

General Protocol for the construction of zinc finger libraries for selection using the omega-B1H system:

Using the on-line tools (<http://pgfe.umassmed.edu/ZFPsearch.html>) that we have developed for the identification of ZFN sites in a gene of interest favorable target sites can be chosen by the user that meet their criteria of position and quality and desired library size. The “construct” button supplies potential library oligos that can be used to construct libraries within the B1H system to select fingers and then ZFPs with the desired DNA-binding specificity.

Overview:

The zinc finger library oligos are designed to be cloned between 5' overhangs that remain after the plasmids are digested with the restriction enzyme Bbs1. Each of the three different finger library plasmids (in a pBS2SK+ backbone) upon digestion creates cloning sites into a different finger backbone (fingers 1, 2 or 3 of Zif268). Each plasmid contains a Kanomycin resistance cassette inserted between the Bbs1 sites within the finger backbone of about 1.2 kb that allows the easy separation of double cut plasmid backbone from single cut plasmid. We recommend cutting ~10 ug of plasmid for 90' with 20 units of bbs1 following NEBs recommendations (make sure the Bbs1 and NEB buffer are fresh).

*Note, because the ends of the F2 plasmid have a tendency to self-ligate at a modest rate, we recommend treating this backbone with CIAP for 15' prior to purification.*

Gel purify the backbone, where the double cut backbone runs at about 4 kb. The gel purification should be run such that the singly- and doubly-cut backbones can be well-resolved on the 1.5% agarose gel. Doing so can reduce background considerably, especially for non-CIAP'd F1 and F3 backbones. We also recommend purifying the kanomycin cassette to use as a positive control for your ligations (although this will not work for the F2 insert since it is CIAPed).

Order the desired library oligos (either those suggested by the program or your own randomization scheme) as well as the necessary complementary oligos for each library cassette. The complementary oligos once annealed leave 4 bp 5' overhangs complementary with the plasmid backbone and a single stranded gap within the library oligo where the randomized region is found.

Complementary oligos (**note there was an error in the 3' complementary oligos for finger 1 and 2 previously**, as they were too long and covered some of the library):

**Finger 1 library complementary oligos:**

5' complement: 5'-GCTGAAGCGGCGGTCA-3'

3' complement: 5'-GCCGGTGTGAATCCGGATGTG-3'

**Finger 2 library complementary oligos:**

5' complement: 5'- GCTGAAGTTCCTCAT-3'

3' complement: 5'- GCCGGTGTGGGTGCGGATGTG -3'

**Finger 3 library complementary oligos:**

5' complement: 5'- CGCGAACTTCCTGCC -3'

3' complement: 5'- CCCGTGTGGATCTTGGTGTG -3'

Separately dissolve the library and complementary oligos to a concentration of 50 uM and kinase the oligos with T4 PNK:

Per 12.5 uL reaction:

1 uL 50 uM oligo  
1.25 uL 10X T4 DNA ligase buffer  
10 uL ddw  
0.25 uL T4 PNK (NEB)

Generally we make a PNK/10Xbuffer/ddw stock mixture for (n+1) reactions that are planned, then aliquot out 11.5 uL/rxn into an eppendorf, and add 1 uL of the appropriate oligo.

Typically we do this reaction in a thermocycler, but it can be done with water baths as well. Hold the reaction at 37 deg C 45 min. Once the oligos have been kinased the library oligo can be annealed with the corresponding complementary oligos at a 1:1:1 ratio (final concentration of duplex ~1.33 uM) at the same time that the t4 PNK is heat killed. Combine the library oligo and the corresponding complementary oligos at a 1:1:1 ratio and anneal by heating to 95 deg C for 3 min, then slowly cooling thereafter. (We use a 5 deg decrement/3 min down to 25 deg, then rapidly down to 4 deg C).

Pilot ligations should be set up with the digested backbone (~100 ng) and the phosphorylated inserts (5-fold molar excess) as well as control reactions where either the insert is omitted (background reclosure rate) or the Kan cassette positive control is used (if desired). Ideally the annealed library oligos should provide a 5 to 10 fold boost in viable colonies on Amp plates when the ligation mix is transformed into chemically competent cells. If desired this test can be done with electrocompetent cells to provide an estimate of the library construction efficiency, and to what degree the pilot reactions will need to be scaled to meet your library requirements where 3 to 5 fold oversampling is desired to provide nearly complete library coverage.

The efficiency of the build can be determined by titration of the transformed cells after the traditional 1 hr recovery in SOC:

1. Make eight 5-fold serial dilutions of the recovered cells in 2xYT in separate tubes or in a 96-well plate.

2. Place 5- $\mu$ l drops of each dilution on a prewarmed (to 37 °C) 2xYT plate with a dry surface that contains one of 3 different markers:

1) No marker: Important for troubleshooting (phage contamination, poor survival after electroporation/recovery, poor cell quality/survival overall)

2) 100  $\mu$ g ml<sup>-1</sup> Amp or carbanicillin: provides estimate of library build.

3) 30  $\mu$ g ml<sup>-1</sup> kanamycin: provides estimate of background due to reclosure.

(Typically four or more 5- $\mu$ l drops from each dilution will fit on a single plate.)

3. Allow the drops to air-dry near a flame and then incubate the plates upside down at 37 °C, overnight.

4. Determine the average number of colonies per microliter at a dilution that provides well-separated colonies (count the number of colonies and divide by the total volume in microliters used to place the cells on the plate).

5. Calculate the total number of transformed cells by multiplying the average number of colonies per microliter at the dilution evaluated (from Step 4) by the dilution factor and by the total volume of recovered cells.

This analysis will establish the ligation efficiency of the various insert-to-plasmid ratios relative to the background rate of plasmid reclosure (no insert) and will provide an estimate of the library complexity that can be constructed per microgram of ligated plasmid.

Libraries should be plated on Amp plates at a density no higher than 10<sup>7</sup> Amp<sup>R</sup> cells/ 150mm x 150mm round plate with colonies grown overnight. The next day count (or estimate by serial dilution of the plated cells the previous day if large libraries are anticipated) the number of colonies on the plate to determine the library size and then wash the colonies from the plate using 2xYT or LB and glass beads or a cell scraper. Pellet the cells, save half as a glycerol stock at -80°C, and harvest the DNA from the remaining cells by dividing between a suitable number of Qiagen minipreps. This DNA should be ethanol ppted, and then can be used for zinc finger selections.

Primers for the assembly of the individual finger pools from the 3 parallel finger selections for the final three finger selection against the ultimate target site have been further optimized from the published primers to improve selectivity. They are:

Amplification primers for individual fingers:

### **Finger 1**

UnivF1forward GCGATGGGTACCCGCCCATATGCTTGCCC  
UnivF1reverse CACTGGAAGGGCTTCTGGCCTGTGTGAATCCGGATGTG

**Finger 2**

UnivF2forward CATCCGGATTACACAGGCCAGAAGCCCTTCCAGTGTCGCATCTGC  
UnivF2reverse ATGTCGCATGCAAAAGGCTTCTCGCCTGTGTGGGTGCGGATGTG

**Finger 3**

UnivF3forward CAGGCGAGAAGCCTTTTGCATGCGA  
UnivF3reverse GCGTAGGATCCACCTGTGTGGATCTTGGTGTG

If you find errors in this protocol or have concerns or questions about it please contact Scot Wolfe at [scot.wolfe@umassmed.edu](mailto:scot.wolfe@umassmed.edu)