

Chapter 2

Targeted Porcine Genome Engineering with TALENs

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2.1 Introduction

The domestic pig, as an economically and biologically important animal in agricultural, pharmacological, and biomedical applications, is well known for its difficulty in targeted genome engineering. This is mostly due to a lack of established authentic embryonic stem cells and the relatively low efficiency of targeted engineering by homologous recombination (HR). The first targeted gene (alpha-1,3-galactosyltransferase) knockout (KO) pig was generated by conventional gene targeting (vector-mediated HR) and nuclear transfer one decade ago (Lai et al. 2002). However, the conventional targeting method is inefficient and laborious. Another method, based on engineered recombinant adeno-associated virus (rAAV) vector, has greatly improved the targeting efficiency and has been applied to produce several genetically modified (GM) pig models for, e.g., cystic fibrosis (Rogers et al. 2008), breast cancer (Luo et al. 2011b), and hereditary tyrosinemia type 1 (Hickey et al. 2011). Nevertheless, the broad use of rAAV for generating targeted GM pig models for biomedical research is still hampered by its relatively low targeting frequency, although ~1000-fold higher than the conventional targeting method, along with its high rate of random insertion and laboratory requirement such as a biosafety level 2 laboratory.

During the last few years, DNA nuclease technologies, including, e.g., zinc-finger nucleases (ZFNs; Kim et al. 1996) and particularly the transcription activator-like

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effector nucleases (TALENs; Boch et al., 2009; Bogdanove and Voytas, 2011), have emerged as exciting tools for targeted genome editing in a wide range of cell types and organisms. To date, the efficacy of TALEN-mediated genome engineering has been proven in approximately 20 species (Table 2.1), also see review by Joung and Sander (2013). Moreover, the feasibility of creating biallelic KO F0 founders has greatly facilitated the production procedure and reduced the cost for generating GM large animals such as pigs and cows that have longer pregnancy periods than rodents (Hauschild et al. 2011; Yu et al. 2011; Carlson et al. 2012). In this chapter, we focus on describing TALENs and the use of TALENs for targeted genome editing in primary porcine fibroblasts.

As illustrated in Fig. 2.1a, one TALEN comprises three domains: (i) a modular sequence-specific DNA-binding domain (DBD) core, which is engineered from the bacterial TAL effector protein; (ii) a catalytic domain (CD), which is derived from the *FokI* nuclease and confers double-stranded DNA breaks (DSBs) upon dimerization with another TALEN; and (iii) a nuclear localization signal, which homes the fusion proteins to the nucleus (see also review by Joung and Sander (2013)). The nucleotide-binding specificity of the TAL effector protein is determined by a modularly repeated domain, which employs a simple protein–DNA code (Boch et al. 2009; Fig. 2.1b 2.1a), and thus greatly facilitated the generation of synthetic TALENs (Cermak et al. 2011; Li et al. 2011b; Reyon et al. 2012). Several methods have been developed for engineering TALENs based on modular assembling (Li et al. 2011b), ligation-independent cloning (Schmid-Burgk et al. 2013), “Golden Gate” cloning (Cermak et al. 2011), or high-throughput solid-phase assembling (Reyon et al. 2012). Unlike ZFNs, the engineering of TALENs by these methods can be accomplished with general laboratory reagents, limited budget, and time (i.e., within a few days).

Several kinds of genomic alterations, such as mutations, small and/or large deletions and insertions (indels), translocations, and inversions, have been reported in a wide range of cells and organisms using TALENs (Bogdanove and Voytas 2011; Miller et al. 2011; Carlson et al. 2012; Joung and Sander 2013). The principle that DSBs enhance cellular DNA damage repairing machineries by error-prone nonhomologous end joining (NHEJ) or error-free HR has been known for three decades (Szostak et al. 1983). TALEN-mediated genomic manipulation shares the same principle as DSBs. Upon being introduced into targeted cells, two TALENs bind to their recognition sites (TS1 and TS2 as illustrated in Fig. 2.1a 2.1b). Upon dimerization of the *FokI* CD, an active endonuclease is formed which creates DSBs within the spacer region. An efficient spacer is between 12 and 31 bp depending on the choice of TALE architectures (Cermak et al. 2011; Li et al. 2011a; Miller et al. 2011; Mussolino et al. 2011). Random mutations, indels, and insertions will be introduced at the DSB sites by NHEJ, while site-specific mutagenesis, indels, and/or insertion can be introduced in or near the DSB sites by using a donor oligo or template (Fig. 2.1b). More advanced genomic alterations such as deletions of large fragments, translocations, or inversions can be achieved by using multiple pairs of TALENs (Carlson et al. 2012).

Table 2.1 List of TALEN-mediated genome engineering (until July 2013)

| Species | TALEN delivery (cell type) | Genes | Reference |
|--|---|---|---|
| <i>Human (Homo sapiens)</i> | | | |
| Human (<i>Homo sapiens</i>) | Electroporation (myoblasts) | <i>DMD</i> | (Ousterout et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (HEK293) | <i>VKOR</i> | (Tie et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (HEK 293T) | <i>miR-155*</i> , <i>miR-155</i> , <i>miR-146a</i> and <i>miR-125b</i> | (Hu et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (Jurkat and RPE-1 cells) | <i>NPM1</i> and <i>ALK</i> | (Piganeau et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (HEK 293) | <i>Genome wide</i> | (Kim et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (HEK293), electroporation (iPSCs) | <i>HPRT</i> , <i>CFTR</i> , <i>eGFP</i> | (Sakuma et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transduction (HeLa cells) | <i>AAVS1</i> | (Holckers et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (fibroblasts) | <i>15 genes (AKT2, ANGPTL3, APOB, ATGL, C6orf106, CIITA, CELSR2, CFTR, GLUT4, LINC00116, NLRC5, PLIN1, SORT1, TRIB1, TTN)</i> | (Ding et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (hESCs) | | |
| Human (<i>Homo sapiens</i>) | Transfection (iPSCs) | | |
| Human (<i>Homo sapiens</i>) | Ligation-independent cloning (LIC) | <i>Genome wide</i> | (Schmid-Burgk et al. 2013) |
| Human (<i>Homo sapiens</i>) | Transfection (HEK 293T) | <i>NDUFA9</i> | (Stroud et al. 2013) |
| Human (<i>Homo sapiens</i>) | FLASH assembly | <i>Genome wide</i> | (Reyon et al. 2012) |
| Human (<i>Homo sapiens</i>) | Transfection (Hela cells) | <i>HBB</i> | (Sun et al. 2012) |
| Human (<i>Homo sapiens</i>) | Transfection (human ESCs and iPSCs) | <i>PPP1R12C</i> , <i>OCT4</i> , and <i>PITX3</i> | (Hockemeyer et al. 2011) |
| <i>Cow (Bos taurus) and pig (Sus scrofa)</i> | | | |
| Livestock: Cow (<i>Bos taurus</i>) and pig (<i>Sus scrofa</i>) | Transfection (fibroblasts) and microinjection (embryos) | <i>Porcine: Ldlr</i> , <i>DMD</i> , and <i>Ghrhr</i> <i>Bovine: Acan</i> and <i>GDF8</i> , | (Carlson et al. 2012) |
| <i>Rabbit (Oryctolagus cuniculus)</i> | | | |
| Rabbit (<i>Oryctolagus cuniculus</i>) | Microinjection (pronuclear) | <i>Rag1</i> and <i>Rag2</i> | (Song et al. 2013) |

Table 2.1 (continued)

| Species | TALEN delivery (cell type) | Genes | Reference |
|---------------------------------------|--|--|-------------------------|
| <i>Mouse (Mus musculus)</i> | | | |
| Mouse (<i>Mus musculus</i>) | Microinjection (pronuclear or zygote) | <i>Mkl1</i> | (Wu et al. 2013) |
| Mouse (<i>Mus musculus</i>) | Microinjection (zygote) | <i>Lepr, Pak1ip1, Gpr55, Rprm, Fbxo6, Smurf1, Dcaf13, Fam73a, Wdr20a, and Tmem74</i> | (Qiu et al. 2013) |
| Mouse (<i>Mus musculus</i>) | Microinjection (CD1, C3H, and C57BL/6J oocyte) | <i>Zic2</i> | (Davies et al. 2013) |
| Mouse (<i>Mus musculus</i>) | Microinjection (one-cell embryo) | <i>Rab38</i> | (Wefers et al. 2013) |
| <i>Rat (Rattus norvegicus)</i> | | | |
| Rat (<i>Rattus norvegicus</i>) | Electroporation (fibroblasts) and microinjection (zygotes) | <i>Try</i> | (Mashimo et al. 2013) |
| Rat (<i>Rattus norvegicus</i>) | Transfection (ES cells) | <i>Bmpr2</i> | (Tong et al. 2012) |
| Rat (<i>Rattus norvegicus</i>) | Microinjection (1-cell-stage embryos) | <i>IgM</i> | (Tesson et al. 2011) |
| <i>Xenopus</i> | | | |
| Xenopus (<i>Xenopus laevis</i>) | Microinjection (one-cell stage) | <i>Tyr and Pax6</i> | (Suzuki et al. 2013) |
| Xenopus (<i>Xenopus tropicalis</i>) | Microinjection (one-cell stage) | <i>Tyr, noggin, and MMP-9TH</i> | (Nakajima et al. 2013) |
| Xenopus (<i>Xenopus tropicalis</i>) | Microinjection (2-cell-stage blastomere) | <i>Tyr</i> | (Ishibashi et al. 2012) |
| Xenopus (<i>Xenopus laevis</i>) | Microinjection (1-cell-stage embryos) | <i>Noggin, Ptfla/p48, and Ets1</i> | (Lei et al. 2012) |
| Xenopus (<i>Xenopus laevis</i>) | Microinjection (1-cell-stage embryos) | <i>eGFP</i> | (Sakuma et al. 2013) |
| <i>Zebra fish (Danio rerio)</i> | | | |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (one-cell stage) | <i>Sema3fb, dre-mir-126a, dre-mir-126b, Cluster Chr. 1, from dre-mir-17a-1 to dre-mir-92a-1, Cluster Chr. 9, from dre-mir-17a-2 to dre-mir-92a-2</i> | (Xiao et al. 2013) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (one-cell stage) | <i>idh1, flt3, npm1b, jak2a, npm1a,</i> | (Ma et al. 2013) |

Table 2.1 (continued)

| Species | TALEN delivery (cell type) | Genes | Reference |
|--|---|---|----------------------|
| Zebra fish (<i>Danio rerio</i>) | Microinjection (one-cell-stage embryos) | <i>apoea</i> , <i>flt4</i> , <i>lepa</i> , <i>linc-birc6</i> [mega-mind] <i>lincRNA</i> gene and the <i>globin</i> locus control region | (Gupta et al. 2013) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1- to 4-cell-stage embryos) | <i>eGFP</i> | (Sakuma et al. 2013) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>ponzr1</i> , <i>msna</i> , <i>ppp1cab</i> and <i>cdh5</i> | (Bedell et al. 2012) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>golden</i> | (Dahlem et al. 2012) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>elmo1</i> , <i>epas1b</i> , <i>fh</i> , <i>hif1ab</i> , <i>ptpmt1</i> , and <i>scl6a3</i> | (Cade et al. 2012) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>bmi1</i> , <i>ikzf1</i> , <i>phf6</i> , <i>jak3</i> , and <i>myoD</i> | (Moore et al. 2012) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>tnikb</i> | (Huang et al. 2011) |
| Zebra fish (<i>Danio rerio</i>) | Microinjection (1-cell-stage embryos) | <i>gria3a</i> and <i>hey2</i> | (Sander et al. 2011) |
| <i>Fruit fly (Drosophila melanogaster)</i> | | | |
| Fruit fly (<i>Drosophila melanogaster</i>) | Microinjection (embryos) | <i>egfp</i> | (Sakuma et al. 2013) |
| Fruit fly (<i>Drosophila melanogaster</i>) | Microinjection (embryos) | <i>yellow</i> | (Liu et al. 2012) |
| <i>Medaka (Oryzias latipes)</i> | | | |
| Medaka (<i>Oryzias latipes</i>) | Microinjection (fertilized eggs) | <i>DJ-1</i> | (Ansai et al. 2013) |
| <i>Silkworm (Bombyx mori)</i> | | | |
| Silkworm (<i>Bombyx mori</i>) | Microinjection (syncytial-preblastoderm-stage embryo) | <i>BmBlos2</i> | (Sajwan et al. 2013) |
| Silkworm (<i>Bombyx mori</i>) | Microinjection (oviposited embryos ≤ 2 h) | <i>BmBlos2</i> | (Ma et al. 2012) |
| <i>Plants</i> | | | |
| Rice (<i>Oryza sativa</i>) | Polyethylene glycol (protoplast) | <i>OsDEP1</i> (LOC_ Os09g26999), <i>OsBADH2</i> (LOC_ Os08g32870), <i>OsCKX2</i> (LOC_ Os01g10110), and <i>OsSD1</i> (LOC_ Os01g40720) | (Shan et al. 2013) |

Table 2.1 (continued)

| Species | TALEN delivery (cell type) | Genes | Reference |
|---|---|--|---------------------|
| Brachypodium (<i>Brachypodium distachyon</i>) | Polyethylene glycol (protoplast) | <i>BdABA1</i> (<i>Bra-di5g11750</i>), <i>BdCKX2</i> (<i>Bradi2g06030</i>), <i>BdSMC6</i> (<i>Bradi4g08527</i>), <i>BdSPL</i> (<i>Bra-di2g03740</i>), <i>BdSBP</i> (<i>Bra-di4g33770</i>), <i>BdCOI1</i> (<i>Bra-di2g23730</i>), <i>BdRHT</i> (<i>Bra-di1g11090</i>), and <i>BdHTA1</i> (<i>Bradi1g25390</i>) | (Shan et al. 2013) |
| Barley (<i>Hordeum vulgare</i> L.) | Transformation (12–14-day embryos) | <i>PAPhy_a</i> | (Wendt et al. 2013) |
| Tobacco (<i>Nicotiana tabacum</i>) | Transformation (protoplasts) | <i>ALS</i> | (Zhang et al. 2013) |
| <i>Other species</i> | | | |
| Mosquitoes (<i>Aedes aegypti</i>) | Microinjection (1-h-old preblastoderm embryo) | <i>kmo</i> | (Aryan et al. 2013) |
| Nematode (<i>Caenorhabditis elegans</i> and <i>C. briggsae</i>) | Microinjection (1–12 h after the L4 adult molt) | <i>ben-1</i> | (Wood et al. 2011) |
| Yeast (<i>Saccharomyces cerevisiae</i>) | Transformation | <i>URA3</i> , <i>LYS2</i> , and <i>ADE2</i> | (Li et al. 2011b) |

Highly efficient TALEN-mediated gene-specific manipulations have been reported in both primary porcine fibroblasts and porcine zygotes (Carlson et al. 2012). In Carlson et al.'s study, the authors achieved modifications up to 75 % of oocytes injected with TALENs as well as a high frequency of monoallelic and biallelic modifications in selected/nonselected fibroblasts clones (Carlson et al. 2012). In addition, the authors further showed that the TALEN-mediated GM cells are suitable for cloning: Biallelic *Ldlr* modified pigs were generated as a model for atherosclerosis (Carlson et al. 2012). Herein, we briefly describe the protocol for engineering TALENs using the “Golden Gate” assembling method adapted from Cermak et al. (2011) and the delivery of TALENs together with a donor plasmid into primary porcine fibroblasts by the 4D-Nucleofector System (Lonza, Basel, Switzerland). As a detailed protocol for assembling TALENs by Golden Gate cloning was introduced by Cermak et al. in their article (Cermak et al. 2011), we mainly focus on describing important *notes* with respect to the design of TALENs, increase of the Golden Gate cloning efficiency, and the efficient delivery of TALENs with or without donor template.

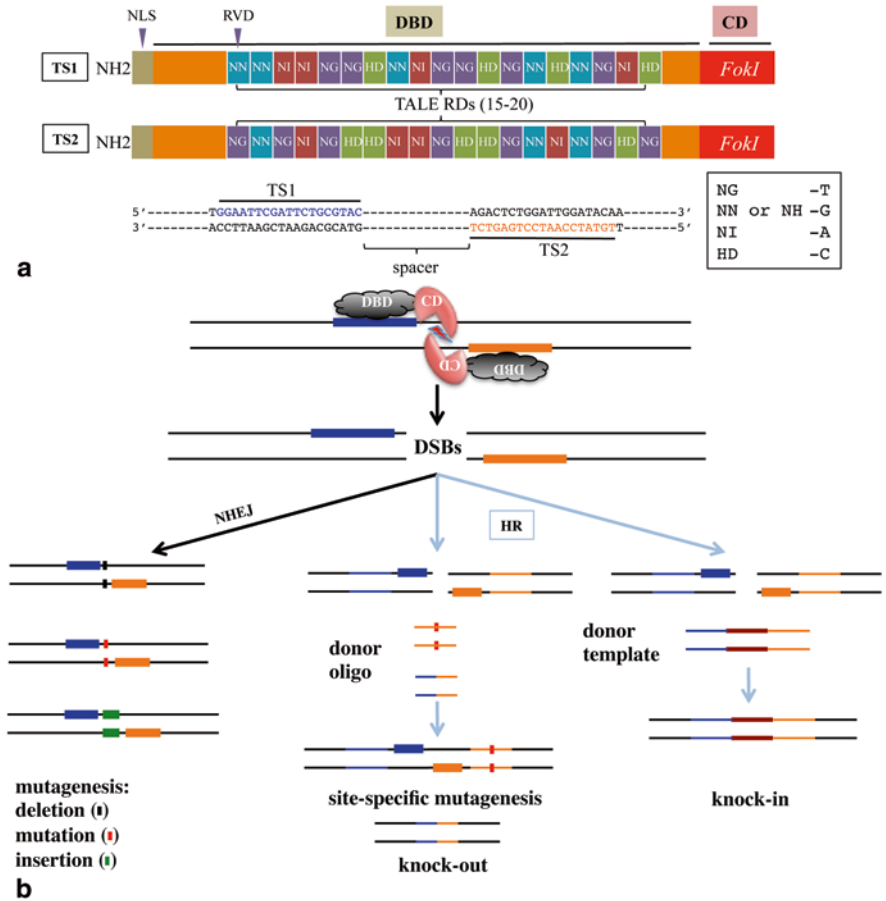


Fig. 2.1 TALEN-mediated genome editing. **a** Schematic diagram of one pair of transcription activator-like effector nuclease (TALEN) that specifically binds to a user-designed genomic locus. One engineered TALEN contains three functional domains: a nuclear localization signal (NLS) at the amino terminus, a truncated transcription activator-like effector (TALE) DNA-binding domain (DBD), and a catalytic domain (CD) consisting of a ubiquitous DNA endonuclease (*FokI*) at the carboxy terminus. The repeat variable di-residues (RVDs), which confer nucleotide specificity, are represented with the two hypervariable residues at position 12–13 (NG, NN or NH, NI, HD). The TALE repeat domain (RD) usually contains 15–20 repeats (however, also longer and shorter versions can be produced). The RD is indispensable for mediating sequence-specific binding of TALENs (TS1 and TS2) to the targeted sites (highlighted in blue and orange). Binding can be predicted using a simple protein–DNA code: NG, NN or NH, NI, and HD recognizing thymine (T), guanine (G), adenine (A), and cytosine (C), respectively. The functionally efficient spacer between the two TALEN recognition sites (TS1 and TS2) ranges from 12 to 31 bp depending on the architecture of TALE used (Cermak et al. 2011; Li et al. 2011a; Miller et al. 2011; Mussolino et al. 2011). **b** Once TALENs bind to the targeted DNA strands, the *FokI* nuclease domains dimerize. This leads to catalytic function of the *FokI* nuclease domains which creates double-stranded DNA breaks (DSBs) within the spacer. DSBs activate the cellular DNA damage repair machinery either preferentially by nonhomologous end joining (NHEJ) or by homologous recombination (HR) in the presence of a homologous donor oligo or template. Many genomic alterations, e.g.,

2.2 Materials

Unless indicated otherwise, all chemicals should be ordered from standard suppliers.

1. Plasmids: a. Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene ID1000000024); b. GoldyTALEN (Addgene ID: 38143).
2. Restriction enzymes: BsaI (Eco31I), AflIII (BspT1), XbaI, BsmBI (Esp3I), AatII, and StuI. Note: Enzymatic activity of BsaI is crucial for the first-round Golden Gate assembly reaction.
3. T4 DNA ligase.
4. Plasmid-safe DNA nuclease (Epicentre Biotechnologies, cat# E3110K).
5. T7 Endonuclease I (T7E1; NEB, M0302).
6. Chemically competent *Escherichia coli* cells.
7. Gel and polymerase chain reaction (PCR) purification kits.
8. LB medium and plates with tetracycline (10 mg/l), spectinomycin (50 mg/l), or ampicillin (50 mg/l).
9. X-gal and dependent on the type of competent cells used also IPTG, if needed.
10. Plasmid mini/midiprep kit. *Note:* Plasmid quality will affect the assembling efficiency; use, e.g., QIAprep® Miniprep from Qiagen.
11. Platinum *pfx* DNA polymerase (Life Technologies, Cat# 11708039).
12. Primary porcine fibroblasts.
13. Cell culture wares: 25 cm² and 75 cm² flasks, 6-well, 24-well, and 96-well plates.
14. Gelatin (0.1 %).
15. Complete medium for primary porcine fibroblasts (DMEM supplemented with 15 % FBS, 1 % P/S, 1 % glutamine).
16. G418 selection medium (complete medium supplemented with 800 ng/ml G418 and 5 ng/ml bFGF2). *Note:* The use of bFGF2 is to increase the doubling time of primary porcine cells in vitro.
17. Cell lysis buffer: (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl, pH 8.5, 0.5 % Nonidet P40, 0.5 % Tween, 400 µg/ml Proteinase K PCR grade).
18. P1 Primary Cell 4D-Nucleofector® X Kit S (V4XP-1032, Lonza).
19. PCR thermal cycler.
20. 4D-Nucleofector™ System with X unit (Lonza).

deletions, insertions, and/or mutations, can be created at the DSB site by NHEJ. In the presence of a short donor oligo, user-designed mutations can be introduced such as site-specific mutations or deletion of predetermined sequences (knockout). In the presence of a DNA template comprising two homology arms and a functional transgene cassette, the transgene can specifically be inserted into or near the DSBs with or without disrupting the endogenous gene function (knockin)

2.3 Engineering of TALENs

Herein, we provide notes that we found important for increasing the TALEN engineering efficiency by Golden Gate cloning. Refer to Cermak's study for detailed TALENs engineering protocols (Cermak et al. 2011).

Note 1 Before designing TALENs for your targets sites, make sure to analyze your potential targeted genomic region with RepeatMasker (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>), and choose a targeted region with no repetitive sequences for designing TALEN target sites using the TALEN targeter 2.0 (<https://tale-nt.cac.cornell.edu/>). The average success rate of TALENs is approximately 60%. We select in average four pairs of TALENs for each targeted locus (gene).

Note 2 To lower the handling time for the first Golden Gate reaction, dilute all plasmids from the Golden Gate kit to 150 ng/μl.

Note 3 For the first Golden Gate assembling reaction, alternatively, we predigest pFUS_A plasmid with *BsaI* and gel purify the plasmid backbone (2564 bp) to increase the efficiency.

Note 4 If FastDigest enzyme from Fermentas are used, we noticed that using double amounts (2 μl per reaction) of *BsaI* (*Eco31I*) and increasing the digestion–ligation reaction to 13 cycles can increase the frequency of correct clones (proportion of white colonies in the blue/white selection).

Note 5 To check the success of the first Golden Gate assembling reaction, run 3 μl of the plasmid-safe nuclease (PSN)-treated ligation reaction on 1% agarose gel as presented in Fig. 2.2a.

Note 6 It is crucial that your final TALEN plasmids are checked by restriction enzyme digestion (Fig. 2.2b) and sequencing before used for transfection.

2.4 Delivery of TALENs into Primary Porcine Fibroblasts

We use the 4D-Nucleofector™ System with X unit for transfection of primary porcine fibroblasts established from two breeds of miniature pigs (Yucatan and Göttingen).

1. Culture primary porcine fibroblasts in complete medium until 80% confluence.
2. Dissociate cells by trypsinization (0.05% trypsin-EDTA at 37 °C for 3 min) and stop trypsin with complete medium.
3. Count cells and use 5 × 500,000 cells per reaction.
4. Spin down cells at 200 g for 5 min.
5. Aspirate the medium as complete as possible without disturbing the cell pellet.
6. Resuspend cells in reconstructed P1 solution using 20 μl P1 solution per 5 × 500,000 cells.

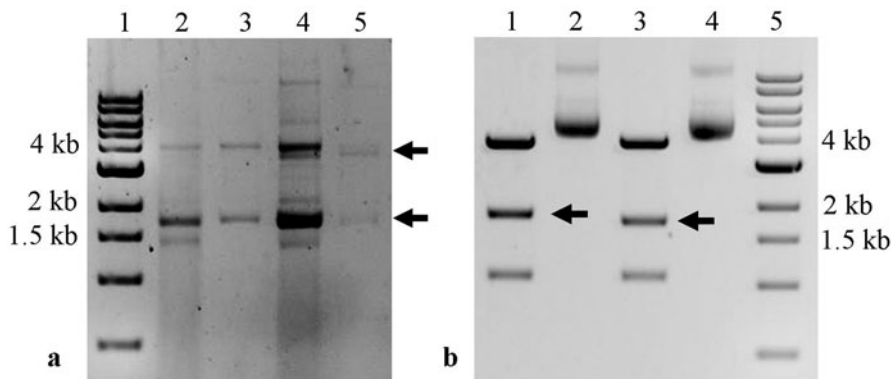


Fig. 2.2 Engineering TALENs by Golden Gate Reaction. **a** Gel electrophoresis of first Golden Gate reaction product. Three-microliter PSN-treated ligation products were separated in a 1% agarose gel. Lane 1, size marker; Lane 2–5, pFUS_A_10RVDs, pFUS_B5_5RVDs, pFUS_A_10RVDs, pFUS_B4_4RVDs; Expected ligation patterns are indicated by arrows. **b** pcGoldyTALEN plasmids digested with *AtaII* and *StuI*. Lane 1–4, digested pcGoldyTALEN_GOI_16RVDs, undigested pcGoldyTALEN_GOI_16RVDs, digested pcGoldyTALEN_GOI_15RVDs, undigested pcGoldyTALEN_GOI_15RVDs; Lane 5, size marker. *Arrows* indicate RVDs containing fragments. *TALEN* transcription activator-like effector nuclease, *PSN* plasmid-safe nuclease, *RVD* repeat variable di-residue

7. Aliquot 20 μ l P1 cell solution to sterilized 1.5 ml microcentrifuge tubes which are prefilled with 400 ng TALENs (per pair). Note: If a donor template is needed, we add 400 ng donor plasmid (linear or circular) plus 400 ng TALENs (per pair), though the recommended amount of total substrate DNA by the supplier is 400 ng.
8. Transfer the P1 cell–DNA mixture to a 20 μ l Nucleocuvette™ Strip.
9. Run nucleofection using the Nucleofector program CM-137. Note: We tested the nucleofection efficiency in primary fibroblasts with the primary cells optimization kit and found that CM-137 gave the highest transfection efficiency (~60%) and with a viability of ~20% (unpublished results). Although this optimization was only tested in Yucatan primary fibroblasts, this protocol should be compatible for fibroblasts from other breeds.
10. Following nucleofection, remove the cuvette from the nucleofector and incubate the transfected cells for 10 min at room temperature.
11. Add 80 μ l pre-warmed complete medium to each reaction and transfer to appropriate cell culture wares. Note: For analysis of TALEN cleavage, we plate the cells to one well of a 6-well plate coated with 0.1% gelatin, followed by culturing in 3 ml complete medium. For selection of gene KO and/or knockin (KI) clones, resuspend the nucleofected cells in 10 ml pre-warmed complete medium (supplemented with 5 ng/ml bFGF2) and plate the cells in one 96-well plate coated with 0.1% gelatin.
12. Culture cells at 37°C or 30°C (cold shock) for 72 h. Note: Changing medium during the first 72-h incubation period is not necessary.

Somatic Genome Manipulation

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