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Engineering Designer Nucleases with Customized Cleavage Specificities

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Abstract

Engineered designer nucleases can be used to efficiently modify genomic sequence in a wide variety of model organisms and cell types. Zinc finger nucleases (ZFNs), consisting of an engineered zinc finger array fused to a non-specific cleavage domain, have been extensively used to modify a broad range of endogenous genes. Here we describe protocols for engineering ZFNs targeted to specific gene sequences of interest using the Context-Dependent Assembly (CoDA) method.

Keywords

engineered zinc finger nucleases; engineering zinc finger proteins; context-dependent assembly; protein engineering; DNA-binding domains

Introduction

Engineered zinc finger nucleases (ZFNs) can be used to introduce site-specific genome alterations in a wide variety of model organisms and a range of different mammalian cell types (Rahman et al., 2011; Urnov et al., 2010). ZFNs are customizable restriction enzymes that can be used to create targeted double-stranded breaks (DSBs) in cells. ZFNs consist of an array of engineered zinc fingers fused to the non-specific cleavage domain of the *Fok*I Type IIS restriction enzyme (Kim et al., 1996). The engineered zinc finger portion of the ZFN directs the nuclease activity to a specific location in the genome. Because the *Fok*I domain must dimerize to cleave DNA, a pair of ZFNs must be engineered to cleave a given target site of interest. Each monomer in a ZFN dimer pair binds to a “half-site” with cleavage of the DNA induced in an intervening 5–7 bp “spacer” sequence.

The repair of ZFN-induced DSBs by normal mechanisms used by nearly all cells can be exploited to make targeted genomic alterations. For example, non-homologous end-joining mediated repair of a ZFN-induced break can lead to the efficient introduction of insertion or

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Key references

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Internet Resources: ZiFiT (http://zifit.partners.org)

deletion mutants, providing a means to create frameshift and knockout mutations if introduced into coding sequences of endogenous genes (Bibikova et al., 2002; Santiago et al., 2008). In addition, ZFN-induced DSBs can also be repaired by homologous recombination with an investigator-supplied “donor template” (Bibikova et al., 2003; Urnov et al., 2005). This template consists of DNA sequences homologous to endogenous sequence surrounding the DSB but with mutations or insertions of interest also incorporated. Repair of the ZFN-induced DSB with this donor template can thus be used to introduce particular mutations or insertions into specific genomic loci.

Three methods have been described (Beerli and Barbas, 2002; Maeder et al., 2008; Sander et al., 2011) for engineering zinc finger arrays required to make ZFNs (Figure 1). These methods are known as (1) modular assembly, (2) oligomerized pool engineering (OPEN), and (3) context-dependent assembly (CoDA). Each of these methods possesses different advantages and disadvantages that we discuss briefly here.

With modular assembly (Figure 1C), individual pre-selected fingers are joined together to create a zinc finger array (Beerli and Barbas, 2002). This method relies on archives of pre-selected individual zinc fingers and assumes that the behaviors of fingers in an array are independent of one another. The advantage of this method is the ease with which it can be practiced - arrays can be engineered in as little as a week. However, the failure rate of this method for constructing pairs of three-finger ZFNs has been reported to be very high (>94%) (Kim et al., 2009; Ramirez et al., 2008). In addition, three-finger arrays produced by modular assembly have also been shown to have low affinities, low specificities, and low activities as ZFNs in cells (Cornu et al., 2008; Hurt et al., 2003; Ramirez et al., 2008). Various detailed protocols for practicing modular assembly have been previously described (Carroll et al., 2006; Gonzalez et al., 2010; Wright et al., 2006).

The OPEN method, unlike modular assembly, explicitly accounts for the context-dependent activities of individual zinc fingers in a three-finger array (Maeder et al., 2008; Maeder et al., 2009). The method relies on the creation of a combinatorial library of multi-finger arrays derived from “pools” of pre-selected fingers for individual three bp “subsites”. Three pools are randomly recombined together to create a library and then a bacterial two-hybrid selection system is used to identify specific library members that bind to a target site of interest (Figure 1A). The OPEN method is highly efficient and has yielded ZFNs that function efficiently in zebrafish, plants, and human somatic and pluripotent stem cells (Foley et al., 2009; Maeder et al., 2008; Townsend et al., 2009; Zhang et al., 2010; Zou et al., 2009). Although OPEN represents a substantially simpler protocol to practice over previously described selection-based methods, it remains lengthy and challenging for most laboratories to practice. The method takes approximately 2 months to complete and requires a substantial investment of time (typically 6 to 12 months) to successfully master. A detailed protocol for practicing OPEN has been previously described (Maeder et al., 2009).

The CoDA method is an assembly-based method that attempts to account for the context-dependent activities of adjacent zinc fingers in an array (Sander et al., 2011). With CoDA, three-finger arrays are engineered by assembling together fingers that have been pre-selected to function well together. As shown in Figure 1B, an amino-terminal finger (F1) is joined to a particular middle finger (F2) with which it is known to work well. A third carboxy-terminal finger (F3) is then added that is known to work well with the same F2. The resulting three-finger arrays are then screened for DNA-binding activity using a bacterial two-hybrid assay. This method is as simple to practice as modular assembly but is also highly efficient with approximately 50% of ZFN pairs showing activities in zebrafish and plants (Curtin et al., 2011; Sander et al., 2011).

Strategic Planning

Researchers interested in engineering their own ZFNs face the initial challenge of choosing from among the three methods described above. In general, we recommend the use of CoDA as a first-line approach. CoDA is simple to practice and has a high success rate for yielding functional ZFNs. CoDA ZFNs have been used successfully in zebrafish and plants and unpublished data from our lab shows that these nucleases also function in transformed human cell lines. The one exception to our recommendation of CoDA as the initial method of choice is for those investigators requiring ZFNs with the highest possible activities and specificities. Although CoDA does account for context-dependent effects between adjacent fingers, in our experience the OPEN method generally yields ZFNs with higher activities. Presumably OPEN ZFNs also possess higher specificities than those made by CoDA because the selection process interrogates a larger number of combinations of fingers and identifies those arrays that can find their target site in the context of competing *E. coli* genomic DNA.

The CoDA method will not yield ZFNs for every target gene of interest. CoDA currently has a targeting range of approximately 1 in every 500 bps of random DNA sequence. If one is unable to identify ZFNs using CoDA, we recommend that users attempt to use OPEN, which has a targeting range of approximately 1 in every 200 bps of random DNA sequence. If no potential OPEN ZFN target sites can be identified or if OPEN selections fail to yield ZFNs, users can consider using modular assembly with the caveat that the success rate may be low.

Basic Protocol 1: Identifying and Synthesizing CoDA ZFN Targets

This section describes in detail how to practice the Context Dependent Assembly (CoDA) for engineering zinc finger proteins. CoDA reagents are currently capable of targeting one site in every 500 bp of random sequence, a range sufficient for generating non-homologous end joining (NHEJ) frame shift mutations for most genes in many organisms. CoDA ZFs are designed using zinc finger domains that have been selected to work well together. The resulting DNA sequences encoding CoDA ZF arrays are less than 300 bp in length and can be synthesized and subsequently cloned into standardized expression plasmids for testing binding affinity in the bacterial two-hybrid (B2H) system or for use as site-specific nucleases.

Materials

DNA sequence of genomic region to target with ZFNs

A computer with internet access

1) Open a web browser and visit http://www.repeatmasker.org. Paste the sequence for the genomic region of interest into the text box. Select your host genome from the drop-down menu labeled “DNA source.” Leave the other options as their default settings and click the “submit sequence” button. If the output states “No repetitive sequences were detected” continue to step 2. If repeat elements are detected, consider varying the target region.

Repeat elements will not be identified if the submitted sequence is shorter than the repeat element. To ensure that the repeat elements are identified submit the genomic sequence that includes several hundred base pairs on both sides of your region of interest.

Use genomic sequence (not CDS) to ensure the ZFN targets are present in adjacent sequence.

It is also recommended to use actual DNA sequence obtained from the host organism to ensure there are no polymorphic variations from the published sequence.

2) Redirect the web browser to the ZiFiT web server (http://zifit.partners.org). Click ZiFiT on the menu below the Zinc Finger Consortium banner then select "Design Zinc Finger Nucleases" under the section labeled "CoDA (Context Dependent Assembly)". Paste either the FASTA or raw DNA sequence into the text box labeled "Sequence" and click the "Submit" button to scan your sequence for CoDA ZFN sequences. White space and numbers are ignored. Genomic sequence entered should be limited to 50,000 bps or less.

By default, ZiFiT defines any sequences in uppercase as exons and sequences in lowercase as introns. This can be used later to aid in picking targets. This option can be deactivated by unchecking the box labeled "Exon/Intron Case Sensitivity" below the lower right corner of the sequence box.

3) ZiFiT displays targets in a condensed format comprising individual target sequences flanked by their start and stop positions and labeled according to their FASTA identifier (if provided), the spacer length, and an index number. Scroll through the ZiFiT output in the main window to view CoDA targets within the submitted sequence. Expand information for individual targets by clicking the "+" adjacent to the label.

Alternatively, select targets by clicking the blue, green, and gold bars representing targets color coded by spacer size (5bp=Blue; 6bp=Green; 7bp=Gold) above the red bars (thin bar = introns; thick bar = exon) from the graphic summary pop-up window. Pop-ups must be enabled in your web browser for this feature to work. Use of the back button can disrupt the link between the popup and the main window. Restore functionality by closing the popup and selecting the ZiFiT link from the menu.

4) Select the link labeled "ZF DNA Sequence" under the table of an expanded target to display the DNA sequences encoding each of the CoDA arrays. Order each zinc finger sequence (<300bp each) through a commercial gene synthesis provider.

Use the following criteria to maximize success rates 1) Choose target sites where each of the two half-sites are composed of two or three GNN subsites 2) Avoid targets with half-sites that are composed of four or more T's 3) Click on the color-coded array in the double-strand DNA target sequence to query ZiFDB and determine whether a zinc finger array for a half-site has already been assayed for DNA-binding activity. Use arrays that have been previously shown to activate three-fold or more in the bacterial two-hybrid assay. 4) Pick targets early in coding sequence to maximize likelihood of generating a knockout mutation. 5) Pick target sites within 100bp of the location of the alteration to be introduced by homologous recombination. 6) Pick two or three ZFN target sites per gene of interest.

Support Protocol 1 (optional): Bacterial two-hybrid (B2H) Assay to Quantify DNA-Binding Activities of Zinc Finger Arrays

To confirm that a zinc finger array can bind to its target site, zinc finger arrays are expressed in B2H reporter strains and assayed for their abilities to activate expression of a beta-galactosidase reporter gene. The B2H reporter strain harbors a single-copy plasmid with the zinc finger array target site positioned upstream of a weak promoter which, in turn, controls expression of a *lacZ* reporter gene (encoding beta-galactosidase). Binding of the zinc finger array (fused to a Gal11P protein fragment) to its target site will recruit RNA polymerase to the promoter, leading to an increase in expression of beta-galactosidase (Figure 2). To perform this experiment, a B2H reporter strain is transformed with two plasmids; one expressing a zinc finger array-Gal11P fusion protein and one expressing an RNA polymerase-alpha-Gal4 hybrid protein. Because the *lacZ* gene encodes β-galactosidase, its expression can be measured by a simple quantitative assay. By comparing β-galactosidase expression in the presence and absence of a given zinc finger array, a “fold-activation” value can be calculated which can guide the choice of which arrays to carry forward for testing as ZFNs.

Materials

pGP-FF plasmid (plasmid, restriction map, and full sequence are freely available online from Addgene; see http://www.addgene.org/zfc)

Synthesized zinc finger plasmid (from above Basic Protocol 1)

*Xba*I restriction enzyme (NEB)

*BamH*I-HF restriction enzyme (NEB)

QIAquick Gel Extraction Kit (Qiagen)

Quick Ligation Kit (NEB)

Chemically competent XL1-Blue cells (Stratagene)

Carbenicillin

LB agar

LB broth

QIAprep Spin Miniprep Kit (Qiagen)

Primer OK61: 5'-GGGTAGTACGATGACGGAACCTGTC-3'

pBAC-LacZ plasmid (plasmid, restriction map, and full DNA sequence are freely available online from Addgene; http://www.addgene.org/zfc)

*Bsa*I restriction enzyme (NEB)

dCTP nucleotide

Cloned Pfu Polymerase and associated 10X reaction buffer (Stratagene)

10X Annealing Buffer (see Reagents and Solutions)

Chemically Competent Bacterial Strain Transformax Epi300 (Epicentre)

Chloramphenicol

Chemically Competent Bacterial Strain KJBAC1 (available from Addgene)

Expand High Fidelity PCR Kit (Roche Applied Science)

Primer OK5: 5'- AAAATAGGCGTATCACGAGGCCCT -3'

Primer OK163: 5'- CGCCAGGGTTTTCCCAGTCACGAC -3'

1M $MgCl_2$

Solution A + Glycerol (see Reagents and Solutions)

96-well microtiter plates

pAC-Kan-alpha-Gal4 plasmid (available from Addgene)

Kanamycin

2ml assay blocks (Corning)

10mM $ZnSO_4$

500mM IPTG

Microtiter plate reader with temperature control option (for example, Biorad Model 680 Microplate Reader)

Popculture reagent (Novagen, cat. no. 71092)

R-lysozyme (30,000 units/ml) and associated dilution buffer (Novagen, cat. no. 71110)

Lysis Master Mix (see Reagents and Solutions)

Z-buffer with β-mercaptoethanol (see Reagents and Solutions)

4mg/ml ONPG

Construct the zinc finger-Gal11P fusion protein plasmid

1) Digest 1 μg of pGP-FF plasmid as follows. Combine the following reagents in a microcentrifuge tube (total reaction volume, 20 μl):

1 μg of pGP-FF plasmid

0.5 μl *Xba*I (10U)

0.5 μl *BamHI*-HF (10U)

2 μl NEB Buffer 4

Sterile water to final volume of 20 μl

Digest for 3 hours at 37C and gel isolate the backbone (~3.5kb) using QIAquick gel extraction kit.

2) Digest 1 μg of each of the synthesized zinc finger plasmids in separate reactions. Combine the following reagents in a microcentrifuge tube (total reaction volume, 20 μl):

1 μg synthesized zinc finger plasmids

0.5 μl *Xba*I (10U)

0.5 μl *BamHI*-HF (10U)

2 μl NEB Buffer 4

Sterile water to final volume of 20 μl

Digest for 3 hours at 37C and gel isolate the zinc finger fragment (~280bp) using QIAquick gel extraction kit.

If your zinc finger proteins contain a Not*I site instead of a* BamH*I site for cloning into the vectors pMLM800 or pMLM802, you will need to first amplify them by PCR (CPMB UNIT 15.1) to generate a BamHI site using the following primers:*

Forward: 5'- GAAAAAAATCTAGACCCGGGGAGC -3'

Reverse: 5'- CGCGGATCCCCTCAGGTGGGTTTTTAGGTG-3'

3) Ligate each purified zinc finger-encoding fragment into vector backbone for 15 minutes at room temperature as follows. Combine the following reagents in a microcentrifuge tube (total reaction volume, 13 µl):

5 µl zinc finger fragment (or nuclease-free water for control)

1 µl pGP-FF backbone

6 µl 2X quick ligase buffer

1 µl T4 DNA Ligase

4) Transform ligation reactions from step 3) into *E. coli* by adding 150 µl XL1-blue chemically competent cells to the ligation. Incubate at 4C for 5 minutes then heat shock for 1 minute at 42C and return to 4C for 1 minute. Add 700 µl of LB and shake at 37C, 250 r.p.m. for 60 minutes. Plate 300 µl of each transformation on LB/Carb plates (LB agar supplemented with 100 µg ml^{-1} carbenicillin) and grow overnight at 37C.

5) If zinc finger transformations have greater than 10× the number of colonies of the control plates use single colonies from the zinc finger transformation plate to inocµlate 4-ml cultures of LB/Carb100µg ml-1 and grow overnight at 37C, 250 r.p.m. Purify plasmids the next day from these cultures using a QIAprep Spin Miniprep Kit.

6) Verify the DNA sequence of the cloned zinc finger array fragment using sequencing primer OK61. Note that a restriction map and the full sequence of the parental plasmid pGP-FF is available freely online through Addgene (http://www.addgene.org).

Generate B2H Reporter Plasmid

7) Digest pBAC-LacZ plasmid. Combine the following reagents in a microcentrifuge tube (total reaction volume, 30 µl):

1 µg pBAC-LacZ plasmid

3 µl NEB Buffer 3

1.5 µl *Bsa*I (5U/µl)

Sterile water to final volume of 30 µl

Incubate at 50°C for 2 hrs. Gel purify (CPMB UNIT 2.5A or 2.6) ~11kb vector backbone and elute in 20 µl of nuclease-free water.

8) Create DNA overhangs by treating the digested vector backbone from step 7) with *Pfu* polymerase and dCTP. Combine the following reagents in a microcentrifuge tube (total reaction volume, 40 µl):

20 µl *Bsa*I digested, gel purified pBAC-LacZ plasmid (from step 7, above)

4 µl 10X Pfu Buffer

4 μl 10 mM dCTP

2.4 μl *Pfu* polymerase

9.6 μl Sterile water

Incubate at 72°C for 15 minutes, followed by 4°C for 2 minutes. Store indefinitely at −20°C.

9) Prepare target site oligonucleotides. On the ZiFiT output page, click on the link titled “ZF DNA Sequence” and order the two displayed target site oligos for each zinc finger protein to be tested.

10) Anneal target site oligos. Combine the following reagents in a microcentrifuge or PCR tube:

1 μl 10 μM top oligo

1 μl 10 μM bottom oligo

20 μl 10X annealing buffer

178 μl sterile water

Heat reaction to 95°C for 2 minutes. Then slowly cool to 35°C either by shutting off the heat-block and letting it cool or by programming a thermocycler to reduce temperature by 1°C per minute. Store indefinitely at −20°C.

11) Insert target site oligos using ligation-independent cloning into pBAC-LacZ vector backbone. Combine the following in a microcentrifuge tube (total reaction volume, 21 μl):

1 μl pBAC-LacZ vector backbone from step 8 above

9 μl annealed oligos from step 10 above

10 μl 2X Quick Ligase Buffer

Incubate reaction at room temperature for 15 minutes, then place on ice.

12) Transform ligation into Transformax Epi300 cells as follows: Add 200 μl chemically competent Transformax Epi300 cells to ligation mix from step 11. Chill on ice for 10 minutes then heat shock at 42°C for 2 minutes, followed by 2 minutes on ice. Add 700 μl of LB and incubate at 37°C, with agitation, for 45 minutes. Plate 300 μl on LB plate supplemented with 12.5 μg/ml chloramphenicol. Incubate at 37°C overnight.

13) Pick single colonies from transformation plates (from step 12) and inoculate each colony into 3 ml of LB supplemented with 12.5 μg/ml chloramphenicol. Incubate overnight, with agitation, at 37°C.

14) Subculture 1ml of overnight culture into 9ml of LB containing 14 μg/ml chloramphenicol and 1.1 mM arabinose. Grow cultures at 37°C, with agitation, for 5–6 hours. Harvest cells and isolate plasmid DNA using a QIAprep spin miniprep kit.

Subculturing the cells containing pBAC-LacZ plasmids in arabinose induces expression of the trfA *gene product, which increases the copy number of the plasmid.*

Generate B2H Reporter Strain

15) Transform reporter plasmids into bacterial strain KJBAC1. Combine 1 µl of miniprep reporter plasmid DNA (from step 14) with 100 µl chemically competent KJBAC1. Chill on ice for 10 minutes then heat shock at 42°C for 2 minutes, followed by 2 minutes on ice. Add 350 µl of LB and incubate at 37°C, with agitation, for 45 minutes. Plate 300 µl on LB plate supplemented with 12.5 µg/ml chloramphenicol. Incubate at 37°C overnight.

16) Inoculate single colonies from transformation plates into 3 ml of LB supplemented with 12.5 µg/ml chloramphenicol. Incubate overnight, with agitation, at 37°C.

17) PCR amplify region of reporter plasmid bearing the target site for sequence verification. Combine the following in a PCR tube (total reaction volume, 25µl):

2 µl bacterial overnight culture

2 µl primer OK5 (10 µM)

2 µl primer OK163 (10 µM)

2.5 µl Expand Buffer 2

2 µl 10 mM dNTPs (2.5 mM each)

0.5 µl Expand Enzyme

14 µl Sterile water

Amplify using the following PCR protocol:

1 cycle:	5 minutes	95°C	(initial denaturation)
35 cycles:	30 seconds	95°C	(denaturation)
	30 seconds	55°C	(annealing)
	30 seconds	72°C	(extension)
1 cycle:	5 minutes	72°C	(final extension)
1 cycle:	indefinitely	4°C	(hold)

18) Sequence PCR product with primer OK163. Note that a restriction map and the full sequence of the parental reporter plasmid pBAC-LacZ is available freely online through Addgene (http://www.addgene.org).

19) Subculture 1 ml of overnight culture into 50 ml of LB supplemented with 200 µl of 1M $MgCl_2$ (final concentration of 15 mM) and 12.5 µg/ml chloramphenicol. Shake at 37°C, 250 rpm for 1 hr.

20) Centrifuge cells in a sterile 50 ml conical tube at 3000–6000×g for 20 minutes.

21) Resuspend cell pellet in 3ml of SolutionA + glycerol, aliquot into microcentrifuge tubes, and freeze at −80°C.

Perform Quantitative B2H Assay

22) Transform pAC-Kan-alpha-Gal4 plasmid and ZF-Gal11P plasmid (from step 6 above) into B2H reporter strain competent cells (from step 22 above). In one well of a 96-well plate, combine 1 µl of ZF-Gal11P plasmid (or pGP-FF plasmid for the basal level control), 100 ng of plasmid pAC-Kan-alpha-Gal4 and 50 µl competent cells (from step 21 above). Chill on ice for 10 minutes then heat

shock at 42°C for 2 minutes, followed by 2 minutes on ice. Add 200 µl of LB to each well and incubate at 37°C for 1 hr.

23) Make two serial ten-fold dilutions of each transformation and spot 5µl of each dilution (10^0, 10^{-1} and 10^{-2}) three times on LB plates supplemented with 100 µg/ml carbenicillin, 12.5 µg/ml chloramphenicol and 30 µg/ml kanamycin. Incubate at 37°C overnight.

24) For each zinc finger protein to be assayed, inoculate three overnight cultures (from 3 independent colonies) as well as three basal controls (pGP-FF and pAC-Kan-alpha-Gal4 plasmids transformed into the B2H reporter strain). Inoculate each colony into 1 ml of LB containing 50 µg/ml carbenicillin, 30 µg/ml kanamycin, 12.5 µg/ml chloramphenicol, 10 µM $ZnSO_4$, and 500 µM IPTG in a 2ml 96-well block. Grow overnight at 37°C with agitation.

Shaking on a platform with a small throw radius of no more than a few millimeters (for example, Microtitertron orbital shaker, Appropriate Technical Resources) will ensure uniform growth of all wells. Overnight culture time should not exceed 18 hours.

26) Subculture saturated overnight cultures by diluting 25µl into 1ml of pre-warmed culture medium (same media used for overnight cultures prepared in step 22). Grow subcultures with agitation at 37°C.

27) Monitor the growth of subcultures by measuring the OD_{600} (relative to a media-only blank) on a spectrophotometer. Harvest cells for lysis when they reach log phase (OD_{600} = 0.3–0.5). Record the OD_{600} at which each well was lysed.

For best results lyse as close to and OD_{600} of 0.3 as possible.

28) Lyse log-phase cultures by mixing 100 µl of culture with 11 µl of Lysis Master Mix in a 96-well plate. Allow lysis reactions to proceed at room temperature for a minimum of 15 minutes.

The activity of β-galactosidase is stable in the cell lysates for at least 18 hours when stored sealed at room temperature.

29) Set up β-galactosidase assay by adding 15 µl of lysate to a 96-well microtiter plate well containing 135 µl of Z buffer with β-mercaptoethanol and 30 µl of 4 mg/ml ONPG and mixing well.

30) Place microtiter plate containing β-galactosidase assay reactions in a microtiter plate reader with temperature control. Incubate reactions at 28° C and take timed serial measurements of absorbance at 415 nm. Calculate the velocity of ONPG cleavage (v) by plotting A_{415} vs. time and calculating the slope of the line.

Many microtiter plate readers can be programmed to take absorbance measurements at fixed intervals and to calculate the velocity of ONPG cleavage. We typically take measurements every 10–30 seconds. Reactions should not be allowed to proceed for more than 30 minutes as substrate can become limiting.

31) Calculate the units of β-galactosidase for each assay using the following formula:

$$V \times 1000/(OD_{600})$$

32) Calculate the fold-activation mediated by each zinc finger protein tested by comparing β-galactosidase units obtained in the presence of the zinc finger-

Gal11P hybrid protein with the units obtained in the presence of the Gal11P control.

Representative Data:

Target: GCAGAAGGT

CoDA Helices: F1: TKQRLEV - F2: QQTNLTR - F3: QGNTLTR

Colony Sample	OD600	Velocity A	Velocity B	Average Velocity	V × (1000) / OD600	Fold Activation
Zinc Finger	0.2827	51.80	59.80	55.80	5635.27	9.39
Zinc Finger	0.3075	59.30	66.00	62.65	5816.18	
Zinc Finger	0.3075	59.90	66.20	63.05	5853.31	
Basal	0.2999	5.90	6.40	6.15	585.49	
Basal	0.3075	6.60	7.30	6.95	645.21	
Basal	0.2961	6.00	6.70	6.35	612.33	

Basic Protocol 2: Construction of ZFN expression vectors

In this step, each ZF array is cloned into a nuclease expression vector. Expression vectors differ based on the type of promoter, nuclease domain, and linker between the ZF and nuclease domain (see Table below). The linker determines the number of nucleotides (5, 6, or 7) that are permitted in the “spacer” sequence between the ZF target half-sites (Handel et al., 2009). The wild-type *Fok*I nuclease monomer permits ZF monomers from the same half-site to form homodimers. This increases the likelihood that the ZFNs will cleave at unintended positions. Alternatively, the heterodimer variants require two different ZFNs to assemble on a full target site to mediate cleavage (Miller et al., 2007). However, the heterodimer variants used in the expression plasmids described below are less active than the wild-type *Fok*I domains.

Materials

Synthesized zinc finger constructs (Basic Protocol 1)

*Xba*I (NEB)

*BamH*I (NEB)

*Not*I (NEB)

Quick Ligation Kit (NEB)

Nuclease free water

LB medium (Difco. Cat. No. 244620)

LB agar medium (Difco. Cat. No 244520)

QIAquick gel extraction kit (Qiagen)

QIAprep spin miniprep kit (Qiagen)

Carbenicillin (Sigma, cat. no T4625; 50 mg/ml^{-1} stock solution in ddH20)

LB/Carb plates (LB agar supplemented with 100 μg/ml carbenicillin.

Chemically competent Bacterial strain XL-1 Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIq lacZDM15 Tn10 (TetR)*]; Stratagene cat. no. 200249)

Primer OK567: 5’- CGCAAATGGGCGGTAGGCGTG -3’

Nuclease Expression Plasmids: (see Table below for description; plasmids, restriction maps, and full DNA sequences of all of these plasmids are freely available online through Addgene: http://www.addgene.org/zfc)

- pST1374 (or)
- MLM290 and MLM292 (or)
- MLM800 and MLM802

Plasmid name	For expression in	*Fok*I cleavage domain type	ZFN spacer length
pST1374	Mammalian cells, zebrafish (RNA)	Wild-type	5 or 6
pMLM290	Mammalian cells, zebrafish (RNA)	'+' obligate heterodimer	5 or 6
pMLM292	Mammalian cells, zebrafish (RNA)	'−' obligate heterodimer	5 or 6
pMLM800	Mammalian cells, zebrafish (RNA)	'+' obligate heterodimer	7
pMLM802	Mammalian cells, zebrafish (RNA)	'−' obligate heterodimer	7
pDW1775	Plants	Wild-type	5 or 6

1) Digest 1 μg of each nuclease expression plasmid(s).

ZFN targets with spacer lengths of 5 or 6 nucleotides

0.5 μl *Xba*I (10U)

0.5 μl *BamH*I-HF (10U)

2 μl NEB Buffer 4

17 μl nuclease free water

ZFN targets with spacer lengths of 7 nucleotides

0.5 μl *Xba*I (10U)

1 μl *Not*I (10U)

2 μl NEB Buffer 3

17 μl nuclease free water

Digest for 3 hours at 37C and gel isolate (CPMB UNIT 2.6) the nuclease backbone (~5.5kb) using QIAquick gel extraction kit.

2) Digest 1 μg of each of the synthesized zinc finger plasmids.

ZFN targets with 5 or 6 nucleotides

0.5 μl *Xba*I (10U)

0.5 μl *BamH*I-HF (10U)

2 μl NEB Buffer 4

17 μl nuclease free water

ZFN targets with 7 nucleotides

0.5 μl *Xba*I (10U)

1 µl *Not*I (10U)

2 µl NEB Buffer 3

17 µl nuclease free water

Digest for 3 hours at 37C and gel isolate (CPMB UNIT 2.6) the fragment encoding the zinc finger fragment (~280bp) using a QIAquick gel extraction kit.

3) Ligate each purified zinc finger fragment with the corresponding vector backbone for 15 minutes at room temperature.

3 µl zinc finger fragment (use nuclease free water for ligation control)

2 µl nuclease expression vector backbone

6 µl 2X quick ligase buffer

1 µl T4 DNA Ligase

4) Transform ligation reaction by adding 6 µl of the ligation reaction to 60 µl XL1-blue chemically competent cells. Incubate at 4C for 5 minutes then heat shock for 1 minute at 42C and return to 4C for 1 minute. Add 300 µl of LB and shake 37C, 250 r.p.m. for 60 minutes. Plate 300 µl of each transformation on LB/Carb plates (LB agar supplemented with 100 µg ml^{-1} carbenicillin) and grow overnight at 37C.

5) If zinc finger transformations have greater than 10× the number of colonies of the control plates use single colonies from the zinc finger transformation plate to inoculate 4-ml cultures of LB/Carb100 µg ml-1 and grow overnight at 37C, 250r.p.m. Purify plasmids using a QIAprep Spin Miniprep Kit.

6) Sequence-verify the portion of the plasmid encoding the zinc finger array using sequencing primer OK567.

REAGENTS AND SOLUTIONS

10X Annealing Buffer

To prepare 1ml of 10X annealing buffer, combine the following sterile solutions:

400 µl 1M Tris, pH8

200 µl 1M $MgCl_2$

100 µl 5M NaCl

20 µl 0.5M EDTA, pH8

280 µl water

Store indefinitely at −20°C.

Solution A+Glycerol

To prepare 300ml of Solution A + Glycerol, combine the following sterile solutions:

3 ml 1 M MnCl2

15 ml 1 M CaCl2

60 ml 50 mM MES, pH6.3 (with KOH)

45 ml 100% glycerol

177 ml water

Store at 4°C wrapped in aluminum foil to protect from light. Solution is usable until it acquires a brown discoloration.

Lysis Master Mix

R-Lysozyme is supplied at 30,000 U/μl and can be diluted to 400U/μl and stored indefinitely at −20°C. To prepare lysis master mix, dilute R-Lysozyme to 4 U/μl and make a 10:1 mixture of Popculture reagent to diluted lysozyme. Prepare lysis master mix fresh before each use.

Z-buffer with β-mercaptoethanol

To prepare 1 L of Z-buffer, dissolve the following in water to a final volume of 1L:

16.1 g Na_2HPO_4-$7H_2O$

5.5 g NaH_2PO_4-H_2O

0.75 g KCl

0.246 g $MgSO_4$-$7H_2O$

Filter sterilize and store indefinitely at room temperature. Prepare Z-buffer with β-mercaptoethanol fresh before use by adding 2.7μl of β-mercaptoethanol per 1 ml Z-buffer.

Commentary

Background Information

A) Application: Zinc finger nucleases can be used to cleave specific targets within complex genomes. Subsequent repair of these double-strand breaks via the imperfect non-homologous end joining (NHEJ) repair pathway generates frame-shift mutations that can be used to abolish gene function. CoDA ZFNs have been shown to generate NHEJ -induced mutations in less than 1% to as high as 19% in zebrafish or plant somatic cells and these mutations can be passed on through the germ line to progeny. Alternatively, the homologous recombination pathway can be exploited by providing an exogenous donor template to introduce specific base changes, insertions and deletions. It has also been shown that short fragment of dsDNA can be inserted at the site of a break via NHEJ.

B) Critical Parameters and Troubleshooting

1) It is critical to submit genomic DNA sequence to ZiFiT. Submitting non-adjacent sequence, such as cDNA, may result in the identification of a site that is formed only after intron splicing and does not actually exist as contiguous sequence in genomic sequence.

2) The heterodimeric *Fok*I variants have been specifically engineered to function only with their obligate partners. It is critical that one half-site is cloned into "+" vector and the other into the "−" vector.

3) The amount of ZFN DNA/RNA required to efficiently generate double strand breaks in cells can vary by target and ZFN pair. Using too little DNA/RNA can lead to suboptimal rates of ZFN-induced gene modification. Using too much DNA/RNA can be toxic to the host cells. Titration may be necessary to determine the amount of template that is appropriate for the specific target or ZFN pair.

4) Some ZFN pairs do not efficiently cleave their genomic targets (See Anticipated Results). Possible explanations for poor activity include 1) insufficient specificity of the ZFN pair and 2) the context of the DNA target such as an inaccessible chromatin state. To maximize the change of success it is recommended to target multiple sites within the region of interest.

5) ZFNs may cleave at unintended off-target sites. Picking targets that are dissimilar to other genomic sequence may help to minimize potential off target cleavage. When possible in model organisms, outcrossing can be used to reduce the effects of potentially unwanted off-target effects.

C) Anticipated Results

Users can expect approximately 75% of CoDA ZFs will activate three-fold or more in the B2H reporter assay (Sander et al., 2011). Approximately 50% of ZFN pairs made from such ZF arrays are expected to induce NHEJ mutations with rates of 1% or higher in zebrafish and plants using the heterodimeric *Fok*I nucleases (Curtin et al., 2011; Sander et al., 2011). Actual cleavage rates will be organism-/cell line-dependent. CoDA ZFs that exhibit B2H reporter assay fold-activation values between 1.57 and 3 fold activations may also show activity as ZFNs in zebrafish and plants. ZFs that activate transcription by 1.57-fold or less in the B2H reporter assay have been shown to have a high failure rate and are not expected to function well as nucleases (Ramirez et al., 2008).

D) Time Considerations

1 Construction of ZFN-expression vector:

Cloning into the ZFN-expression vector takes 3–4 days with 2 overnight incubation steps and a few additional days for sequence verification.

2 B2H Assay (Optional):

The optional B2H protocol requires 8–10 days total. Construction of the zinc finger-Gal11P fusion protein plasmid takes 3–4 days with 2 overnight incubation steps and can be performed concurrently with construction of the B2H reporter plasmid. Construction of the B2H reporter plasmid and strain takes 5–7 days with 4 overnight incubation steps. The B2H assay takes 1 day for transformation, 1 day for starting overnights and the assay is run on the 3rd day. With practice, a full 96-well plate can easily be assayed at one time. For this reason, it is logical to construct 16 proteins and 16 reporter strains concurrently because picking 3 colonies of the protein and 3 of the basal control transformation for each strain will result in 96 samples.

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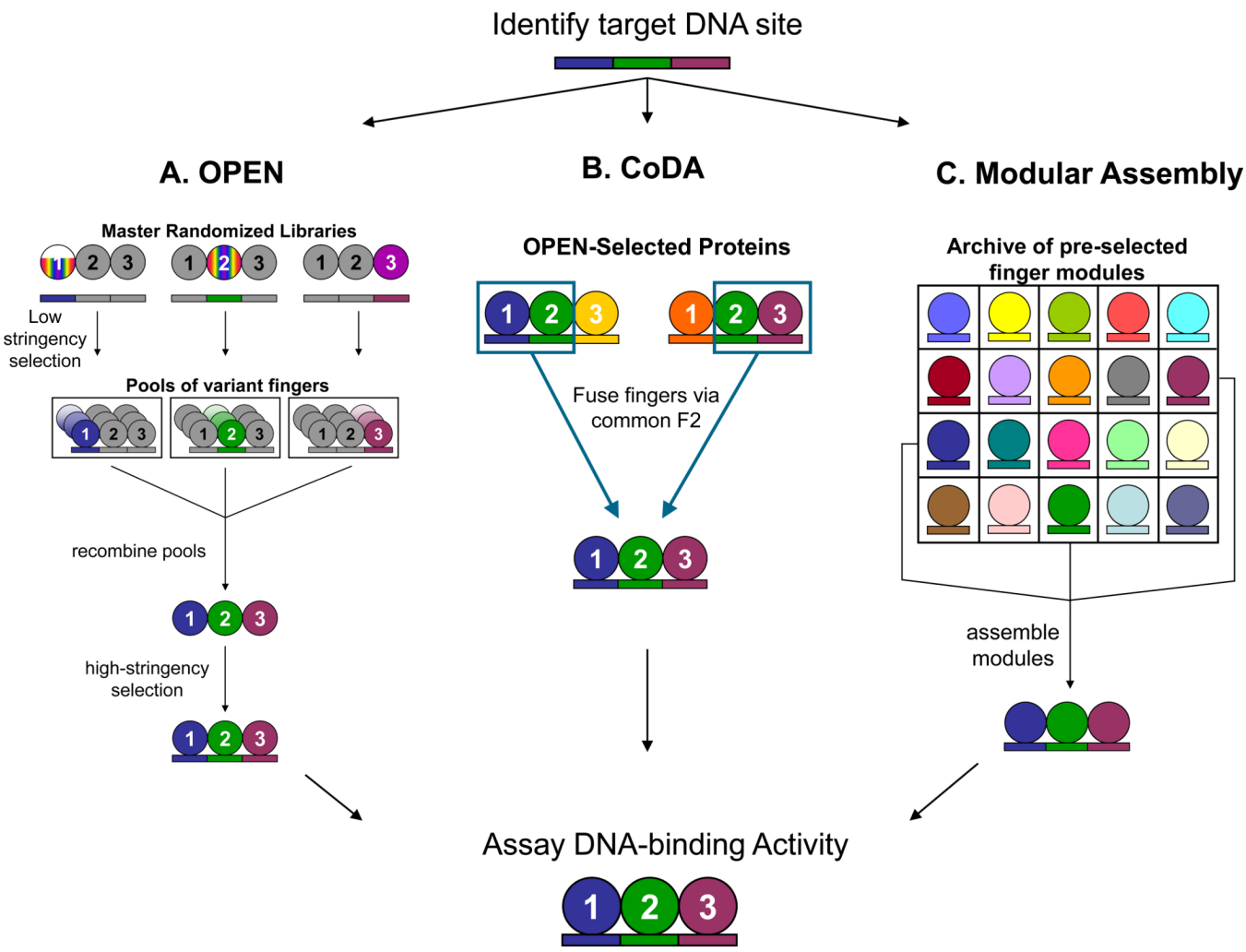

Figure 1. Publicly available methods for engineering zinc finger arrays

A. The Oligomerized Pool Engineering (OPEN) method utilizes master randomized libraries in which up to 95 different zinc fingers have been selected to bind to a 3 bp subsite at a specific position within a 3-finger array. Appropriate combinatorial libraries composed of randomly recombined pools for a 9 bp target site of interest are constructed and then interrogated using a bacterial two-hybrid selection system to yield three-finger proteins selected to bind the 9bp site of interest.

B. The Context Dependent Assembly (CoDA) method takes context-dependent effects between neighboring fingers into account by identifying three-finger arrays selected in the bacterial two-hybrid system that share a common middle finger. These fingers can then be combined, via the common middle finger, to yield a three-finger protein targeted to the 9 bp site of interest.

C. Modular Assembly utilizes an archive of single-finger modules that have been characterized to bind to a specific 3 bp DNA site. These modules are then combined to generate a 3-finger protein.

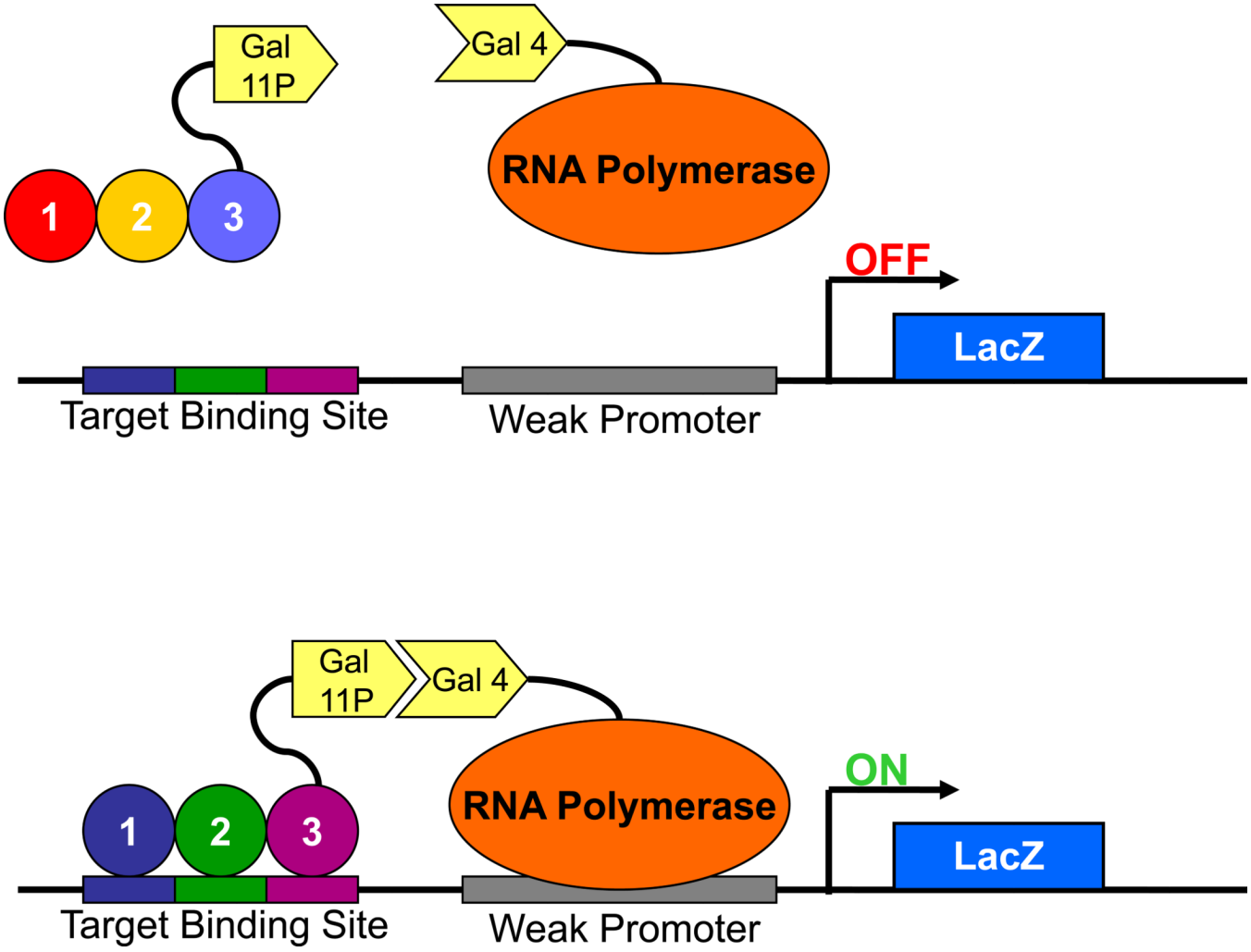

Figure 2.

Bacterial Two-Hybrid Reporter System. A DNA target site of interest is positioned upstream of a weak promoter driving *lacZ* expression. In the absence of a zinc finger protein that binds to this target site, *lacZ* is not expressed. However, when a fusion protein consisting of Gal11P and a zinc finger capable of binding the target site are introduced, they will recruit the RNA polymerase complexes containing alpha-Gal4 fusions to the weak promoter via interaction between the Gal11P and Gal4 proteins. This recruitment results in increased *lacZ* expression, thereby enabling a quantitative read-out of the zinc finger protein's ability to bind the target site.