

Genome Engineering with Custom Recombinases

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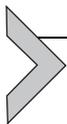
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Abstract

Site-specific recombinases are valuable tools for myriad basic research and genome engineering applications. In particular, hybrid recombinases consisting of catalytic domains from the resolvase/invertase family of serine recombinases fused to Cys₂-His₂ zinc-finger or TAL effector DNA-binding domains are capable of introducing targeted modifications into mammalian cells. Due to their inherent modularity, new recombinases with distinct targeting specificities can readily be generated and utilized in a "plug-and-play" manner. In this protocol, we provide detailed, step-by-step instructions for generating new hybrid recombinases with user-defined specificity, as well as methods for achieving site-specific integration into targeted genomic loci using these systems.



1. INTRODUCTION

Hybrid recombinases composed of catalytic domains derived from the resolvase/invertase family of serine recombinases fused to engineered

Cys₂-His₂ zinc-finger (Akopian, He, Boocock, & Stark, 2003; Gordley, Smith, Graslund, & Barbas, 2007) or TAL effector DNA-binding domains (Mercer, Gaj, Fuller, & Barbas, 2012) are powerful tools for targeted genome engineering (Fig. 4.1A). Unlike classical site-specific recombination systems, such as Cre-*loxP*, Flp-*FRT*, and phiC31-*att*, hybrid recombinases are modular chimeric proteins that are capable of introducing genomic modifications at user-defined sites in human cells (Gaj, Sirk, & Barbas, 2014). Depending on the length of the custom DNA-binding domain, these enzymes can recognize target sites between 44- and 62-bp in length. In general, each target site consists of a central 20-bp core sequence recognized by the recombinase catalytic domain, flanked by two inverted

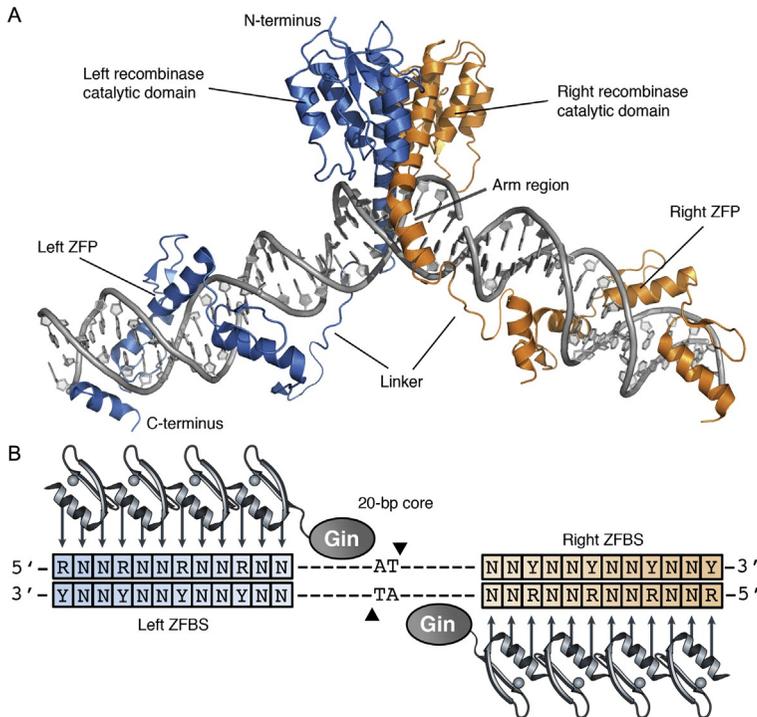
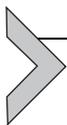


Figure 4.1 Structure of the zinc-finger recombinase (ZFR) dimer bound to DNA. (A) Each ZFR monomer (blue or orange) consists of an activated serine recombinase catalytic domain fused to a custom-designed Cys₂-His₂ zinc-finger DNA-binding domain. (B) Cartoon of the ZFR dimer bound to DNA. ZFR target sites consist of two inverted zinc-finger binding sites flanking a central 20-bp core sequence recognized by the serine recombinase catalytic domain. Abbreviations are as follows: N = A, T, C, or G; R = G or A; and Y = C or T. Adapted from Gaj, Mercer, et al. (2013).

zinc-finger or TAL effector binding sites (Fig. 4.1B). These enzymes catalyze recombination via a concerted mechanism in which the recombinase catalytic domain cleaves all four DNA strands before ultimately promoting strand exchange and religation (Grindley, Whiteson, & Rice, 2006). For both zinc-finger and TAL effector recombinase platforms, enzyme specificity is the cooperative product of modular, site-specific DNA recognition, and sequence-dependent catalysis (Gordley, Gersbach, & Barbas, 2009). As such, advances in the design and synthesis of modular zinc-finger (Gersbach, Gaj, & Barbas, 2014) and TAL effector DNA-binding proteins (Joung & Sander, 2013) have enabled the possibility of generating new custom recombinases capable of recognizing a wide range of DNA sequences. In addition, new recombinase variants with distinct catalytic specificities can readily be generated via a “plug-and-play” manner using a collection of preselected recombinase catalytic domains derived from an activated mutant of the DNA invertase Gin from bacteriophage Mu (Gaj, Mercer, Gersbach, Gordley, & Barbas, 2011; Gaj, Mercer, Sirk, Smith, & Barbas, 2013; Gersbach, Gaj, Gordley, & Barbas, 2010; Klippel, Cloppenborg, & Kahmann, 1988; Proudfoot, McPherson, Kolb, & Stark, 2011). These catalytic domain variants, referred to here as Gin α , β , γ , δ , ϵ , and ζ , were generated by targeted saturation mutagenesis of the Gin recombinase C-terminal arm, a region of the enzyme that connects the catalytic and DNA-binding domains, and also contacts substrate DNA. These redesigned catalytic domains demonstrate high specificity for their intended DNA targets and can be used in a “mix and match” approach to recognize highly diverse DNA sequences. Indeed, our laboratory and others have demonstrated that hybrid recombinases composed of these reengineered catalytic domains are capable of targeted integration of therapeutic factors into endogenous genomic loci at specificities of >80% (Gaj, Sirk, Tingle, et al., 2014) and excision of transgenes with efficiencies of >15% in human cells (Gordley et al., 2007).

Here, we provide a step-by-step protocol for the design and validation of hybrid recombinases based on zinc-finger protein technology. We include assays for evaluating zinc-finger recombinase (ZFR) activity in mammalian cells and describe how to achieve site-specific integration into endogenous genomic loci using this technology.



2. TARGET IDENTIFICATION

Putative ZFR target sites can be identified using the consensus sequences provided in Fig. 4.2. Engineered ZFRs are fully modular enzymes

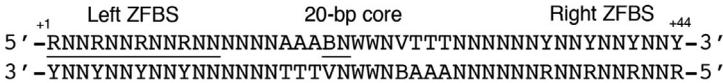
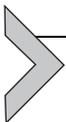


Figure 4.2 The consensus 44-bp target sequence used to identify potential ZFR target sites. Underlined bases indicate zinc-finger DNA-binding sites and core positions 3 and 2. Abbreviations are as follows: N = A, T, C, or G; R = A or G; Y = T or C; B = T, C, or G; V = A, C, or G; and W = A or T.

composed of an N-terminal recombinase catalytic domain fused to a C-terminal zinc-finger DNA-binding protein. Using the collection of preselected recombinase catalytic domains described in Fig. 4.3 and Table 4.1, we estimate that one potential ZFR target site can be identified for every $\sim 160,000$ bp of random sequence. The specificity profile of each reengineered Gin catalytic domain is presented in Fig. 4.3B. Currently, the presence of adenine at core positions 6, 5, and 4 is the only major targeting requirement for ZFRs containing the Gin recombinase catalytic domain. In cases that require recognition of nonadenine bases at these positions, ZFRs derived from the Sin and β recombinase catalytic domains (Sirk, Gaj, Jonsson, Mercer, & Barbas, 2014), which recognize both guanine and thymine at these sites, can be used. In general, 20-bp core sequences that display $>50\%$ sequence identity to the native recombinase target site typically show the highest level of activity. Following the identification of a specific target site, ZFRs with the intended complementary specificity can be generated from a preselected library of modular parts.



3. RECOMBINASE CONSTRUCTION

We have designed vectors to facilitate ZFR assembly and expression: ZFR construction requires the assembly of two zinc-finger protein assays that recognize the DNA sequences flanking the central 20-bp core sequence. We note that our laboratory (Gonzalez et al., 2010) and others (Carroll, Morton, Beumer, & Segal, 2006; Maeder, Thibodeau-Beganny, Sander, Voytas, & Joung, 2009; Wright et al., 2006) have described a number of protocols for zinc-finger assembly, which are described in detail elsewhere.

Gin catalytic domains engineered to recognize $\sim 10^7$ distinct core sequences can be obtained from the SuperZiF-compatible subcloning plasmids pBH-Gin- α , β , γ , δ , ϵ , and ζ (Gaj, Mercer, et al., 2013). These vectors are easily modified for compatibility with zinc-finger proteins constructed by OPEN (Maeder et al., 2008), CoDA (Sander et al., 2011), and the other

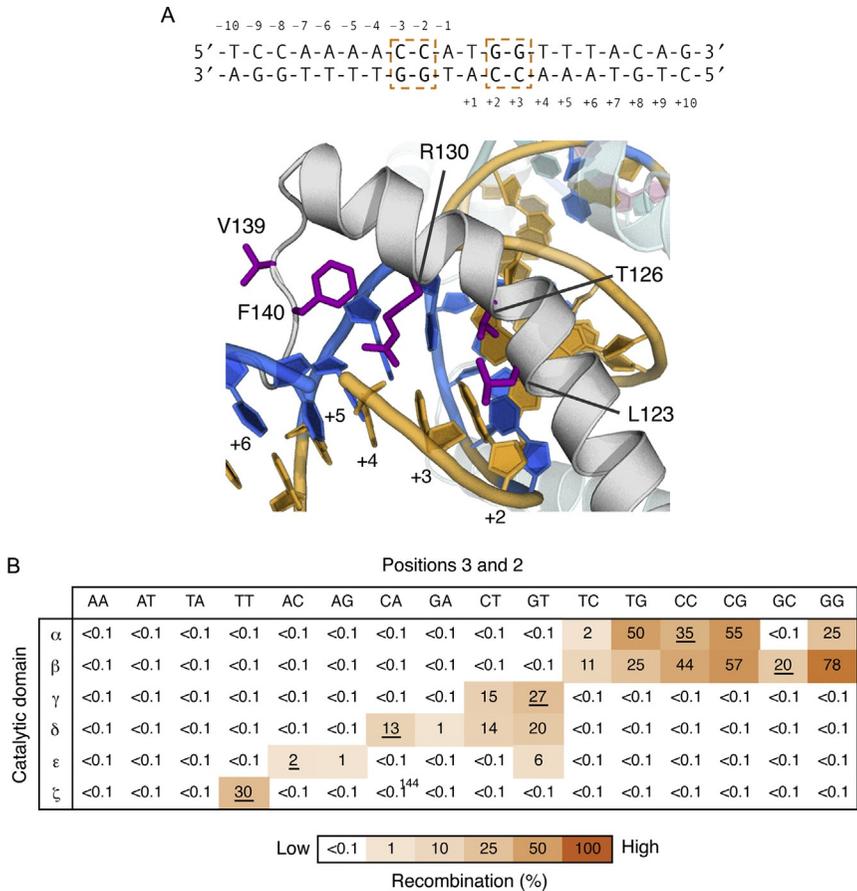


Figure 4.3 Gin catalytic domain specificities. (A, top) The native 20-bp core sequence recognized by the Gin catalytic domain. All base positions within the core site are numbered. Positions 3 and 2 are boxed. (A, bottom) Structure of a serine recombinase catalytic domain in complex with DNA. Residues subject to reprogramming are shown as magenta (black color in the print version) sticks. (B) Recombination specificity of the Gin α , β , γ , δ , ϵ , and ζ catalytic domains for each possible two-base combination at positions 3 and 2. Intended DNA targets are underlined. Adapted from *Gaj, Mercer, et al. (2013)*.

open-source assembly methods (Bhakta et al., 2013), as well as alternative serine recombinase catalytic domains. The following is a protocol for constructing ZFR heterodimers that recognize the user-defined target site identified in Section 2, using SuperZiF-assembled zinc-finger proteins. Importantly, ZFR targeting of asymmetric DNA sequences requires both the presence of “left”- and “right”-ZFR monomers. We note that the following protocol describes the generation of a single ZFR monomer.

Table 4.1 Reengineered Gin catalytic domain substitutions

Catalytic domain	Target	Positions				
		120	123	127	136	137
α	CC ^a	Ile	Thr	Leu	Ile	Gly
β	GC	Ile	Thr	Leu	Arg	Phe
γ	GT	Leu	Val	Ile	Arg	Trp
δ	CA	Ile	Val	Leu	Arg	Phe
ϵ ^b	AC	Leu	Pro	His	Arg	Phe
ζ ^c	TT	Ile	Thr	Arg	Ile	Phe

^aIndicates wild-type DNA target.

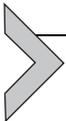
^bThe ϵ catalytic domain also contains the substitutions E117L and L118S.

^cThe ζ catalytic domain also contains the substitutions M124S, R131I, and P141R.

Note: Carbenicillin (i.e., Carb) is a more stable analog of ampicillin and is recommended, but not required, for this protocol.

1. Digest the appropriate subcloning vector (pBH-Gin- α , β , γ , δ , ϵ , or ζ) with the restriction enzymes *AgeI* and *SpeI* in recommended buffer for 3 h using 10 Units (U) of enzyme per 1 μ g of vector DNA. Visualize DNA by agarose gel electrophoresis using a fluorescent intercalating dye, such as ethidium bromide.
2. Purify the digested vector by gel extraction using the QIAquick Gel Extraction Kit, according to the manufacturer's instructions.
3. Release SuperZiF-assembled zinc-finger proteins from pSCV with the restriction enzymes *XmaI* and *SpeI* in appropriate buffer for 3 h using 10 U of enzyme per 1 μ g of DNA and isolate via gel electrophoresis using the QIAquick Gel Extraction Kit.
4. Ligate the purified zinc-finger protein DNA into 25–50 ng of purified pBH-Gin- α , β , γ , δ , ϵ , or ζ vector using 1 U T4 DNA ligase for 1 h at room temperature. For best results, perform the ligation reaction using a 6:1 insert-to-vector ratio.
5. Transform 10–20 ng of ligated pBH-Gin- α , β , γ , δ , ϵ , or ζ into any competent laboratory strain of *Escherichia coli*, such as TOP10 or XL1 Blue, by electroporation and recover in 2 mL SOC for 1 h at 37 °C with shaking at 250 rpm.
6. Spread 100 μ L of recovery culture on an LB agar plate with 100 μ g/mL carbenicillin to determine ligation/transformation efficiency, and for possible use in Step 8. Inoculate remaining \sim 2 mL recovery culture

- for overnight growth by adding 4 mL SB medium and 100 µg/mL carbenicillin. Incubate cultures for 16–24 h at 37 °C with shaking at 250 rpm.
7. The following day, purify plasmid from overnight culture with any commercially available Miniprep kit, according to the manufacturer's instructions.
 8. Release ZFR from miniprepmed pBH by restriction digestion with *SfiI* for 3 h using 10 U of enzyme per 1 µg of DNA and visualize DNA by agarose gel electrophoresis. If unable to recover ZFRs, we recommend performing colony PCR using the plated recovery culture from Step 6 to screen for individual clones containing full-length ZFR gene inserts.
 9. Purify the ZFR insert by gel extraction using the QIAquick Gel Extraction Kit.
 10. Ligate the purified full-length ZFR insert into 25–50 ng of *SfiI*-digested pcDNA 3.1 (Invitrogen). Transform 10–20 ng of the ligation reaction into any competent laboratory strain of *E. coli* and recover in 1–2 mL of SOC. After 1 h, spread 100 µL of cells on LB agar plates containing 100 µg/mL of carbenicillin and incubate overnight at 37 °C.
 11. The following day, inoculate 6 mL of SB medium containing 100 µg/mL of carbenicillin with one colony from the LB agar carb plate and culture overnight at 37 °C with shaking at 250 rpm.
 12. The next day, harvest the overnight culture and purify pcDNA-ZFR plasmid by Miniprep. Confirm ZFR identity by standard DNA sequencing using the primer T7 Universal (5'-TAATACGACTCACTATAGGG-3').



4. MEASUREMENTS OF RECOMBINASE ACTIVITY

Our laboratory has developed a transient reporter assay for use in mammalian cells that links ZFR-mediated recombination to reduced luciferase expression (Gaj, Mercer, et al., 2013; Gaj, Sirk, Tingle, et al., 2014). To achieve this, recombinase target sites are introduced both up- and downstream a Simian vacuolating virus 40 (SV40) promoter that drives expression of a luciferase reporter gene. Active recombinases excise the promoter, resulting in reduced luciferase expression. The following protocol describes the generation and application of luciferase-based reporter vectors, but is also broadly adaptable to other types of reporter genes, including EGFP and β-galactosidase.

4.1. Reporter plasmid construction

1. The SV40 promoter sequence can be amplified from many different sources. In our studies, we used the primers SV40-ZFR-*Bgl*III-Fwd and SV40-ZFR-*Hind*III-Rev to PCR amplify the SV40 promoter from pGL3-Prm (Promega). These primers also encode flanking ZFR target sites.

SV40-ZFR-*Bgl*III-Fwd:

5'-TTAATTAAGAGAGATCTGCTGATGCAGATACAG
AAACCAAGGTTTTCTTACTTGCTG CTGCGCGATCTGC
 ATCTCAATTAGTCAGC-3'

SV40-ZFR-*Hind*III-Rev:

5'-ACTGACCTAGAGAAGCTTGCAGCAGCAAGTAAG
AAAACCTTGTTTTCTGTATCTGCA TCAGCTTTGC
 AAAAGCCTAGGCCTCCAAA-3'

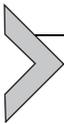
Note: ZFR target sites are underlined. Restriction sites are italicized.

2. Purify the PCR product by gel electrophoresis using the QIAquick Gel Extraction Kit, according to the manufacturer's instructions.
3. Digest the purified PCR product and pGL3-Prm with the restriction enzymes *Bgl*III and *Hind*III and purify both DNA fragments by gel electrophoresis.
4. Ligate the purified SV40-ZFR insert into 25–50 ng of purified pGL3 vector for 1 h at 25 °C, creating pGL3-target.
5. Transform 10–20 ng of ligated plasmid into *E. coli* by electroporation. Recover cells for 1 h in 1–2 mL of SOC, and spread 100 μ L of cells on LB agar plates containing 100 μ g/mL of carbenicillin. Incubate plates overnight at 37 °C.
6. The following day, inoculate 6 mL of SB medium containing 100 μ g/mL of carbenicillin with one colony from the LB agar plate and culture overnight at 37 °C with shaking at 250 rpm.
7. Purify pGL3-target plasmid by Miniprep and confirm reporter plasmid identity by DNA sequencing using SV40-Mid-Prim-Fwd (5'-ACCATAGTCCC GCCCTAACTCC-3') and SV40-Mid-Prim-Rev (5'-GGAGTTAGGGGCGGGACTATGGT-3').

4.2. Luciferase assay

8. Seed human embryonic kidney (HEK) 293 T cells in a 96-well plate at a density of 4×10^4 cells per well in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Gibco).

9. Transfect cells 24 h after seeding. For best results, cells should be 80–90% confluent at time of transfection.
 - a. Use 25–50 ng of each pcDNA-ZFR monomer expression vector, 25 ng of pGL3-target reporter vector, and 1 ng of pRL-CMV (*Renilla* luciferase transfection control; Promega), according to the manufacturer's instructions. We typically transfect each well with 0.8 μ L Lipofectamine 2000 (Invitrogen).
 - b. To accurately assess recombinase activity, include the following samples:
 - i. *Experimental*: reporter construct with ZFR expression vectors.
 - ii. *Background luciferase activity*: reporter plasmid only.
 - iii. *Negative control*: mock transfected cells that receive no reporter plasmid or ZFR expression vectors.
10. Evaluate the fold reduction in luminescence using a Microplate luminometer and dual-luciferase reporter assay 48 h after transfection, normalizing to cotransfected *Renilla* luciferase control.
11. Recombinases that induce a >20-fold decrease in luminescence are considered sufficiently active for endogenous genomic targeting studies. In our experience, recombinases that lead to a >60-fold reduction in luminescence yield the best results for downstream applications.



5. SITE-SPECIFIC INTEGRATION

Site-specific integration is one core application of ZFR technology. We have developed a donor plasmid backbone (pDonor) based on the pBABE vector system (Morgenstern & Land, 1990) that facilitates ZFR-mediated transgene insertion into the human genome (Gaj, Sirk, Tingle, et al., 2014; Gordley et al., 2009). This vector contains a constitutively expressed puromycin-resistance gene for enrichment of ZFR-modified cells, and a multiple-cloning site upstream of the cloned ZFR target site for gene of interest (GOI) placement. The following protocol describes a step-by-step procedure for constructing ZFR donor plasmids and achieving site-specific integration with ZFRs.

5.1. Donor plasmid construction

1. PCR amplify the cDNA sequence of the GOI using primers that encode a 5' *Pst*I and 3' *Bam*H1 restriction site. The 3' primer must also encode the ZFR target site selected in Section 2 upstream of the *Bam*H1 restriction site. *Note*: pDonor does not contain a universal promoter or polyA

region, and therefore these components should be amplified with the GOI.

2. Gel purify the PCR product using QIAquick Gel Extraction Kit, according to the manufacturer's instructions.
3. Digest both the purified PCR product and pDonor (empty) with the restriction enzymes *Pst*I and *Bam*H1. Purify both DNA fragments by gel electrophoresis.
4. Ligate the purified GOI insert into 25–50 ng of purified pDonor vector for 1 h at room temperature.
5. Transform 10–20 ng of ligated plasmid into any competent laboratory strain of *E. coli* by electroporation. Recover cells for 1 h in 1–2 mL of SOC, and spread 100 μ L of cells on LB agar plates containing 100 μ g/mL of carbenicillin. Incubate plates overnight at 37 °C.
6. Purify plasmid DNA by Miniprep and confirm pDonor plasmid identity by DNA sequencing.

5.2. Cell culture methods

7. Seed HEK293 cells in a 24-well plate at a density of 2×10^5 cells per well in DMEM containing 10% FBS. *Note:* we test the ability of our recombinases to integrate donor plasmid in HEK293 cells; however, these enzymes should demonstrate high activity in most cell types.
8. At 24 h after seeding, transfect cells with 80 ng of pDonor plasmid, 10 ng each of pcDNA-ZFR-L and pcDNA-ZFR-R expression vectors and (optionally) 10 ng of pCMV-EGFP for transfection control using any desired transfection protocol. We note that the efficiency of ZFR-mediated integration is dependent on the amount of plasmid transfected and thus may require subsequent optimization, e.g., less specific recombinases may require up to 100 ng of each L- and R-ZFR plasmids.
9. At 72 h after transfection, perform the following evaluations and expand clonal populations.

5.2.1 PCR confirmation of integration

- i. At 72 h after transfection, harvest cells, and isolate bulk genomic DNA using Quick Extract DNA Extraction Reagent (Epicentre), according to manufacturer's instructions.
- ii. Design primers complementary to the 5' and 3' junctions of the genomic region targeted and PCR amplify this region by nested PCR. As an

internal control, we recommend PCR amplifying the GAPDH gene from all harvested cell types.

Note: ZFR-mediated integration can occur in both the forward and reverse orientation. We thus recommend designing internal nested primers for detecting both the forward and reverse integration of the GOI.

- iii. Gel purify the PCR product and confirm the identity of the donor vector by DNA sequencing.

5.2.2 Measurements of modification efficiency

- iv. At 72 h after transfection, split cells into 6-well plates at a density of 1×10^4 cells per well and maintain in DMEM/FBS with or without 2 $\mu\text{g}/\text{mL}$ puromycin.
- v. At 14–18 days after seeding, stains cells with 0.2% crystal violet staining solution and calculate genome-wide integration rates by dividing the number of colonies in puromycin-containing media by the number of colonies in the absence of puromycin.

5.2.3 Isolation and expansion of modified clones

- vi. At 72 h after transfection, split 1×10^4 cells into a 100 mm dish and maintain cells in DMEM/FBS with 2 $\mu\text{g}/\text{mL}$ puromycin. Individual colonies can be isolated with 10 mm \times 10 mm open-ended cloning cylinders with sterile silicone grease and expanded in 96-well plates in the presence of puromycin. Alternatively, modified cells can be isolated and expanded by harvesting 72 h after transfection and reseeded in 96-well plates at limiting dilution with 2 $\mu\text{g}/\text{mL}$ puromycin. Sample gel illustrating positive integration events among expanded clones in the forward and reverse directions are shown in Fig. 4.4.

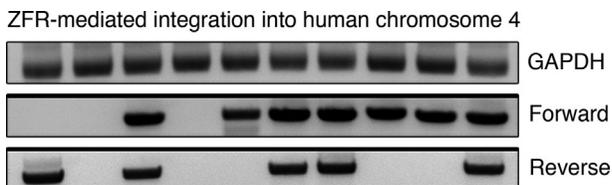
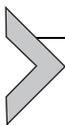


Figure 4.4 ZFR-mediated integration into the human genome. Example clonal analysis of puromycin-resistant cells transfected with a pair of ZFR expression vectors (left and right) and pDonor plasmid.



6. CONCLUSIONS

The protocol provided here details the generation of engineered ZFRs capable of recognizing user-defined sites. The recombinases described here have the capacity to catalyze a diverse range of genome modification outcomes, including targeted gene integration, excision, and cassette exchange. These chimeric enzymes can also be used in tandem with other genome engineering technologies, including site-specific nucleases (Gaj, Gersbach, et al., 2013) and transposases (Gersbach, Gaj, Gordley, Mercer, & Barbas, 2011), for even more diverse editing outcomes. While this protocol does not include guidance for the generation of recently described custom TAL effector recombinases (Mercer et al., 2012), it should be easily adapted for the creation of any type of hybrid recombinases.

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