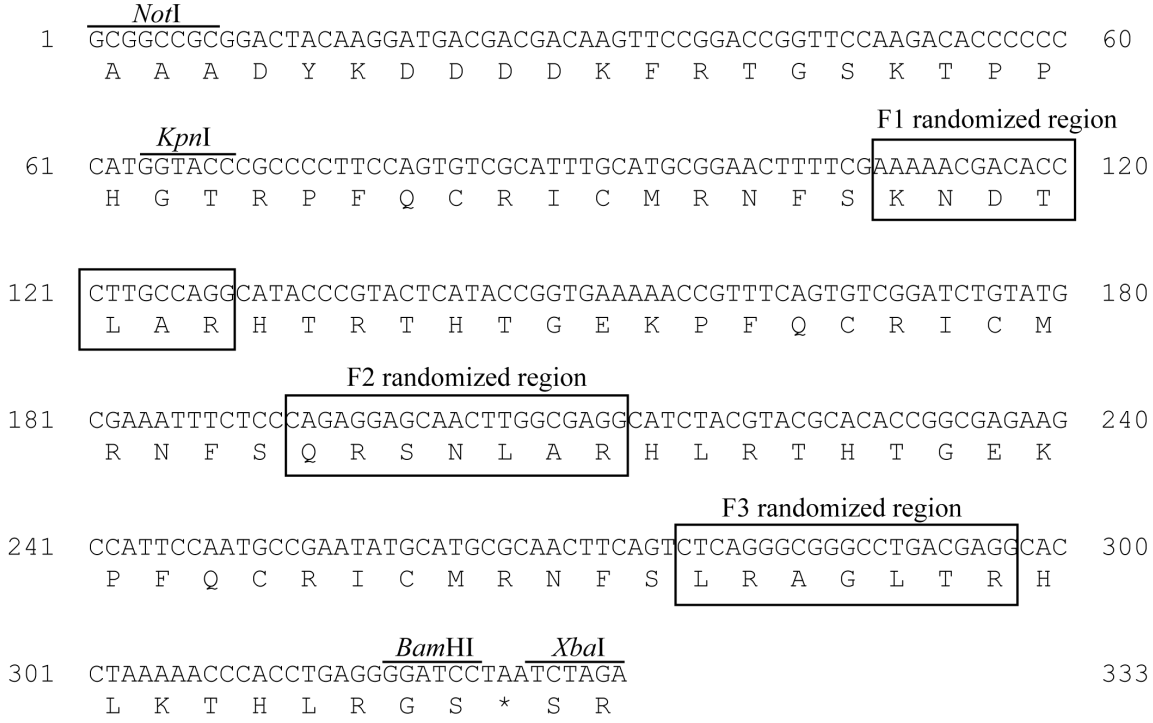
library vector: pB1H2w2-mcs [3,722bp]

ZFP PCR fragment was inserted at KpnI/BamHI sites

insert length: 261bp (KpnI-BamHI)

analyzed sequence: 333bp (NotI-XbaI)

randomized regions: originally XXXXLXX



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-FokI 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

- 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, 1 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
H_2O	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
H_2O	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	-
H_2O	-	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
H_2O	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 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 (VWR, #90003-330)	500g	RT
SOB		Becton Dickinson, #244310 (VWR #90003-336)	500g	RT
Bacto Agar		Becton Dickinson, #214010 (VWR #90000-760, 454g)	454g	RT
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 (3-amino-1, 2, 4-triazole)	84.08	Sigma, #A-8056	100g	-20°C
Uracil	112.1	Sigma, #U-1128	25g	RT
IPTG (isopropyl b-D-1-thiogalactopyranoside)	238.3	Sigma, #I-6758	10g	4°C
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

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

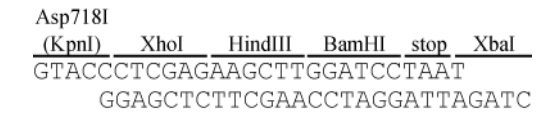
Plasmid Name	size	resistance	copy#	host strain
pB1H2w2-mcs	3,722bp	amp	(high)	DH5a

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)

GAATTCCGGGCTTTACACTTTATGCTTCCGGCTCGCATTATGTGTCGACTTGTGAGCGGATAACAATTT
CACACAGGAAACAGCTATGCCTCAACAGCAGCAAATGCAACCTCCCAATTCAAGTGCGGACAACAAC
CCTTTGCAACAGCAATCATCACAAAATACCGTACCAAACGTCCTCAACCAAATTAACCAAATCTTTTCT
CCAGAGGAGCAACGCAGCTTATTACAAGAAGCCATCGAAACCTGCAAGAATTTTGAAAAAACACAAT
TGTAAGTCTCGGCTCCAAGAAGGAGATATACCCATGGCACGCGTAACTGTTCAGGACGCTGTAGAGAA
AATTGGTAACCGTTTTGACCTGGTACTGGTCGCCGCGCGTCGCGCTCGTCAGATGCAGGTAGGCGGAA
AGGACCCGCTCGTACCGGAAGAAAACGATAAAACCACTGTAATCGCGCTGCGCGAAATCGAAGAAGG
TCTGATCAACAACCAGATCCTCGACGTTCGCGAACGCCAGGAACAGCAAGAGCAGGAAGCCGCTGAA
TTACAAGCCGTTACCGCTATTGCTGAAGGTCGTGCGGCCGCGGACTACAAGGATGACGACGACAAGTT
CCGGACCGGTTCCAAGACACCCCCCCATGGTACCCTCGAGAAGCTTGGATCCTAATCTAGAGACTAGA
AAAAGGCCGACAAGTCCCGCTCCGCTGAAGATCCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGC
CCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGC
GGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTT
CTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGG
TTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGG
CCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGT
TCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTT
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACG
CTTACAATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATAC
ATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAG
AGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGC
TCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATC
GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAG
CACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCG
CCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATG
GCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTT
CTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCG
CCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCT
GTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACA
ATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCT
GGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCA
GATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAA
TAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCAT
ATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATA
ATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATC
AAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTA
CCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGA
GCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGC

ACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT
TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCG
TGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAG
AAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAG
GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCAC
CTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAA
CGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTG
ATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAG
CGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTG
CGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTA
TACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGC
GCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCA
TGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGG
TCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTT
AATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCC
GTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATAC
GGGTTACTGATGATGAACATGCCCGGTTACTGGAACGG

10. MAKING ELECTROCOMPETENT BACTERIA

Contents

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 OD_{595} 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 OD_{595} to reach to 0.8.
- After growing the cells to OD_{595} = 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 ($carb^r$) or any $carb^r$ = amp^r 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