Constructing CoDA ZFNs

Colby Starker, Nick Baltes & Shaun Curtin 2011

Step #1 Query ZiFiT with genomic DNA to obtain putative target sites and ZFN arrays

```
Eg. RdR2ab   gAGCGTCCTCGAATCCTGGGAGGGCg   GCT  GAC  GAG   VRQGLTR   DRGNLTR   REDNLGR
             cTCGCAGGAGCTTAGGACCCTCCCGc   GGC  GAG  TGG   SPSKLVR   RQDNLGR   RMDHLAG
```

Step #2 Design F1 and F3 primers with incorporated RHs

Design primers for the appropriate RH using the soybean codon usage chart Table1.

```
F1_template TTGCATGCGGAACTTTTCGaacacttctctgctgaggaggCATACCCGTACTCATACCGG
F3_template TCAGGTGGGTTTTTAGGTGcctcttcaggtgagacatttgACTGAAGTTGCGCATGCATA
```

Example:

F1_VRQGLTR → GTGAGACAAGGACTGACTAGA

F1_VRQGLTR - TTGCATGCGGAACTTTTCGGTGAGACAAGGACTGACTAGACATACCCGTACTCATACCGG

F3_REDNLGR → AGAGAGGATAACCTGGGAAGA (R/C) → TCTTCCCAGGTTATCCTCTCT

F3_REDNLGR - TCAGGTGGGTTTTTAGGTGTCTTCCCAGGTTATCCTCTCTACTGAAGTTGCGCATGCATA

Table 1. Soybean codon usage

Soybean	UGA	*
Soybean	GCU	A
Soybean	UGC	C
Soybean	GAU	D
Soybean	GAG	E
Soybean	UUC	F
Soybean	GGA	G
Soybean	CAC	H
Soybean	AUC	I
Soybean	AAG	K
Soybean	CUG	L
Soybean	AAC	N
Soybean	CCA	P
Soybean	CAA	Q
Soybean	AGA	R
Soybean	UCC	S
Soybean	ACU	T
Soybean	GUG	V
Soybean	UAC	Y

Table 2. F2 templates

Target	RH
GGG	RREHLVR
GGA	QSAHLKR
GGC	LKEHLTR
GGT	EAHHLSR
GAG	RQDNLGR
GAA	QQTNLTR
GAC	DRGNLTR
GAT	VRHNLTR
GCG	RTDTLAR
GCA	QSTTLKR
GCC	DSSVLRR
GCT	QRSDLTR
GTG	RREVLEN
GTA	QRSSLVR
GTC	DHSSLKR
GTT	HKSSLTR
TGG	RREHLTI
TGA	QREHLTT

Step #3 Select F2 template for the ZFA PCR

Eg. RdR2ab F2 for the Left ZFA (L_ZFA)

DRGNLTR

F2 template

CATACCCGTACTCATACCGGTGAAAAACCGTTTCAGTGTCGGATCTGTATGCGAAATTTCTCCGACCGGGGCAACTTGACCCGCCATCTACGTACGCACACCGGCGAGAAGCCATTCCAATGCCGAATATGCATGCGCAACTTCAGT

HTRTHTGEKPFQCRICMRNFSDRGNLTRHLRTHTGEKPFQCRICMRNFS

Step #4 Perform PCR using the F1 & F3 primers and the F2 as template.

Step #5 After 10 cycles spike reaction with 1.5µL (10µM) of oCS276 & oCS218 primer pair. This reaction extends the ZFA to include restriction sites for future ligations (see below).

```
oCS276 - AGTGGTTGGTCTAGACCCGGGGAGCGCCCCTTCCAGTGTCGCATTTGCATGCGGAACTTT
oCS218 - TTCAGATTTCACTAGCTGGGATCCCCTCAGGTGGGTTTTTAGGTG
```

Table 3. PCR reaction to amplify fingers and ZFAs

Reagent	Vol.
10X *Pfu* buffer	2.5µL
Template (~10-100ng)	0.5µL
Primer Pair F1&F3 (10µM)	0.2µL
Pfu Turbo (Stratagene)	1µL
dNTPs (10mM)	0.5µL
dH_2O (25µL)	20.3µL

PCR conditions

Step	Temp	Time
Denature	95°	1 min
Denature	94°	30 sec
Anneal	55°	30 sec
Extension	72°	30 sec
Extension	72°	5 min
Soak	10°	∞

Cycle steps 2-4 x30

Primary PCR

F1 forward primer

TTGCATGCGGAACTTTTCGGTGAGACAAGGACTGACTAGACATACCCGTACTCATACCGG

F2 template

CATACCCGTACTCATACCGGTGAAAAACCGTTTCAGTGTCGGATCTGTATGCGAAATTTCTCCGACCGGGGCAACTTGACCCGCCATCTACGTACGCACACCGGCGAGAAGCCATTCCAATGCCGAATATGCATGCGCAACTTCAGT

F3 reverse primer

TCAGGTGGGTTTTTAGGTGTCTTCCCAGGTTATCCTCTCTACTGAAGTTGCGCATA

Spike with oCS276& oCS218

oCS276

AGTGGTTGGTCTAGACCCGGGGAGCGCCCCTTCCAGTGTCGCATTTGCATGCGGAACTTT

TTGCATGCGGAACTTTTCGGTGAGACAAGGACTGACTAGACATACCCGTACTCATACCGGTGAAAAACCGTTTCAGTGTCGGATCTGTATGCGAAATTTCTCCGACCGGGGCAACTTGACCCGCCATCTACGTACGCACACCGGCGAGAAGCCATTCCAATGCCGAATATGCATGCGCAACTTCAGTAGAGAGGATAACCTGGGAAGACACCTAAAAACCCACCTGA

oCS218

TTCAGATTTCACTAGCTGGGATCCCCTCAGGTGGGTTTTTAGGTG

Completed ZFA

XbaI *BamHI*

AGTGGTTGGTCTAGACCCGGGGAGCGCCCCTTCCAGTGTCGCATTTGCATGCGGAACTTTTCGGTGAGACAAGGACTGACTAGACATACCCGTACTCATACCGGTGAAAAACCGTTTCAGTGTCGGATCTGTATGCGAAATTTCTCCGACCGGGGCAACTTGACCCGCCATCTACGTACGCACACCGGCGAGAAGCCATTCCAATGCCGAATATGCATGCGCAACTTCAGTAGAGAGGATAACCTGGGAAGACACCTAAAAACCCACCTGAGGGGATCCCAGCTAGTGAAATCTGAA

SGWSRPGERPFQCRICMRNFSVRQGLTRHTRTHTGEKPFQCRICMRNFSDRGNLTRHLRTHTGEKPFQCRICMRNFSREDNLGRHLKTHLRGSQLVKSE

Step #6 Repeat steps 2 → 5 to construct right ZFA (R_ZFA).

Step #7 PCR purify product and double digest with *Xba*I/*Bam*HI. (297-bp; 268-bp after digestion)

Step #8 Linearize 10µL of pFZ50 *Fok*I expression vector with *Xba*I/*Bam*HI (Table 5.)

Step #9 Ligation of left ZFA in to linearized pFZ50 expression vector (Table 6.)

Step #10 Transform into DH5q competent cells and select on spec (50mg/L) plates

Step #11 Select three colonies and culture overnight at 37°C in LB broth (Spec 50)

Step #12 Mini-prep cultures and confirm ligation by diagnostic restriction assay (Table 5.)

Step #13 Linearize L_ZFA/pFZ50 with *Nhe*I/*Bgl*II (Table 7.) and ligate right ZFA (Table 6.)

Step #14 Repeat steps #11-13 and confirm ligation with (*Eco*RI/*Kpn*I) ~624bp fragment

Step #15 Confirm L_ZFA & R_ZFA by sequence using ZFN R1 & ZFN R2 primers

Step #16 PCR amplify ZFN cassette (L_ZFA/R_ZFA/pFZ50) with Xho.ZFA.F1 & NheI.ZFA.R1 primers using reaction (Table 8). Confirm PCR by gel electrophoresis (~ 2020bp)

Step #17 PCR purify product and double digest with *Xho*I/*Nhe*I.

Step #18 Linearize 10µL of Inducible 218 binary vector with *Xho*I/*Spe*I.

Step #19 Ligate ZFN cassette (*Xho*I/*Nhe*I) into linearized binary vector (*Xho*I/*Spe*I) (Table 6.)

Step #20 Transform into DH5q competent cells and select on Kan (50mg/L) plates

Step #21 Select three colonies and culture overnight at 37°C in 10mL LB broth (Kan 50)

Step #22 10mL mini-prep cultures and confirm ligation by *Eco*RI diagnostic restriction assay (Fig2.)

```
XhoI.ZFA F1 - ACTCGAGATGGATTATAAGGATCACGATG
NheI.ZFA R1 - TGCTAGCTCAATAAAAGTTTATCTCGCCGT
```

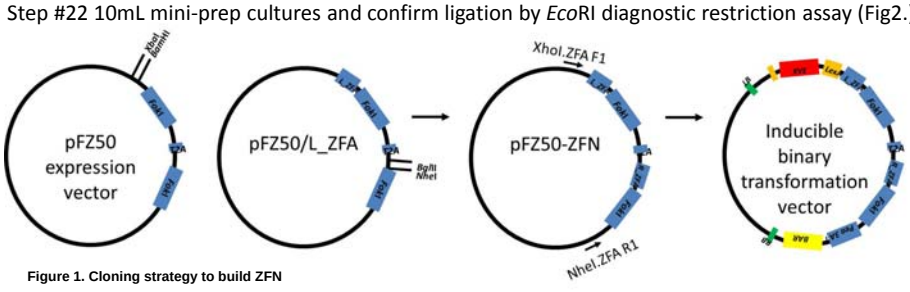

Figure 1. Cloning strategy to build ZFN

Table 4. PCR digestion reaction

NEB#4	3.0µL
BSA 10x	3.0µL
Purified PCR product	23.0µL
*Xba*I	0.5µL
*Bam*HI	0.5µL

Incubate @37°C for 6 hours

Table 5. Plasmid digestion reaction

NEB#4	3.0µL
BSA 10x	3.0µL
Plasmid	10.0µL
*Xba*I	0.5µL
*Bam*HI	0.5µL
dH20	13µL

Incubate @37°C for 6 hours

Table 6. Ligation reaction

2x ligation buffer	5.0µL
Linearized plasmid	2.0µL
Insert	2.5µL
T4 ligase	0.5µL

Incubate @ RT for an hour or overnight

Table 7. Plasmid digestion reaction

NEB#3	3.0µL
BSA 10x	3.0µL
Plasmid	10.0µL
*Nhe*I	0.5µL
*Bgl*II	0.5µL
dH20	13µL

Incubate @37°C for 6 hours

Table 8. PCR reaction to amplify ZFN

Reagent	Vol.
10X *Pfu* buffer	5.0µL
Template (~10-100ng)	1.0µL
Primer Pair F1&F3 (10µM)	2.0µL
Pfu Turbo (Stratagene)	1µL
dNTPs (25mM)	0.4µL
dH_2O (25µL)	40.6µL

PCR conditions

Step	Temp	Time
Denature	95°	1 min
Denature	94°	30 sec
Anneal	60°	30 sec
Extension	72°	30 sec
Extension	72°	5 min
Soak	10°	∞

Cycle steps 2-4 x30

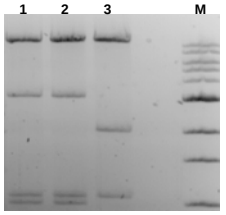

Figure 2. *Eco*RI diagnostic digestion of ZFN in binary vector. Lanes #1 and 2 have the correct pattern