

ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool

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ABSTRACT

ZiFiT (Zinc Finger Targeter) is a simple and intuitive web-based tool that provides an interface to identify potential binding sites for engineered zinc finger proteins (ZFPs) in user-supplied DNA sequences. In this updated version, ZiFiT identifies potential sites for ZFPs made by both the modular assembly and OPEN engineering methods. In addition, ZiFiT now integrates additional tools and resources including scoring schemes for modular assembly, an interface with the Zinc Finger Database (ZiFDB) of engineered ZFPs, and direct querying of NCBI BLAST servers for identifying potential off-target sites within a host genome. Taken together, these features facilitate design of ZFPs using reagents made available to the academic research community by the Zinc Finger Consortium. ZiFiT is freely available on the web without registration at <http://bindr.gdcb.iastate.edu/ZiFiT/>.

INTRODUCTION

Engineered zinc finger proteins (ZFPs) are important tools for gene regulation and genome modification because they can be used to target functional domains to virtually any desired location in a complex genome (1,2). Zinc finger nucleases (ZFNs) consist of an engineered ZFP fused to a non-specific nuclease domain and can be used to create double-stranded breaks (DSBs) in specific endogenous genes (3). These DSBs can be exploited to induce highly efficient insertion or alteration of DNA sequences via homologous recombination at the targeted locus (4–11). Alternatively, imperfect repair of a ZFN-induced DSB by

non-homologous end joining can lead to highly efficient generation of gene-specific knockouts (7,12–17).

Engineered C2H2 ZFPs comprise multiple (usually three to six) ZF domains joined together by a fixed amino-acid linker sequence(s), typically TGEKP. Each individual domain conforms to the zinc-finger motif X₂–C–X_{2–4}–C–X₁₂–H₃₅–H which, when chelated with a zinc ion, forms a ββ α fold. This structure presents a stabilized α -helix (the recognition helix) capable of making base specific contacts with approximately three bases in the major groove of double-stranded DNA. Adjacent ZF domains in a ZFP typically specify adjacent DNA triplets, establishing specificity for an extended target site (18).

ZFPs can be engineered to recognize new DNA sequences by altering as many as six important residues in the recognition helix. Because ZFP libraries covering this sequence space (20¹⁸ variants for a three finger protein) cannot be built or adequately sampled using existing molecular biology techniques, much effort has been placed on developing alternative methods for engineering multi-finger proteins (7,19–29). These methods typically involve identifying individual zinc-finger domains that recognize specific DNA triplet ‘subsites’ and then joining these domains together to create multi-finger proteins. The two most common ZFP engineering methods, modular assembly and oligomerized pool engineering (OPEN), both use variations on this general approach (2). Modular assembly usually assumes that a single domain (module) can recognize a specific DNA triplet regardless of the position of the triplet within the target site or the identities of adjacent neighboring fingers (i.e. it assumes binding of the ZF module is context-independent). Appropriate modules are simply joined together to create a ZFP that should recognize the target

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sequence. Modular assembly is relatively simple to accomplish; however, ZFPs generated using this method have been shown to have a high failure rate *in vivo* (30–32). In contrast, OPEN uses customized ‘pools’ of ZF modules selected to recognize triplets in a specific sequence context. These pools can be assembled to create combinatorial libraries (with up to one million unique solutions for a three-finger protein) from which the ZFPs best able to bind the chosen target DNA site are identified. Although OPEN is somewhat more labor intensive, it is more robust, with a higher success rate than modular assembly (7).

ZiFiT provides a simple interface for scanning a DNA sequence to identify potential ZFP and ZFN binding sites. The updated version (3.2) identifies target sites for proteins engineered using either OPEN or modular assembly. ZiFiT 3.2 also provides several new tools to help researchers evaluate ZFP targets, including validated scoring schemes for ranking potential target sites, a tool for querying NCBI BLAST servers for potential off-target sites, and a seamless interface with the Zinc Finger Database [ZiFDB, a database of engineered ZFPs (33)].

MATERIALS AND METHODS

Modular assembly

The modular assembly engineering approach employs individual zinc-finger domains (modules) that have been pre-characterized (in the middle position of a three-finger array) to bind a specific DNA triplet subsite. Several (three to six) of these modules can be arranged and linked together to generate a ZFP that recognizes an extended DNA sequence corresponding to the desired target site. ZiFiT provides support for the three most commonly used module sets developed by three independent research groups (21,23,27).

Oligomerized pool engineering

OPEN utilizes pools of zinc-finger domains pre-characterized to bind a specific DNA triplet subsite in the first, second or third position of the target site for a three-finger ZFP (7). Appropriate pools are combined to generate hundreds of thousands of distinct solutions for a given 9-bp target DNA sequence. The best solutions are subsequently identified using a bacterial two-hybrid (B2H) assay (34). Although this method requires considerable effort, it reliably generates ZFPs that bind with high affinity and specificity to their intended target site (7,9,11,16).

GNN scoring

The GNN score is an empirical estimate of the probability that a modularly assembled three-finger ZFP will provide >1.6-fold activation in the B2H assay [proteins that fail to meet this cutoff have been shown to fail to function in mammalian cells (30)]. Using probabilities based on evaluation of a set of 168 three-finger ZFPs generated by modular assembly, ZFPs designed to bind target sites containing 3, 2, 1 and 0 triplets of the form GNN (where N is

any nucleotide) are predicted to have success rates of 59, 29, 12 and 0%, respectively (30).

Affinity scoring

The affinity score is an energy-based parameter that predicts which modularly assembled three-finger ZFPs are most likely to function by inferring the contributions of the individual modules. Scores are calculated by estimating the relative free energy contributions of individual modules from dissociation constants reported for modules in the middle (F2) position of a three-finger ZFP (27). These affinity scores have been calibrated to B2H fold-activation values for modularly assembled ZFPs tested *in vivo*. ZFPs with affinity scores less than five are expected to have adequate affinity to function in the B2H assay (35). These scores do not directly address specificity and are available only for ZFPs composed exclusively of Barbas GNN and TGG modules.

Program input

The ZiFiT 3.2 interface enables customizable searches for potential ZFP and ZFN-binding sites that can be targeted using either the modular assembly or OPEN engineering methods. After selecting *ZiFiT* from the menu bar, users select their preferred engineering method (modular assembly or OPEN) and target type (ZFP or ZFN), e.g. OPEN – ZFN. In all interfaces, users enter their DNA query sequence into the *Sequence* input box near the top of the page (Figure 1). Sequences can be submitted either in FASTA format or raw text. Ideally, sequences should be entered using uppercase characters to denote exons and lowercase characters to denote introns. Entering information in this format can facilitate target selection for certain experimental applications, such as generation of knockout mutations via ZFN-induced DSBs within a protein-encoding region. This function can be disabled by de-selecting the *Exon/Intron Case Sensitivity* check box immediately below the *Sequence* input box.

The engineering method chosen by the user determines which sequences can be targeted. For modular assembly, users can choose one or more of the three available module sets by selecting the *Barbas*, *ToolGen* and *Sangamo* check boxes at the top of the page (21,23,26,27,36). Several studies have generated functional zinc fingers by combining modules from different sets (21). For OPEN, users indicate which pools they wish to use by choosing the corresponding target DNA triplet for each finger position in a three-finger array. All published OPEN pools currently available from the Zinc Finger Consortium are checked by default (7).

Users can specify the number of ZF modules to include in the *Left Array* and *Right Array* using dropdown menus below the *Sequence* input box. ZiFiT restricts the number of modules in concordance with the available reagents. OPEN reagents and Sangamo modules are specific to three-finger ZFPs or ZFNs, whereas practitioners of modular assembly implementing only Barbas and Toolgen modules may scan for individual target sites for ZFPs consisting of three to eight fingers, or for dimeric target sites for ZFNs consisting of three or four fingers for

ZiFiT Version 3.2
IOWA STATE UNIVERSITY

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Sequence:

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>IGF1R FASTA sample input

TTCCTTCCATTCCCTTGGACGTTCTTTTCAGCATCGAACTCCTCTTCTCAGTTAATCGTGAAGTGGAACCCCTCCCTCTCTG
CCCAACGGCAACCTGAGTTACTACATTGTGCGCTGGCAGCGGCAGCCTCAGGACGGCTACCTTTACCGGCACAATTACTG
CTCCAAAGgtaagggtgcagcagcggcctggacggaggggtgtgaccgttcattcctgtggttgaatgtgacctgagccct
aatattacagctatcagacaacagtgtagttctccattggaaaccagctatcctctggtttttttttttttttttttttgaga
cagagtctcactctgttggccaggtggagtgacgtggcagcatcatggctcactgcaattctgcctcccaggttcaag
catttctcattccccagcattccagtagctgggattacaggcatgcaccaccatgccagctaattttttgtatttttag
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ttttccttacaagcatgtataacggctttcattcccactctgttttggcttttctttccgagaagACAAAATCCCCA
TCAGGAAGTATGCCGACGGCACCATCGACATTGAGGAGGTCACAGAGAACCCCAAGACTGAGGTGTGTGGTGGGGAGAAA
GGGCCTTGCTGCGCTGCCCAAAACTGAAGCCGAGAAGCAGGCCGAGAAGGAGGAGGCTGAATACCGCAAAGTCTTTGA

```

Left Array
Spacer
Right Array
 Exon/Intron

Position 1

<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GGG	GGA	GGT	GGC	GAG	GAA	GAT
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GAC	GTG	GTA	GTT	GTC	GCG	GCA
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
GCT	GCC	AGG	AGA	AGT	AGC	AAG
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
AAA	AAT	AAC	ATG	ATA	ATT	ATC
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
ACG	ACA	ACT	ACC	TGG	TGA	TGT
<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
TGC	TAG	TAA	TAT	TAC	TTG	TTA
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Position 2

<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GGG	GGA	GGT	GGC	GAG	GAA	GAT
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GAC	GTG	GTA	GTT	GTC	GCG	GCA
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
GCT	GCC	AGG	AGA	AGT	AGC	AAG
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
AAA	AAT	AAC	ATG	ATA	ATT	ATC
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
ACG	ACA	ACT	ACC	TGG	TGA	TGT
<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
TGC	TAG	TAA	TAT	TAC	TTG	TTA
<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Position 3

<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GGG	GGA	GGT	GGC	GAG	GAA	GAT
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
GAC	GTG	GTA	GTT	GTC	GCG	GCA
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
GCT	GCC	AGG	AGA	AGT	AGC	AAG
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
AAA	AAT	AAC	ATG	ATA	ATT	ATC
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
ACG	ACA	ACT	ACC	TGG	TGA	TGT
<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
TGC	TAG	TAA	TAT	TAC	TTG	TTA
<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Figure 1. ZiFiT Input Window. In this example the user has chosen to scan for ZFN sites using OPEN. The sequence has been entered in FASTA format, with exon sequences in uppercase and intron sequences in lowercase. Published sets of OPEN finger pools available through the Zinc Finger Consortium are checked by default.

each target 'half-site'. Users should note that although increasing the number of modules might be expected to confer enhanced specificity, this is not always the case because deformation of the DNA upon ZFP binding may limit the number of ZF domains that bind concurrently (37). In addition, because three domains are often sufficient to bind DNA with high affinity, longer ZFP arrays may require disrupted linkers between domains to prevent binding at unintended sub-sequences within the target site (24).

For ZFN targets, users must also specify the length of the spacer region between the binding sites for the left and right ZF arrays using the *Spacer* dropdown menu immediately below the *Sequence* input box. An active dimeric ZFN cleaves within the spacer region and its preferred length is dependent on the sequence and length of the amino-acid linker between the ZF and nuclease

domains. Zinc Finger Consortium vectors harbor a linker that works with spacers of 5 or 6 bp of DNA (38–40). An additional linker permitting spacers with 6 or 7 bp of DNA has also been identified (40). By default, ZiFiT scans for ZFN targets with spacers of 5, 6, or 7 bp. Users can choose to limit the scan to only one or two spacer lengths.

Advanced search options are accessible by selecting the *Advanced* link in the lower right hand corner (this link then toggles to *Basic*, which hides the Advanced options). Advanced options allow users to customize their scan by adjusting additional constraints. For example, users can restrict the minimum and maximum number of GNN, ANN, CNN and TNN triplets for reported targets. This feature can help users identify the best sites by restricting searches to more successful GNN-rich sites (13,30). Additional Advanced options

available for a subset of the input interfaces include: (i) *Ignore Asp overlap*: This option is available to users of modular assembly; it refers to ‘target site overlap’ in which an Asp in position +2 of the recognition helix specifies a fourth base (41). This option is useful for troubleshooting why ZiFiT fails to return an expected target site; its use during design should be restricted to advanced users. (ii) *Search both strands*: Because ZFPs bind directionally in a 5′–3′ manner, they can be

engineered to bind either strand of DNA. ZiFiT searches both strands by default. Additional guidance for using advanced options is provided on the ZiFiT Instructions and FAQ pages.

Program output

ZiFiT scans the user-supplied input DNA sequence for potential ZFP binding or ZFN cleavage sites based on

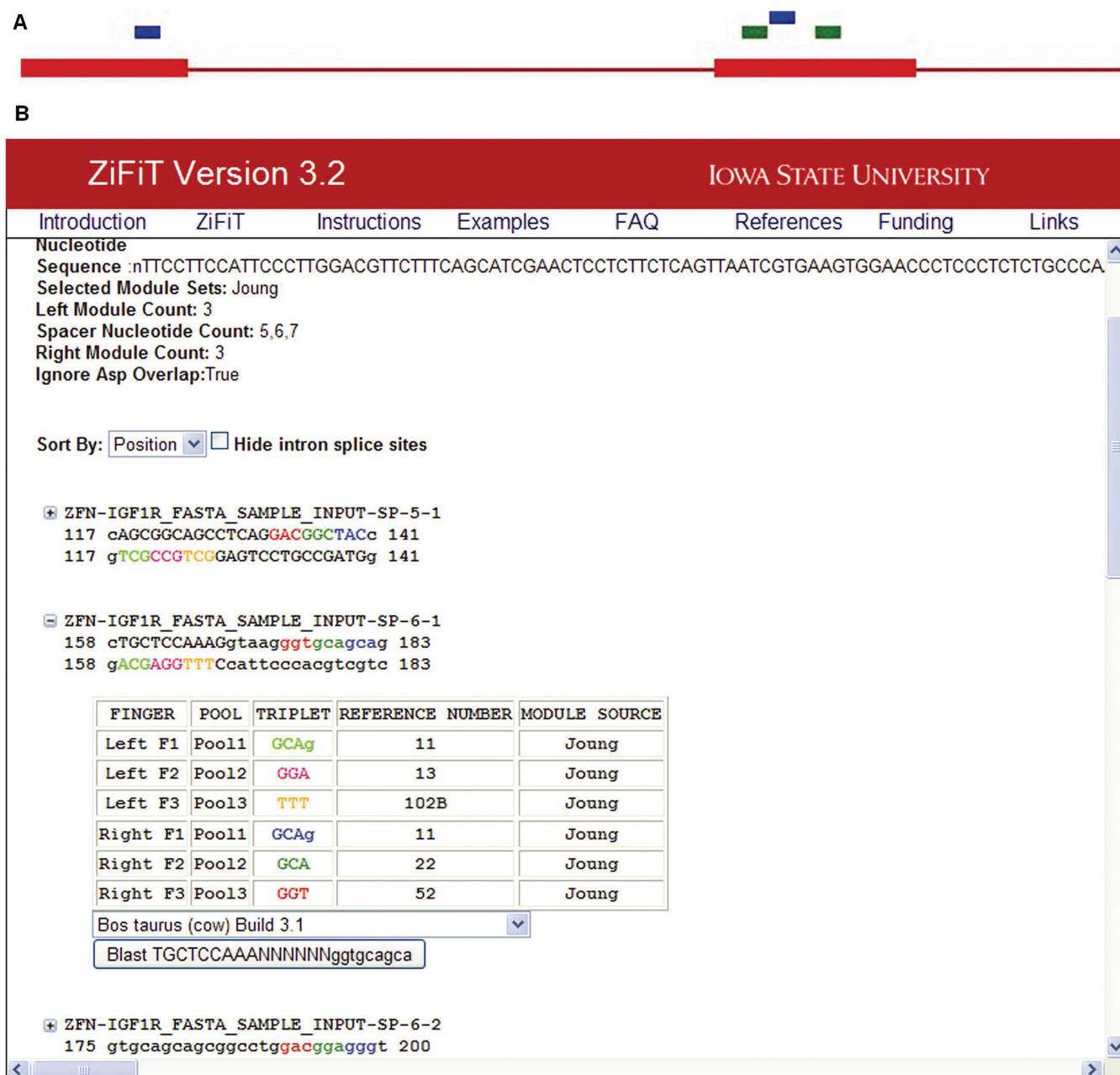


Figure 2. ZiFiT Output Windows. (A) The Graphic Summary window (top) displays potential ZFN-binding sites (‘hits’) identified in the input sequence. Exons (denoted by uppercase characters in the input sequence) are displayed as thick red bars and introns as thin red lines. Hits are represented by short colored bars above the input sequence track; these serve as bookmarks that are linked to individual target sites in the Detailed Target List. ZFN hits are color-coded based on spacer size (5 bp = Blue; 6 bp = Green; 7 bp = Gold). (B) The Detailed Target List window (bottom) provides in-depth information about each hit. Hits are presented as double-stranded DNA sequences and labeled according to the FASTA description, spacer size, and index. Each hit can be expanded to reveal a reagent list for generating the corresponding ZFP. Hits can be sorted according to various criteria as detailed in the text.

the selected engineering method and any additional user-defined restrictions. For each user submission, ZiFiT displays a graphic map of the submitted sequence with each target site ('hit') indicated above the sequence (Figure 2A). (Users may need to 'enable pop-ups' in their browser for this feature to function properly.) The submitted sequence is displayed as a red bar at the bottom of the map. When an *Exon/Intron Case-Sensitive* search is performed (see Program Input), exons are represented by thick red bars and introns by thin red lines. Hits are represented as short colored bars above the sequence track, with overlapping hits overflowing vertically into auxiliary tracks. Each bar is a clickable link to detailed target information on the main output page. For ZFN scans, hits are color-coded according to the length of the spacer.

The main output page opens with a summary of the search parameters, followed by a dropdown *Sort By* menu that can be used to sort individual hits based on position or score when available (see 'Materials and Methods' section). Because ZFN targets consist of two ZFP target sites, both of which must be targeted successfully, score-based sorting considers the score of the inferior scoring array site before the better scoring array target. When users implement an 'Exon/intron case-sensitive' scan for ZFN targets, a *Filter intronic splice sites* checkbox is present immediately to the right of the *Sort By* menu. Selecting this box will hide ZFN targets whose spacers occur within (or overlap) an intron (Figure 2B). In addition to its web-based output, ZiFiT also provides a text version of the output which can be downloaded as a .csv file from the top of the output page.

Each hit is named using the description/comment line of the submitted FASTA sequence and an index number. If no sequence name is supplied by the user, this parameter is set to 'Unknown'. Names for ZFN targets also include the spacer length. For example, for a submission with the FASTA description '>ZFN-SAMPLE,' the third ZFN target site with a spacer of 7bp would be labeled 'ZFN-SAMPLE-SP-7-3'. Immediately beneath each hit name, the double-stranded DNA target sequence is displayed, along with its position within the submitted sequence. Individual triplets within this sequence are highlighted with distinct colors denoting the targets of individual ZF domains. Each highlighted ZFP is linked to ZiFDB, a database of engineered ZFPs. Clicking these links automatically queries ZiFDB for available information regarding ZFPs tested against the same or similar target DNA sequences (33). If an expected functional activity score is available for a given ZFP, the score is presented on the same line as the target. ZiFiT currently provides two validated functional scoring schemes for modular assembly targets (30,35). Scoring schemes for OPEN targets are under development.

Individual ZF targets can be expanded using the '+' to the left of the target name. Expanding a target reveals three types of information. (i) A table describing reagents that can be used to generate corresponding ZFPs. Each row in the table describes a reagent (pool or module) corresponding to a *Triplet* DNA sequence, which

is color-coded according to its position in the double-stranded sequence above the table. Entries in the *Reference Number* column of the table signify the names of reagents (either modules or pools) that are currently available to the academic research community through the Zinc Finger Consortium. Modules (and other reagents for performing modular assembly) are available from the non-profit plasmid distribution service Addgene (<http://www.addgene.org/zfc>) (42). OPEN pools are available by request from the Joung lab (jjoung@partners.org) and other reagents for practicing OPEN are also available from Addgene (<http://www.addgene.org/zfc>). (ii) Sequences of oligonucleotides that must be synthesized to create bacterial two-hybrid selection and/or reporter strains needed to screen or select ZFPs for DNA-binding activity (34,42). (iii) An *Organism* dropdown menu for selecting a host genome and BLAST button that can be used to scan the selected host organism genome for exact and similar target matches. The BLAST button submits search parameters to NCBI and via a popup directs users to NCBI website where they can initiate the query by selecting the 'view report' button. This is useful because it is generally desirable to avoid targeting sites that occur frequently in a genome (e.g. sites that fall within repeat regions). When using BLAST to search for genomic ZFN targets, the spacer is replaced with N's to prevent it from positively influencing a scan. Due to the nature of the algorithm (and a fixed spacer size in the case of a nuclease), this query is not guaranteed to identify all similar sites. ZiFiT output may need to complete loading before BLAST queries are accessible. ZiFiT is freely available on the web without registration at <http://bindr.gdcb.iastate.edu/ZiFiT/>.

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REFERENCES

1. Carroll, D. (2008) Progress and prospects: zinc-finger nucleases as gene therapy agents. *Gene Ther.*, **15**, 1463–1468.

2. Cathomen,T. and Joung,J.K. (2008) Zinc-finger nucleases: the next generation emerges. *Mol. Ther.*, **16**, 1200–1207.
3. Kim,Y.G., Cha,J. and Chandrasegaran,S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl Acad. Sci. USA*, **93**, 1156–1160.
4. Bibikova,M., Beumer,K., Trautman,J.K. and Carroll,D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science*, **300**, 764.
5. Hockemeyer,D., Soldner,F., Beard,C., Gao,Q., Mitalipova,M., DeKelver,R.C., Katibah,G.E., Amora,R., Boydston,E.A., Zeitler,B. *et al.* (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat. Biotechnol.*, **27**, 851–857.
6. Lombardo,A., Genovese,P., Beausejour,C.M., Colleoni,S., Lee,Y.L., Kim,K.A., Ando,D., Urnov,F.D., Galli,C., Gregory,P.D. *et al.* (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol.*, **25**, 1298–1306.
7. Maeder,M.L., Thibodeau-Beganny,S., Osiak,A., Wright,D.A., Anthony,R.M., Eichinger,M., Jiang,T., Foley,J.E., Winfrey,R.J., Townsend,J.A. *et al.* (2008) Rapid “open-source” engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell*, **31**, 294–301.
8. Shukla,V.K., Doyon,Y., Miller,J.C., DeKelver,R.C., Moehle,E.A., Worden,S.E., Mitchell,J.C., Arnold,N.L., Gopalan,S., Meng,X. *et al.* (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature*, **459**, 437–441.
9. Townsend,J.A., Wright,D.A., Winfrey,R.J., Fu,F., Maeder,M.L., Joung,J.K. and Voytas,D.F. (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*, **459**, 442–445.
10. Urnov,F.D., Miller,J.C., Lee,Y.L., Beausejour,C.M., Rock,J.M., Augustus,S., Jamieson,A.C., Porteus,M.H., Gregory,P.D. and Holmes,M.C. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, **435**, 646–651.
11. Zou,J., Maeder,M.L., Mali,P., Pruett-Miller,S.M., Thibodeau-Beganny,S., Chou,B.K., Chen,G., Ye,Z., Park,I.H., Daley,G.Q. *et al.* (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell. Stem Cell*, **5**, 97–110.
12. Bibikova,M., Golic,M., Golic,K.G. and Carroll,D. (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics*, **161**, 1169–1175.
13. Perez,E.E., Wang,J., Miller,J.C., Jouvenot,Y., Kim,K.A., Liu,O., Wang,N., Lee,G., Bartsevich,V.V., Lee,Y.L. *et al.* (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 808–816.
14. Meng,X., Noyes,M.B., Zhu,L.J., Lawson,N.D. and Wolfe,S.A. (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 695–701.
15. Doyon,Y., McCammon,J.M., Miller,J.C., Faraji,F., Ngo,C., Katibah,G.E., Amora,R., Hocking,T.D., Zhang,L., Rebar,E.J. *et al.* (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 702–708.
16. Foley,J.E., Yeh,J.R., Maeder,M.L., Reyon,D., Sander,J.D., Peterson,R.T. and Joung,J.K. (2009) Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool Engineering (OPEN). *PLoS ONE*, **4**, e4348.
17. Lee,H.J., Kim,E. and Kim,J.S. (2010) Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res*, **20**, 81–89.
18. Pavletich,N.P. and Pabo,C.O. (1991) Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science*, **252**, 809–817.
19. Carroll,D., Morton,J.J., Beumer,K.J. and Segal,D.J. (2006) Design, construction and in vitro testing of zinc finger nucleases. *Nat. Protoc.*, **1**, 1329–1341.
20. Hurt,J.A., Thibodeau,S.A., Hirsh,A.S., Pabo,C.O. and Joung,J.K. (2003) Highly specific zinc finger proteins obtained by directed domain shuffling and cell-based selection. *Proc. Natl Acad. Sci. USA*, **100**, 12271–12276.
21. Bae,K.H., Kwon,Y.D., Shin,H.C., Hwang,M.S., Ryu,E.H., Park,K.S., Yang,H.Y., Lee,D.K., Lee,Y., Park,J. *et al.* (2003) Human zinc fingers as building blocks in the construction of artificial transcription factors. *Nat. Biotechnol.*, **21**, 275–280.
22. Sera,T. and Uranga,C. (2002) Rational design of artificial zinc-finger proteins using a nondegenerate recognition code table. *Biochemistry*, **41**, 7074–7081.
23. Liu,Q., Xia,Z., Zhong,X. and Case,C.C. (2002) Validated zinc finger protein designs for all 16 GNN DNA triplet targets. *J. Biol. Chem.*, **277**, 3850–3856.
24. Moore,M., Klug,A. and Choo,Y. (2001) Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units. *Proc. Natl Acad. Sci. USA*, **98**, 1437–1441.
25. Isalan,M., Klug,A. and Choo,Y. (2001) A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter. *Nat. Biotechnol.*, **19**, 656–660.
26. Dreier,B., Beerli,R.R., Segal,D.J., Flippin,J.D. and Barbas,C.F.3rd (2001) Development of zinc finger domains for recognition of the 5'-ANN-3' family of DNA sequences and their use in the construction of artificial transcription factors. *J. Biol. Chem.*, **276**, 29466–29478.
27. Segal,D.J., Dreier,B., Beerli,R.R. and Barbas,C.F.3rd (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5'-GNN-3' DNA target sequences. *Proc. Natl Acad. Sci. USA*, **96**, 2758–2763.
28. Greisman,H.A. and Pabo,C.O. (1997) A general strategy for selecting high-affinity zinc finger proteins for diverse DNA target sites. *Science*, **275**, 657–661.
29. Wu,H., Yang,W.P. and Barbas,C.F. 3rd (1995) Building zinc fingers by selection: toward a therapeutic application. *Proc. Natl Acad. Sci. USA*, **92**, 344–348.
30. Ramirez,C.L., Foley,J.E., Wright,D.A., Muller-Lerch,F., Rahman,S.H., Cornu,T.I., Winfrey,R.J., Sander,J.D., Fu,F., Townsend,J.A. *et al.* (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat. Methods*, **5**, 374–375.
31. Kim,H.J., Lee,H.J., Kim,H., Cho,S.W. and Kim,J.S. (2009) Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.*, **19**, 1279–1288.
32. Joung,J.K., Voytas,D.F. and Cathomen,T. (2010) Reply to “Genome editing with modularly assembled zinc-finger nucleases”. *Nat. Methods*, **7**, 91–92.
33. Fu,F., Sander,J.D., Maeder,M., Thibodeau-Beganny,S., Joung,J.K., Dobbs,D., Miller,L. and Voytas,D.F. (2009) Zinc Finger Database (ZiFDB): a repository for information on C2H2 zinc fingers and engineered zinc-finger arrays. *Nucleic Acids Res.*, **37**, D279–D283.
34. Maeder,M.L., Thibodeau-Beganny,S., Sander,J.D., Voytas,D.F. and Joung,J.K. (2009) Oligomerized pool engineering (OPEN): an ‘open-source’ protocol for making customized zinc-finger arrays. *Nat. Protoc.*, **4**, 1471–1501.
35. Sander,J.D., Zaback,P., Joung,J.K., Voytas,D.F. and Dobbs,D. (2009) An affinity-based scoring scheme for predicting DNA-binding activities of modularly assembled zinc-finger proteins. *Nucleic Acids Res.*, **37**, 506–515.
36. Dreier,B., Fuller,R.P., Segal,D.J., Lund,C.V., Blancafort,P., Huber,A., Koksche,B. and Barbas,C.F. 3rd (2005) Development of zinc finger domains for recognition of the 5'-CNN-3' family DNA sequences and their use in the construction of artificial transcription factors. *J. Biol. Chem.*, **280**, 35588–35597.
37. Peisach,E. and Pabo,C.O. (2003) Constraints for zinc finger linker design as inferred from X-ray crystal structure of tandem Zif268-DNA complexes. *J. Mol. Biol.*, **330**, 1–7.
38. Smith,J., Bibikova,M., Whitby,F.G., Reddy,A.R., Chandrasegaran,S. and Carroll,D. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.*, **28**, 3361–3369.
39. Bibikova,M., Carroll,D., Segal,D.J., Trautman,J.K., Smith,J., Kim,Y.G. and Chandrasegaran,S. (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol. Cell. Biol.*, **21**, 289–297.
40. Handel,E.M., Alwin,S. and Cathomen,T. (2009) Expanding or restricting the target site repertoire of zinc-finger nucleases: the

- inter-domain linker as a major determinant of target site selectivity. *Mol. Ther.*, **17**, 104–111.
41. Elrod-Erickson, M., Benson, T.E. and Pabo, C.O. (1998) High-resolution structures of variant Zif268-DNA complexes: implications for understanding zinc finger-DNA recognition. *Structure*, **6**, 451–464.
42. Wright, D.A., Thibodeau-Beganny, S., Sander, J.D., Winfrey, R.J., Hirsh, A.S., Eichinger, M., Fu, F., Porteus, M.H., Dobbs, D., Voytas, D.F. *et al.* (2006) Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. *Nat. Protoc.*, **1**, 1637–1652.