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Article: **Gene editing and clonal isolation of human induced pluripotent stem cells using CRISPR/Cas9**

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Abstract

Human induced pluripotent stem cells (hiPSCs) represent an ideal in vitro platform to study human genetics and biology. The recent advent of programmable nucleases makes also the human genome amenable to experimental genetics through either the correction of mutations in patient-derived iPSC lines or the *de novo* introduction of mutations into otherwise healthy iPSCs. The production of specific and sometimes complex genotypes in multiple cell lines requires efficient and streamlined gene editing technologies. In this article we provide protocols for gene editing in hiPSCs. We presently achieve high rates of gene editing at up to three loci using a modified iCRISPR system. This system includes a doxycycline inducible Cas9 and sgRNA/reporter plasmids for the enrichment of transfected cells by fluorescence-activated cell sorting (FACS). Here we cover the selection of target sites, vector construction, transfection, and isolation and genotyping of modified hiPSC clones.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) represent an ideal platform to study human genetics and biology *in vitro* [1,2]. Induced pluripotent stem cell lines can be established by reprogramming of somatic cells from any healthy or diseased individual [3], being able to embody the diversity of genotypes within the human population. Since hiPSCs are self-renewing they can be propagated indefinitely in the pluripotent state but on demand differentiated into selected cell types such as neurons [4] or cardiomyocytes [5].

The strength of non-human model organisms is based on *in vivo* analysis and the availability of reverse and forward genetic tools enabling functional genomics studies which unravel genotype to phenotype connections, including disease related variants. The recent advent of programmable nucleases makes also the human genome amenable to experimental genetics through either the correction of mutations in patient-derived iPSC lines or the *de novo* introduction of mutations into otherwise healthy iPSCs [6,7]. Some heritable diseases are strictly monogenetic, but observable phenotypes often depend on epistatic interaction from multiple genes. An understanding of disease-specific epistasis will improve the prediction of disease risks and elucidate disease mechanisms, e.g. distinguishing between disease specifiers and modifiers [8,9]. In animal genetics genotypes and backgrounds can be constructed and manipulated by breeding and backcrossing. In hiPSCs the genome is fixed, so studies investigating the effects of human genetic diversity require the use of multiple cell lines side-by-side.

The production of specific and sometimes complex genotypes in multiple cell lines requires efficient and streamlined gene editing technologies. We presently achieve high rates of gene editing at up to three loci using a modified iCRISPR system. This system includes a doxycycline inducible Cas9 and sgRNA/reporter plasmids for the enrichment of transfected cells by fluorescence-activated cell sorting (FACS). In this article we provide our protocols for gene editing in hiPSCs. Here we cover the selection of target sites, vector construction, transfection, and isolation and genotyping of modified hiPSC clones.

1.1 Gene editing in hiPSCs

Due to its efficiency and simplicity, the CRISPR/Cas9 system derived from *Streptococcus pyogenes* [10] has become the preferred method for gene editing in mammalian cells [11–13]. CRISPR/Cas9 gene editing requires two basic components to achieve a DNA double-strand break (DSB) and subsequent gene editing: the Cas9 nuclease protein and a single guide (sg)RNA to home the Cas9 nuclease. A DNA repair template is often additionally provided to allow for homology directed repair (HDR). The first twenty nucleotides of sgRNAs direct Cas9 to a specified complementary DNA target sequence via RNA-DNA hybridization. The target sequence is always located upstream of an invariant protospacer adjacent motif (PAM) sequence (NGG for SpCas9 from Streptococcus pyogenes). Upon correct hybridization the Cas9 nuclease undergoes a conformational change and creates a DSB located 3 bp upstream of the PAM site (Fig.1A, B) [14]. An advantage of the CRISPR/Cas9 system is that the Cas9 protein component is of universal use and new target sites can be easily targeted by addition of a new sgRNA sequence. The gene editing process follows induction of DSBs where DNA repair pathways result in deletion, replacement or insertion of sequences.

Two main DNA repair pathways exist in mammalian cells. In most instances DSBs are repaired by the non-homologous end joining (NHEJ) pathway. NHEJ religates open DNA ends using DNA ligase IV without using a repair template [15]. DSBs processed by the NHEJ pathway frequently exhibit random small deletions and/or insertions of nucleotides (indels) (Fig. 1C). Within coding regions this results in frameshift (knockout) mutations in the majority of cases. Alternative to NHEJ, DSBs can be repaired by HDR and requires a DNA molecule as repair template [16,17]. The natural endogenous use of HDR is for spontaneously occurring DSBs. Cycling cells use the intact homologous region of the sister chromatid as HDR template. To achieve precise sequence modifications at targeted DSBs, the HDR pathway can be exploited to copy in a sequence of interest. This is done through the addition of an artificial DNA template which mimics the sister chromatid by including homology

sequences located up- and downstream of the DSB and concurrently flanking a desired sequence modification or insertion [18]. In the repair process gene conversion extends from the template`s homology regions into the heterologous sequence and also transfers the modified region into the target sequence (knockin), enabling to introduce precise mutations such as codon replacements or the insertion of reporter genes (Fig. 1C). Large sequence insertions require the construction of plasmid-based gene targeting vectors which include homology regions of $\geq$ 1000 bp, whereas small sequence modifications can be introduced by using synthetic single-stranded DNA oligonucleotides (ssODN) with lengths of 100-150 nt. In cycling cells both repair mechanisms work side by side, but most DSBs are repaired by the prevailing NHEJ pathway whereas HDR occurs less frequently. In a population of transfected hiPSCs cells the induction of DSBs leads to a variety of repaired alleles in individual cells. Therefore, single cell-derived subclones are established from this heterogeneous population and genotyped for the presence of the desired mutant alleles. Isolated clones may harbor none, one or two differing mutant alleles and the occasional presence of non-clonal, mixed colonies make precise genotyping an elaborate task to exclude errors. Efficient mutagenesis protocols ensuring that gene editing occurs in the majority of treated cells are important to avoid the time-intensive expansion and genotyping of large numbers of unedited wildtype hiPSC clones.

Sequence deletions leading to gene inactivation (knockout) are commonly used for modelling loss of function alleles. Sequence replacements aim for the introduction or correction of disease-related point mutations. Sequence insertions are often used for the generation of cell type-specific reporter alleles or the ectopic expression of therapeutic proteins [6,7]. Furthermore, the multiplex gene editing approach enables the simultaneous processing of several target genes by the combination of multiple sgRNAs [19].

Frequent gene editing applications include the correction of disease-related mutations in patient-derived hiPSCs. The resulting engineered hiPSCs differ from the parental cells exclusively at the edited loci and are otherwise isogenic. Parallel differentiation of these isogenic cell lines into disease-relevant cell-types provides the basis for the phenotypic analysis of cellular pathologies that are directly attributable to the genetic specific mutations [2,20,21]. This reversion of disease-related phenotypes in cells derived from corrected hiPSCs can serve as a paradigm for the feasibility of future cell based therapies. The CRISPR/Cas9 system can be used for modification of both copies of a target gene, or restricted to induce changes only in alleles of interest by selective gene editing based on haplotype-specific target sites [22–25].

Gene editing in hiPSCs and other cells is now routinely performed using the

unmodified Cas9 nuclease. Effective use of Cas9 is constrained by the dependence on the invariant PAM (NGG) motif and imperfect selectivity resulting in cleavage of unintended genomic, `off-target´ sequences that exhibit one or more mismatches to the desired on-target site [26]. The level of off-target activity complicates Cas9 applications in cell-based disease modeling as well as in clinical translation. The off-target cleavage can be minimized by choosing target sequences showing minimal homology elsewhere in the genome. Whole genome sequencing of hiPSCs does not observe off-target effects if highly specific gRNAs are used [27–29]. Additionally, the specificity of Cas9 has been improved by engineering forms that destabilize the protein`s interaction with the DNA. By decreasing the strength of nonspecific DNA interactions through amino acid substitutions, the resulting Cas9 variants are thermodynamically more dependent on perfect gRNA-DNA pairings, leading to decreases in off-target binding and cleavage. Two of these enzymes have been produced. Zhang et al. [30] focused on residues that interact with the non-target DNA strand to generate `enhanced´ Cas9 (eCas9), whereas Joung [31] altered amino acids that contact the DNA backbone on the target strand obtaining `high-fidelity´ Cas9 (Cas9-HF). Using unbiased genome-wide assays, both groups showed greatly reduced off-target activity below detectable levels. The on-target cleavage of these variants is comparable (eCas9) or approaches (Cas9-HF) the activity of wild-type Cas9. These modified nucleases are not yet broadly used in hiPSCs but provide opportunities for future application.

- Figure 1 -

1.2 Human induced pluripotent stem cell lines

hiPSC lines can be established either from healthy individuals or from patients with inherited or idiopathic diseases. Lines from healthy individuals either serve for studying human developmental processes or as a framework for introduction of *de novo* or patient-derived mutations into disease-associated genes. Patient-derived hiPSCs are used to study an individual disease-prone genotype and the role of mutations identified in disease-related genes. A large resource is the HipSci initiative [32] that offers more than 400 hiPSC lines (www.hipsci.org) which are distributed via the ECACC (www.phe-culturecollections.org.uk). Moreover, a variety of human stem cell lines is available from WiCell (www.wicell.org). Despite the importance of allelic variants as part of the genetic background, it is a shortfall of the present hiPSC research that genome sequences for most cell lines are not available, except for the HipSci resource which offers whole exome sequencing (WES) for all and whole genome sequencing (WGS) data for selected lines. Different hiPSC lines exhibit

individual properties such as susceptibility to various transfection treatments or culture techniques approaches (see section 3.3).

1.3 Gene editing approach

The purpose of gene editing in a hiPSCs workflow may be the insertion of a specific sequence from a HDR donor template, or the deletion of a genomic sequence. Templates for HDR-mediated knockin may use donor vectors that include drug selection or fluorescent marker genes enabling the efficient isolation of targeted colonies harboring stable vector integrations [33,34]. In some cases, especially for gene therapeutic purposes, the marker gene needs to be removed. This can be achieved by flanking the marker gene with recombinase or transposase recognition sites that upon transient expression of recombinase or transposase facilitate the removal of the marker gene. Alternatively, templates without selection marker genes can be used for HDR such as ssODNs [35], providing that gene editing in the population of transfected cells is highly efficient.

Efficient methods would ideally allow the simultaneous introduction of knockout or knockin mutations into multiple genes (multiplex engineering) in different cell lines with limited efforts. A first step into the direction of multiplexed gene editing is the genomic integration of an inducible Cas9 expression vector into the AAVS1 locus. This allows for the delivery of sgRNAs and HDR donors only, while yielding an increased gene targeting efficacy in hiPSC. This approach was first described in hiPSCs as the doxycycline inducible iCRISPR system which just requires the transfection of multiple sgRNAs and allows the knockout of up to three genes in a single step [19]. Depending on the experimental setup, both classical CRISPR/Cas9 and the iCRISPR system feature advantages and drawbacks. The former is applicable to wildtype cells if no further genomic modifications are desired, e.g. experimental (re-)engraftments. The latter was demonstrated to provide improved efficacy when applied to modify multiple loci, although prior cell line generation is required and a modified AAVS1 locus will remain if no strategies for final Cas9 gene removal are applied.

In this protocol we include two ways to achieve gene editing in hiPSCs. Both describe detailed procedures on how to introduce CRISPR/Cas9-mediated, site-specific DSBs followed by suggestions for analysis of resulting indel formations. These methods can further provide a framework for HDR-mediated insertion.

The first approach, enables editing of single genes by transfection of a vector encoding Cas9 and sgRNA (cloned into pU6-(BbsI)sgRNA_CAG-Cas9-bpA_EF1-TagRFP or pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA, section 3.1.1) into unmodified hiPSC lines

(Workflow A, Fig. 2A). Included fluorescent Venus or RFP reporters enable subsequent FACS isolation of transfected cells. The second approach focuses on multiplex gene editing (Workflow B, Fig. 2B, 2C). This multiplexing protocol uses hiPSC lines with a doxycycline inducible Cas9 expression system integrated in the AAVS1 locus. We currently use a modified version of the iCRISPR system [19] which is available upon request. As described in section 3.3, these iCRISPR lines show a strong Cas9 expression when induced with doxycycline. These cells are transfected with a small vector for the expression a fluorescent Venus reporter and of multiple sgRNAs (cloned into plasmid pU6-(BbsI)sgRNA_CAG-Venus-bpA; section 3.1.1). This allows the fraction of successfully transfected hiPSCs expressing sgRNAs and the reporter to be isolated by FACS. The sorted cells are then plated for the establishment of single cell derived colonies (section 3.4).

Further improvements of this technology may be possible. We have found Cas9 to be heterogeneously expressed in iCRISPR cell lines after doxycycline induction. Therefore, we are further testing vectors for the induction of Cas9 and a second fluorescent reporter, enabling the two color FACS enrichment for cells expressing high levels of Cas9 and sgRNAs.

- Figure 2 -

1.4 Guide RNA selection and project planning

The selection of an appropriate RNA target site is determined by the specific aim of each experiment. A frequent application of gene editing is the generation of knockout alleles by the induction of indel formation at the DSB site by error-prone NHEJ repair causing frameshift mutations and premature termination. To achieve gene disruption the sgRNA target site has to be located in a protein coding exon that is included in all splice variants. Despite the presence of a frameshift mutation in an mRNA, ribosomes may be able to reinitiate translation at an ATG codon located downstream, generating a shortened protein [36]. Therefore, coding regions should be analyzed for the likelihood of such illegitimate translation events using e.g. the Netstart prediction server (http://www.cbs.dtu.dk/services/NetStart/) [37]. Besides forming knock-outs by indel formation one can delete large sections of coding sequence or regulatory elements by cutting with two sgRNAs. Deletions not necessarily have to affect a gene itself, but can involve regulatory elements as well. Furthermore, chromosomal rearrangements, that are duplications and inversions of regions up to more than 1 Mb, can be generated by the use of two sgRNAs targeting sites in an appropriate distance [38,39].

Once the genomic region of interest is identified a stretch of 150 – 250 bp can be analyzed for the distribution and quality of Cas9 target sites. Due to their simple structure target sites are found on average every 8–12 bp in the human genome and can be seen by visual inspection for NGG PAM motifs. However, the sequence composition of the 20 nt target sequence (such as GC content) influences the nuclease activity of Cas9 at the target site (on-target activity), as well as the probability that Cas9 cuts other related sites within the genome (off-target activity). To analyze all sgRNAs for their on-target efficiency and off-target sites we prefer to use the CRISPOR website (http://crispor.tefor.net/) [40]. CRISPOR provides scores for the specificity of sgRNAs as well as on-target efficiency using meta-analysis of all published activity ranking tools. Various studies suggest that off-target sites of RNA-guided Cas9 nucleases can be variable in frequency, challenging to predict and it is not possible to predict how many mismatches can be tolerated [26]. Nevertheless, a careful selection among the target sequences addressing a given genomic region of interest will reduce the risk of creating off-target mutations. Therefore, we recommend to use only sgRNAs showing the highest specificity scores and to save the information on potential off-target sites for later PCR analysis (see section 3.5.8).

Genetic editing resulting in the generation of a knockin, being either an insertion or a regional replacement, is achieved by HDR. To generate such a targeted sequence modification, the sgRNA target site should be located close (< 100 bp) to the position of the intended mutation as the frequency of sequence conversion by HDR decreases with distance. Targeted mutations by HDR are guided by repair templates, being either ssODNs or plasmid gene targeting vectors [14,41]. ssODNs are convenient as they are synthetized for a reasonable price so that cloning work is not required. ssODNs used as HDR template contain a short sequence modification (deletion, insertion or substitution) flanked by two homology sequences. Many manufacturers of custom ssODNs offer lengths up to 150 nt or more, enabling the insertion of sequences of up to ~50 nt. When using ssODNs we recommend the selection of target sites that are less than 10 bp distant to the planned mutation. In the standard design, as their length is limited, a desired mutation is located at the center of an ssODN, resulting in similarly-sized homology regions (usually 40-60 nt each) that flank the desired mutation symmetrically on both sides. It is crucial to avoid the recognition and recleaving of the recombined allele by Cas9 by the inclusion of one or more silent nucleotide replacements to destroy the PAM recognition site. Silent mutations can be added to facilitate later screening for successful recombination, e.g. by the introduction of a recognition site for restriction enzymes for restriction fragment length polymorphism (RFLP).

Recent studies suggest that choice of the target strand and an asymmetric design of ssODNs increase the knockin efficiency [42] and that phosphorothioate (PTO) modifications improve stability and HDR efficacy [43]. However, it was not shown if these rules apply to hiPSCs as well. We have performed a side by side evaluation of ssODNs with symmetric or asymmetric structure with or without PTO modifications (Fig. 3). Analogously to Glaser et al. [44], we used human iPS cells heterozygously expressing a CAG promoter driven EGFP from the AAVS1 locus as a reporter system for HDR. The EGFP gene was targeted with a sgRNA to induce a DSB. By homologous recombination of donor templates that substitute 4 bp, the original green fluorescent EGFP is converted to blue fluorescent BFP and the PAM sequence rendered non-functional, as previously reported [44]. Based on this assay, we observed that in hiPSCs unmodified ssODNs without PTO modifications yield higher HDR rates compared to modified ones (Fig. 3). Furthermore, in contrast to HEK293 cells as reported by Richardson et al. [42], a symmetric architecture of ssODNs resulted in higher HDR efficiencies than an asymmetrical in hiPSCs (Fig. 3). However, the efficacy of CRISPR/Cas9 mediated targeting of specific loci depends on the cell line subjected, the localization of the locus within the chromatin architecture and the sequence itself. Therefore, these observations cannot be generalized, but may vary in respect to the individual purpose.

- Figure 3-

For larger and more sophisticated modifications such as the insertion of cDNAs or reporter cassettes, targeting vectors with long homology arms are required. Such vectors include the insertion in between two regions homologous to sequences located up- and downstream of the DSB. For the design and construction of gene targeting vectors, adhere to protocols developed for gene targeting in mouse embryonic stem cells [45,46] with two exceptions: a drug selection cassette is not required and the homology arms used are often shorter ($\geq$ 500 bp) that facilitates cloning by PCR or gene synthesis. For vector construction, homology arms can be PCR-amplified from isogenic genomic DNA and ligated into a cloning plasmid backbone. Alternatively, the insert can be generated by gene synthesis purchased from a commercial provider. In the simplest configuration the selected Cas9 recognition sequence should cover the region of the intended mutation, bridging the homology arms of the targeting vector to prevent its cleaving by Cas9, being specific only to the wildtype genomic DNA.

1.5 Workflow of CRISPR/Cas9 gene editing in hiPSCs

For designing a new experimental setup our workflow for gene editing and clonal isolation of hiPSCs follows a generalized sequence, as shown in Figure 4: 1. Cloning of a suitable sgRNA expression vector. Assuming usage of available combined vectors to express Cas9 and sgRNA (section 2., Vectors) 7 days are estimated for the procedure and quality control. 2. Transfection of fluorescent reporter vectors carrying sgRNAs and Cas9 (Workflow A) or - if using an inducible Cas9 harboring iPS cell line - transfection with fluorescent reporter vectors carrying only sgRNAs (Workflow B) and if desired repair templates. 3. On day 9, 48 h post transfection, the successfully transfected cells are enriched by FACS sorting for the fluorescent reporter. 4. The FACS sorted cells are then plated at low density. To achieve a good viability in feeder-free conditions and to prevent spontaneous differentiation the cells are cultivated for 7 days in 50 % of conditioned medium [47]. 5. The single cell derived colonies can be picked on around day 16, further cultured and split to two separate cultivations each. 6. After expansion for 5 more days, one of the two cultivations is subjected to analysis of gene editing events (Fig. 6, B1-B6). 7. The other cultivation is kept in culture until establishment of the final cell line or preparation of cryopreserved stocks on day 28.
Following these workflows and picking 24 colonies for further processing should yield at least two positive clone on average. However, as CRISPR/Cas9-mediated gene editing depends on the targeted loci as well as on the cell line used efficacies may vary. To promote increased efficacy of gene editing, we suggest performing a second round of transfection and FACS enrichment, and optionally to co-transfect a vector encoding for Trex2, an end-processing enzyme.

- Figure 4-

2. Materials

2.1 Vectors

Workflow A

- pU6-(*BbsI*)sgRNA_CAG-Cas9-bpA_EF1-TagRFP (Addgene ID 86987, www.addgene.org) or
-pU6-(*BbsI*)sgRNA_CAG-Cas9-venus-bpA (Addgene ID 86986)

Plasmids for the cloning of a single sgRNA into the BbsI site and separate the expression of Cas9 and RFP or the expression of a Cas9-venus fusion protein.

Workflow B

- pU6-(*BbsI*)sgRNA_CAG-venus-bpA (Addgene ID 86985)
Plasmid for the cloning of single or multiple sgRNAs into the BbsI site and the expression of Venus.

Optional: Co-expression of Trex2 (pCAG-mTrex2-bpA; Addgene ID 86984) enhances indel formation.

2.2 Cloning sgRNAs

1. Empty sgRNA cloning vectors (section 2.1)
2. A pair of target specific sgRNA oligos with BbsI overhangs, e.g. MWG-Biotech
3. BbsI restriction enzyme, NEB, #R0539S
4. *AscI* restriction enzyme, NEB, #R0558S
5. T4 Ligase, NEB, #M0202S
6. PureYield™ Plasmid Miniprep System, Promega, #A1222
7. Chemical competent *E. coli* DH5α, Thermo Fisher, #18265-017
8. Carbenicillin, Sigma, #C3416
9. LB medium and LB agar plates
10. Agarose gel electrophoresis (setup and consumables)

2.3 General hiPS cell culture

1. hiPS cell lines (e.g. HPSI0114i-kolf_2, www.hipsci.org or MIRJT7i-mND2-0, www.wicell.org)
2. Essential 8 medium, Thermo Fisher Scientific, # A1517001
3. Y-27632 (dihydrochloride), Biomol, # Cay10005583-5
4. DPBS -/- (-Ca^{2+}, -Mg^{2+}), Life Technologies, # 14190169
5. Vitronectin (VTN-N) Recombinant Human, Life Technologies, # A14700
6. Matrigel Matrix, Fisher Scientific, # 10162371
7. DMEM/F-12 Hepes, Life Technologies, # 31330038
8. Cell culture plates (e.g. Corning Costar 6-well plates, # CLS3516-50EA)
9. Accutase solution, Sigma-Aldrich, # A6964
10. Bambanker Serum Free Cell Freezing Medium, BioCat, # BB01-NP
11. DMSO, Sigma-Aldrich, # D5879
12. Doxycycline Hydrochloride, Sigma-Aldrich, # D9891
13. Dissociation buffer: 0.18 % NaCl, 0.5 mM EDTA in DPBS (to 491.92 ml DPBS add 5 ml of 0.5M EDTA pH 8.0 and 3.8 ml of 5M NaCl)

14. ReLeSR, Stemcell Technologies, # 05872

2.4 Lipofection of hiPS cells

1. Lipofectamine 3000 transfection kit, Life Technologies, # L3000001
2. Opti-MEM I reduced serum medium, Gibco, # 31985062

2.5 Electroporation of hiPS cells with the Neon system

1. Neon Transfection system, Thermo Fisher Scientific, # MPK5000
2. Neon transfection kit 100µl, Thermo Fisher Scientific, # MPK10025

2.6 Electroporation of hiPS cells with the Gene Pulser system

1. Gene Pulser Xcell Electroporation Systems, Bio-Rad, # 1652660
2. Gene Pulser electroporation cuvette 0.4 cm, Bio-Rad, # 1652081

2.7 FACS enrichment of transfected hiPS cells

1. 0.45 µm PTFE membrane filter, Millipore, # SLCR025NS
2. FACS tubes with 35 µm strainer cap, Falcon, # 352235
3. Penicillin/Streptomycin (10,000 U/ml), Life Technologies, # 15140122
4. Gentamicin (10 mg/mL), Life Technologies, # 15710049
5. RevitaCell Supplement (100X), Life Technologies, # A26445-01
6. Fluorescence activated cell sorter (e.g. BD FACSAria III, BD Biosciences)

2.8 Analyzing modifications

1. Wizard Genomic DNA Purification Kit, Promega, # A1125
2. Wizard DNA Clean-Up System, Promega, # A9282
3. Herculase II Fusion DNA Polymerase Kit, Agilent, # 600679
4. Q5 High-Fidelity DNA Polymerase, NEB, # M0491S
5. dNTP Set, Thermo Fisher Scientific, # R0181
6. Gene specific primers, e.g. MWG-Biotech
7. Fluorescence labeled gene specific primers, e.g. MWG-Biotech
8. Restriction enzymes, NEB
9. Thermocycler, e.g. Eppendorf
10. Agarose gel electrophoresis (setup and consumables)

3. Methods

3.1 Cloning of sgRNAs

This protocol recommends the usage of either plasmid pU6-(*BbsI*)sgRNA_CAG-Cas9-venus-bpA or pU6-(*BbsI*)sgRNA_CAG-Cas9_EF1-TagRFP (Workflow A), encoding for sgRNA and Cas9 and a fluorescent Venus or RFP reporter, or usage of pU6-(*BbsI*)sgRNA_CAG-venus-bpA, carrying a sgRNA-cassette and a fluorescent reporter only (Workflow B). These vectors allow expression of the sgRNA by the human U6-promoter. This promoter requires a "G" base at the transcription start site. Hence, it is recommended using CRISPR/Cas9 target sites starting with a "G". Otherwise an additional "G" should be added at the start of the sgRNA sequence. It should be noted that wildtype Cas9 is amenable to the inclusion of an extra "G" but other RNA guided nucleases are not (Cas9-HF/eCas9). All three above mentioned plasmids together with their maps and sequences are deposited at Addgene (www.addgene.org) which also provides a wide range of other vector systems for Cas9 expression and sgRNA cloning.

3.1.1 Cloning of a single sgRNA

Annealing of sgRNA oligos

The sgRNA oligos can be cloned into the above mentioned vectors by *BbsI* restriction enzyme overhangs, with N_1-N_{20} as the selected Cas9 target sequence:

sgRNA-oligo-F 5' –CACC(G)N_1NNNNNNNNNNNNNNNNNN$_{20}$ –3'

sgRNA-oligo-R 5' –AAACNNNNNNNNNNNNNNNNNNN(C) –3'

1. Resuspend oligos at 1 µg/µl in 1x TE-buffer.
2. Combine in a microcentrifuge tube
   - 1 µl oligo F (1 µg/µl)
   - 1 µl oligo R (1 µg/µl)
   - 98 µl 1x TE-buffer
3. Incubate 5 min at 98 °C in a heat block.
4. Switch off the heat block and cool down slowly to RT for 1-2 h.
5. Put the annealed oligos on ice or store at -20 °C.

Digestion of sgRNA expression vector

1. Set up digestion reaction as following:
    - X µl of the above mentioned vectors (5 µg)
    - 2.5 µl *BbsI* (store at -80 °C)
    - 10 µl NEB2 buffer
    - Fill up to 100 µl with nuclease free water
2. Incubate at 37°C for 1 h.
3. Inactivate the restriction enzyme for 20 min at 65 °C.
4. Load the digested vector on a 0.9% agarose gel and extract the linearized vector using a DNA gel extraction kit. Expected fragment sizes are: pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA – 10.1 kb; pU6-(BbsI)sgRNA_CAG-Cas9-bpA_EF1-RFP – 11.6 kb; pU6-(BbsI)sgRNA_CAG-venus-bpA – 6.0 kb.

Ligation of sgRNA oligos

1. Set up ligation in a microcentrifuge tube
    - X µl linearized vectors (100 ng)
    - 1.5 µl annealed oligos (1 µg/µl each)
    - 1.5 µl fresh ligase buffer
    - 1 µl T4 DNA Ligase
    - Fill up to 15 µl with nuclease free water and incubate O/N at 16 °C.

Transformation

1. Thaw chemically competent *E. coli* on ice slowly.
2. Pipet the transformation reaction on ice
    - 50 µl chemically competent DH5 alpha *E. coli*
    - 5 µl of the ligation mix.
3. Incubate 30 min on ice.
4. Perform the heat shock in a water bath at 42 °C for 90 sec.
5. Incubate 3 min on ice.
6. Add 1 ml LB medium w/o antibiotics and incubate 30 min at 37°C at 200 rpm.
7. Plate the transformation on LB-Agar plates 50 µg/ml carbenicillin or ampicillin and incubate O/N at 37°C.

The next day pick up to five colonies and inoculate 5 mL LB medium containing 50 µg/ml carbenicillin or ampicillin. Incubate O/N at 37 °C at 200 rpm. Perform plasmid mini preparations and subject for Sanger sequencing to verify the correct sequence using primer hU6-For: GAGGGCCTATTTCCCATG.

Optional: Redigestion of the ligated plasmid using the enzyme *BbsI* improves the cloning

efficacy.

3.1.2 Cloning of multiple sgRNAs

To clone multiple sgRNA expression cassettes in pU6-(*BbsI*)sgRNA_CAG-venus-bpA via Gibson assembly, the individual sgRNAs have to be cloned separately beforehand as previously described in section 3.1.1. These single sgRNA plasmids serve as template for amplifying a specific sgRNA cassettes including overlapping ends for Gibson assembly. The following protocol enables to assemble two or three sgRNA expression cassettes in one vector. If it is intended to clone more than three sgRNAs in one vector primer pairs have to be designed with appropriate assembly overhangs, according to manufacturer guidelines. Following primers can be used for cloning:

Gibson-pU6-A_F	CAGGAAACAGCTATGACCATGAGGGCCCCCTTCACCGAGGGCCTATTTC
Gibson-pU6-A_R	CCGATGGCCAGGCCGATGCTGTGATCAAAAAAAGCACCGACTCGG
Gibson-pU6-B_F	ACAGCATCGGCCTGGCCATCGGGCCCCCTTCACCGAGGGCCTATTTC
Gibson-pU6-B_R	CTTGGCCATCTCGTTGCTGAAGATCAAAAAAAGCACCGACTCGG
Gibson-pU6-C_F	TTCAGCAACGAGATGGCCAAGGCCCCCTTCACCGAGGGCCTATTTC
Gibson-pU6-C_R	GTCAATAATCAATGTCGAATCCGGGATCAAAAAAAGCACCGACTCGG

Use primer combinations A_F/A_R; B_F/B_R and C_F/C_R if three cassettes are desired to be cloned and combinations A_F/A_R and B_F/C_R if it is desired to clone two sgRNA cassettes.

Amplification of guide RNA cassettes with overhangs for assembly

1. Set up PCR reaction in 50 µl reaction volume:
    - 5 – 20 ng plasmid DNA
    - 0.25 µM primer (each)
    - 250 µM dNTPs (each)
    - 0.5 µl Herculase II
    - Fill up to 50 µl with nuclease free water.
2. Carry out the PCR reaction with following conditions: 98 °C, 2 min; [98 °C, 30 sec; 55°C, 30 sec; 72 °C, 30 sec] x30 cycles; 72 °C 2 min.
3. Load the PCR products on a 1% agarose gel and purify the 412 bp fragment using a

DNA gel extraction kit.

Digestion of sgRNA expression vector

1. Set up digestion reaction as following:
   - X µl plasmid DNA, pU6-(*BbsI*)sgRNA_CAG-venus-bpA (5 µg)
   - 2.5 µl *AscI* restriction enzyme
   - 10 µl CutSmart buffer
   - Fill up to 100 µl with nuclease-free water
2. Incubate at 37°C for 1 h.
3. Inactivate the restriction enzyme for 20 min at 65 °C.
4. Load the digested vector on a 0.9% agarose gel and extract the linearized vector backbone using a DNA gel extraction kit. Expected fragment sizes are: 5586 bp for the vector backbone and 383 bp for the empty sgRNA cassette.

Gibson assembly reaction

1. Set up assembly reaction as following:
   - 200 ng AscI linearized plasmid DNA, pU6-(*BbsI*)sgRNA_CAG-venus-bpA
   - 30 ng PCR product (each)
   - 10 µl Gibson assembly master mix (2x)
   - Fill up to 20 µl with nuclease-free water
2. Incubate at 50 °C for 1 h.
3. Perform transformation as described in section 3.1.1
4. Conduct test digests with *NdeI* on five to ten plasmid mini preparations. The expected fragment sizes are: for assembly of two sgRNA expression cassettes 5515 bp; 436 bp and 391 bp and for three sgRNA expression cassettes 5515 bp; 436 bp¸391 bp and 390 bp. Subject two plasmids with the correct digestion pattern for Sanger sequencing using following primers:

   pU6-(BbsI)sgRNAseqF: TTGTGTGGAATTGTGAGCGG

   pU6-(BbsI)sgRNAseqR: GGCTATGAACTAATGACCCCG

Optional: To improve the transformation efficacy perform a clean-up of the assembled reaction using a PCR purification kit, prior to transformation.

3.2 Feeder-free hiPSC culture conditions

Cultivation of high-quality human iPS cells requires optimal and aseptic conditions. The cultures should be free of viral, mycoplasmic and bacterial contaminations and have a normal karyotype. It is recommended to cultivate human iPSCs in antibiotic-free media to avoid the overlooking of mycoplasma infections. Therefore, it is worth to emphasize the importance of aseptic culture conditions. The optimal growth conditions for human iPS cells are in a humidified, hypoxic incubator equipped with a HEPA-filter at 37°C with 1-5 % O_2 and 5 % CO_2. Feeder-free iPS cell culturing requires special culture media and culture plate coatings. Since different hiPSC lines may demand different conditions, it is recommended to test multiple commercially available hiPSC media and coatings. Pluripotent stem cells, such as hiPSCs have a doubling time of 18-20 h and should therefore be passaged every 3 to 4 days. If the culture becomes over-confluent, iPS cells tend to grow as multi-layers and spontaneously differentiate. Induced pluripotent stem cells are sensitive to environmental changes, thus monitor the culture on a daily base. To keep the culture in high-quality condition, it is recommended to reduce the time of cells being outside the incubator to a minimum. When exposed to stress by e.g. dissociating to single cells or transfection, iPS cells induce apoptosis by activation of the ROCK (Rho-associated protein kinase) pathway. To avoid cell death a ROCK inhibitor (Y-27632) should be added to the culture medium overnight, when the cells are transfected or passaged. The addition of ROCK inhibitor changes the morphology of the iPSCs, cells will grow extensions and look fibroblast-like. These changes are reversible and will vanish when Y-27532 is removed.

The following protocol is optimized for the healthy donor-derived hiPS cell lines: MIRJT7i-mND2-0 (WiCell), Kolf-2 (HipSci), XM001 (Helmholtz Center Munich) and BCRT#1 (Berlin-Brandenburg Center for Regenerative Therapies).

3.2.1 General feeder-free human iPSC culture

1. Culture hiPSCs on cell culture plates pre-coated with an appropriate coating (e.g. Matrigel Matrix, Fisher Scientific or truncated rhVitronectin (VTN-N), Life Technologies).
2. Culture in appropriate medium (e.g. Essential 8 Medium).
3. Change medium on a daily basis, when using Essential 8.
4. Passage the cells when they reach 60 % to 80 % confluency.
5. hiPS cells can be passaged either using dissociation buffer as patches for maintenance or using Accutase as single cells, when transfection or FACS enrichment is desired.

Nevertheless, even properly handled hiPSC cultures might start to expose differentiated cells. Such cells appear as fibroblast-like outgrowths at the edge of hiPSC colonies. Contamination by differentiated cells can be eliminated by selectively transferring the hiPSC culture to a new pre-coated cell culture plate using ReLeSR according to the manufacturer's manual.

3.2.2 Passaging hiPSC using dissociation buffer

1. Remove medium and wash the cells once with DPBS.
2. Add 0.5 ml dissociation buffer (see 2.3.13) per well of a 6-well plate and incubate for 3-5 min at 37 °C.
3. When colonies begin to detach at the edges, remove the dissociation buffer and add 0.5 ml of Essential 8 medium.
4. Detach cells by tapping the plate and gently flush them off with medium using a pipette.
5. Pipet carefully 2-3 times to break bigger cell clumps into small ones.
6. Resuspend the cells in appropriate volume of medium (optional: including 10 µM ROCK inhibitor) and plate the cells on cell culture plate with an appropriate coating.
7. Change medium the following day.

3.2.3 Passaging hiPSC using Accutase

1. Remove medium and wash the cells once with DPBS.
2. Add 0.5 ml Accutase per well of a 6-well plate and incubate for 3-5 min at 37 °C.
3. When colonies begin to detach and fall apart, add 2 ml Essential 8 Medium supplemented with 10 µM with ROCK inhibitor.
4. Pipet carefully up and down to break cell patches to single cells and spin down at 300 g for 4 min.
5. Resuspend in appropriate volume of medium including 10 µM Y-27632 and plate the cells on cell culture plates with appropriate coating.
6. Change medium the following day.

3.2.4 Cryopreservation of hiPSC

1. Detach hiPS cells using dissociation buffer as previously described.
2. Very carefully resuspend the colonies in 0.75 ml of Essential 8 medium per well, leaving the colonies as large as possible.

3. Add 0.75 ml cold Essential 8 medium supplemented with 20 % DMSO drop by drop to the freezing vial and swirl gently (final DMSO concentration: 10%).
4. Distribute the cell suspension to three cryopreservation tubes, each with 500 µl.
5. Put cryovials into a freezing container (e.g. Mr. Frosty, Nalgene) and freeze at -80 °C for 24 h before shifting the vials to liquid nitrogen for long term storage.

Optional: Alternatively to growth medium supplemented with 10% DMSO, BamBanker can be used as cryopreservation medium. The usage of BamBanker increases the viability.

3.2.5 Thawing hiPSC

1. Remove the cryovial containing the frozen cells from liquid nitrogen and transport them to the cell culture lab on dry ice.
2. Immediately place it into a 37°C water bath.
3. Thaw cells by gently swirling the vial in the water bath until there is only a small piece of ice left.
4. Wipe the outside of the tube with 70 % ethanol quickly and transfer the vial to the laminar flow hood.
5. Add 1 ml pre-warmed Essential 8 medium in a dropwise manner to the cells and incubate for 1 min.
6. Transfer the thawed cells to a 15 ml centrifugation tube and add another 4 ml Essential 8 medium.
7. Centrifuge the cell suspension at 200 g for 4 min.
8. Aspirate the supernatant without disturbing the cell pellet.
9. Gently resuspend the cells in Essential 8 medium.
10. Plate the cells on cell culture 6-well plates pre-coated with an appropriate coating in Essential 8 medium supplemented with 10 µM Y-27632. Depending on the density the cells were frozen at, we recommend to plate the cells at different dilutions, 1:2 and 1:10.
11. Change the medium the following day to Essential 8 medium without Y-27632.

3.3 Transfection of CRISPR/Cas9 constructs into hiPS cells

We suggest using unmodified hiPSC lines for editing of a single target gene (Workflow A) or using hiPS cell lines expressing Cas9 under a strong doxycycline inducible promoter if one intends to modify multiple genes simultaneously (Workflow B). In our laboratory we generated several different hiPS cell lines carrying an inducible Cas9 in the safe harbor locus AAVS1, based on Gonzales et al. [19] (cell lines available upon request). Instead of using the

M2rtTA transactivator we used the TRE-3G Tet-on transactivator that drives a stronger expression upon doxycycline induction (Fig. 2C). The inducible Cas9 system is a versatile tool to generate complex genotypes in a one-step experiment (Fig. 2B). Since the cells are already equipped with Cas9, smaller plasmids carrying only the sgRNAs and a reporter/selector have to be transfected transiently, which improves the transfection efficiency (Fig. 5).

An efficient transfection of a CRISPR nuclease and the sgRNA is indispensable for successful genome editing. The choice of a certain transfection method depends upon its efficacy in a hiPS cell line of interest as well as on the viability of the cell line, when exposed to the transfection method. Generally small vectors < 8 kb can be more efficiently transfected and induce less cell death than large vectors >8 kb (Fig. 5A and B). However, where some lines can be efficiently transfected by reagent or chemical-based transfection methods such as Lipofectamine 3000, others respond well to electroporation. Thus, transfection conditions have to be optimized for different iPS cell lines as shown in Figure 5.

- Figure 5 -

Here we describe three different transfection methods generally used for CRISPR/Cas9-based gene editing. These methods are based on transient transfection of plasmids expressing Cas9 and/ or sgRNAs. Depending upon the nature of the planned experiment, multiple sgRNAs can be transfected simultaneously to achieve multiplex gene editing. To facilitate generation of gene edited clones we recommend to use either a fluorescent marker if FACS sorting is feasible or selection markers like puromycin or hygromycin on the plasmid encoding for Cas9 and sgRNA to enrich transfected cells.

This protocol is based on hiPS cells grown in feeder-free conditions in Essential 8 culture media in a 6-well cell culture plate.

3.3.1 Reagent-based transient transfection using Lipofectamine 3000

1. Prepare plasmid DNA at a concentration of 0.5-1 µg/µl in deionized water or TE buffer. The DNA used for transfection should be of high quality as poor quality of DNA might decrease the efficacy of transfection.
2. One day prior to transfection dissociate the cells using Accutase and seed $5x10^4$ – $1x10^5$ cells per well of a pre-coated 6-well plate as single cells or in small clumps.

Cultivate the cells in fresh medium containing 10 µM Y-27632 overnight.

3. Change the medium ahead of transfection.
4. Dilute depending on the size of the plasmid between 1 µg and 2.5 µg DNA in 125 µl of Opti-MEM reduced serum medium in tube labeled as A. Add 3.75 µl of p3000 reagent and mix well. Dilute 5 µl Lipofectamine 3000 reagent in 125 µl of Opti-MEM reduced serum medium in tube B. Diluted Lipofectamine 3000 should be used within 15 minutes. Longer times can result in decreased transfection efficacy.
5. Add the content of tube A to tube B, mix well by pipetting and incubate at room temperature for 5 minutes.
6. Add the DNA-lipid complex to one well of a 6-well plate in a dropwise manner and gently rock the plate to ensure its distribution over the whole well. Some cell lines are sensitive to Lipofectamine 3000.
   Optional: The addition of 5 µM Y-27632 to the culture medium for 24 h increases viability.
7. Change medium on the following day. When iPS cells harboring a doxycycline inducible Cas9 were used, feed the cells for 48 h with medium supplemented with 1 µg/ml doxycycline.
8. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis of bulk population (see section 3.5 for the method of choice for analysis).
9.

3.3.2 Electroporation-based transient transfection using the Neon system

The following protocol describes the electroporation procedure using the 100 µl Neon Transfection kit, aiming for a 6-well plate format. The DNA used should be of high quality to achieve the optimal results.

1. Prepare highly concentrated plasmid DNA (1-5 µg/µl) in deionized water or TE buffer. The amount of plasmid DNA should not exceed 10% of the total volume of resuspension buffer.
2. Coat a 6-well plate with appropriate coating.
3. Pre-warm Essential 8 medium containing 10 µM Y-27632.
4. Change the medium ahead of transfection.
5. Dissociate the cells using Accutase and spin down for 4 min at 300 g.
6. Wash the cell pellet once with PBS.
7. Dilute 5.5 µg total plasmid DNA in 110 µl buffer R (part of Neon transfection kit) in a

microcentrifuge tube.

8. Resuspend 6x10^5 cells in 110 µl buffer R containing the DNA. Care should be taken while resuspending for no air bubbles will be formed. Presence of air bubbles will lead to unsuccessful electroporation.
9. Fill 3 ml of electrolytic buffer E2 (provided with the Neon kit) into a Neon tube and insert it into the pipetting station. A single Neon tube can be used up to 10 times.
10. Insert a 100 µl Neon tip into the Neon pipette. Make sure that the tip is adjusted correctly before pipetting the cell/ DNA mix.
11. Load the appropriate Neon program (it is advised to optimize settings for each cell line using a fluorescent plasmid). The electroporation parameters we generally use are 1 pulse at 1300 V and a duration of 30 ms.
12. Press start and wait until the procedure is complete. A single Neon tip can be used twice with the same plasmid/cell line.
13. Transfer the electroporated cells to a microcentrifuge tube containing 500 µL medium supplemented with 10 µM Y-27632 and pellet at 300 g for 4 min.
14. Resuspend the cells in 600 µL fresh medium supplemented with Y-27632. Plate 100 µL cell suspension into one well of a pre-coated 6-well plate containing 2 ml medium supplemented with 10 µM Y-27632.
15. Rock the plate gently and incubate the cells.
16. Change medium the following day. When iPS cells harboring a doxycycline inducible Cas9 were used, feed the cells for 48 h with medium supplement with 1 µg/mL doxycycline.

    Optional: The addition of 5 µM Y-27632 to the culture medium for 24h on the day after transfection improves viability.
17. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis of bulk population (section 3.5).

3.3.3 Electroporation-based transient transfection using the Gene Pulser system

1. Prepare highly concentrated plasmid DNA (1-5 µg/µl) in deionized water or TE buffer.
2. Change the medium ahead of transfection.
3. Coat a 6-well plate with appropriate coating.
4. Pre-warm Essential 8 medium containing 10 µM Y-27632.
5. Pre-chill a 0.4 cm electroporation cuvette.
6. Dilute the appropriate amount of plasmid DNA (20-40 µg per plasmid, if multiple plasmids are transfected we recommend to use up to 100 µg DNA) in 850 µl DPBS

and chill on ice.

7. Dissociate the cells using Accutase and spin down for 4 min at 300 g.
8. Wash the cells once with DPBS, take an aliquot for counting and pellet again at 300 g for 4 min.
9. Resuspend at16x10^6 cells/ ml in ice-cold DNA/ PBS dilution and carefully transfer 800 µl of it to the electroporation cuvette containing the DNA, avoid air bubble formation.
10. Incubate on ice for 5 minutes and proceed with electroporation. We generally use following parameters: single pulse at 300 V and 500 µF, the time constant should be between 10 ms and 15 ms. It is advised to optimize settings for each cell line using a fluorescent plasmid.
11. Immediately after the pulse add 500 µl of pre-warmed culture medium supplemented with ROCK inhibitor Y-27632 to the cells.
12. Transfer the cells to a microcentrifuge tube and pellet them by centrifugation.
13. Resuspend the cells in 600 µl fresh medium containing Y-27632 and plate 100 µl cell suspension into one well of a pre-coated 6-well plate containing 2 ml medium supplemented with 10 µM Y-27632.
14. Rock the plate gently and incubate the cells.
15. Change medium the following day. When hiPS cells harboring a doxycycline inducible Cas9 were used, feed the cells for 48 h with medium supplement with 1 µg/ml doxycycline.

    Optional: The addition of 5 µM Y-27632 to the culture medium for 24 h improves viability.
16. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis of bulk population (see section 3.5).

3.4 FACS enrichment and clonal isolation hiPS cells

Selecting cells that were successfully transfected improves the rate of gene editing cells. In this protocol, we describe the enrichment of transfected cells by FACS (Fig. 6). If there is no opportunity to utilize FACS, other selection methods e.g. using antibiotics like puromycin or hygromycin can be applied. Cultivating hiPS cells in 50 % conditioned medium dramatically increases cell survival of the FACS process [47]. We have observed that the usage of conditioned medium increases the viability of single seeded iPS cells in low density and prevents the cells from spontaneous differentiation. Nevertheless, the procedure of FACS enrichment might induce cell stress therefore we suggest processing the cells as fast as possible. To avoid contaminations during FACS we suggest using penicillin and streptomycin

as well as gentamicin. The dissociated cells should not be kept on ice for longer than 45 minutes.

- Figure 6 -

3.4.1 FACS enrichment of transfected hiPSC

1. Prepare post-FACS medium: 50 % fresh Essential 8 medium, 50 % conditioned Essential 8 medium (medium that was conditioned for 24 h on hiPS cells with 50-80 % confluency of the same line and filtered through a 0.45 µm PTFE membrane filter) supplemented with 10 µM Rock inhibitor Y-27632 , RevitaCell Supplement (1X), Pen/Strep (1X), and Gentamicin (1X).
2. Coat a 6-well plate with appropriate coating.
3. For each FACS sample, prepare 1.5 ml microcentrifuge tube with 1 ml post-FACS medium and keep on ice.
4. Pre-warm Essential 8 medium containing 10 µM Y-27632, Pen/Strep (1X), and Gentamicin (1X).
5. Dissociate cells using Accutase and spin down for 4 min at 300 g.
6. Resuspend the cells thoroughly by pipetting the cells 5-10 times to achieve a single cell suspension. We suggest resuspension in 200 to 300 µl medium per well of a 6-well plate at 70 % - 80 % confluency
7. Strain through a strainer cap with a mesh size of 35 µm into a FACS tube.
8. Put cells on ice and proceed with FACS immediately.
9. Depending on the nature of your experiment and the chosen fluorophore, sort for your desired cell population directly into a cooled 1.5 ml microcentrifuge tube containing 1 ml of post-FACS medium.
10. Seed 300-500 cells per well in 2 ml post-FACS medium in a 6-well format as quickly as possible after sorting. Prepare several wells for each sample.
11. Make sure single cells are evenly distributed throughout the well to ensure emergence of single cell derived colonies.
12. Additionally, seed the remaining cells at a higher density on a separate well. This well can be used after expansion for gDNA extraction from the sorted bulk population and subsequent PCR based genotyping to determine the rough gene targeting efficacy for example by RFLP. This can be very helpful to determine the number of single clones that need to be analyzed for the specific gene targeting event.

In case of low gene targeting efficacy, two rounds of transfection and sorting can be of

advantage to enrich for gene edited single clones. Therefore, seed all positive cells coming out of the first sort. Once the cells have recovered, and have reached an appropriate confluency, transfect them again, followed by a second sorting step.

3.4.2 Clonal isolation of hiPSCs

1. Culture the sorted cells for 5 days in 50 % conditioned and 50 % fresh medium until small colonies emerge. Change medium daily.
   Optional: The addition of 5 µM Rock inhibitor Y-27632 for 1 to 2 days to the medium (50 % conditioned and 50 % fresh medium) improves viability of single cell derived clones.
2. At day 6 switch to regular Essential 8 medium and continue with daily feeding.
3. Closely monitor the growth of the single cell derived colonies to exclude the possibility that neighboring colonies fuse together. This can be done by circling neighboring colonies on the bottom side of the well with a marker pen.
4. After approximately 7 to 10 days colonies are large enough for manual picking.
5. Gently scratch the colonies into smaller, checkered patches either using a small needle or using a 10 µl pipette tip utilizing a stereo microscope in a sterile environment.
6. Transfer the patches of one clone to one well of a pre-coated 12-well plate containing pre-warmed medium supplemented with 10 µM Y-27632.
7. Gently resuspend the cell patches by pipetting up and down with a 1000 µl tip. After a few days colonies may need to be dissociated with EDTA within the plate to break apart the large colony and avoid differentiation of the cells.
8. As soon as the cells build larger colonies, split each clone on 2 separate wells in either 12- or 6-well format in a 20 % to 80 % ratio. Use one plate (80 %) for further expansion and subsequent gDNA extraction, followed by PCR based genotyping to detect CRISPR/Cas9 edited clones.
9. Meanwhile, you can freeze the second plate as a back-up (20 %). It is recommended to use the BamBanker cryopreservation solution instead of 10 % DMSO when freezing iPSCs in a multiwell plate instead of cryovials.
10. Aspirate the medium and cover the wells with dissociation buffer. Incubate the plate for 3-5 min at 37 °C. Aspirate the Dissociation reagent and add 0.3-0.5 ml of BamBanker cryopreservation solution. Gently dissociate the colonies by repeated pipetting. Seal the plate with Parafilm and store the plate at -80 °C in a Styrofoam box until further use.

11. In most cases, cryo-preservation of the single cell derived clones after further expansion is recommended as a back-up (section 3.2.4).

3.5 Analysis techniques of CRISPR/Cas9 induced modifications

There is a variety of methods to analyze genome modifications. Table 1 summarizes the most common methods for analyzing indels or point mutations. Most of them are based on PCR amplification of the targeted region, and in such cases optimization of the PCR reactions is a prerequisite for clear analysis results. For successful PCR we recommend using a high fidelity polymerase such as Herculase II (Agilent) or Q5 (NEB). We suggest to use fragment analysis or Sanger sequencing followed by TIDE [48] analysis for indel detection in clonal derived cells. To detect indels in bulk populations we use deep sequencing of fragments. For evaluation of inserted point mutations we recommend RFLP to pre-screen bulk populations or single cell derived clones and Sanger or deep sequencing for verifying the correct integration of the targeting donor.

- Table 1 -

3.5.1 PCR design

Depending on the analysis assay the requirements on the PCR reaction differ mainly in the size of the PCR product. The major requirement for all analysis methods is a specific amplification of the desired genomic locus. Therefore while designing the primers heterozygous mutations or SNPs as well as repetitive sequences should be avoided. When using Sanger sequencing followed by TIDE analysis the smallest recommended product length is 250 bp. To guarantee adequate sequence decomposition, the sgRNA targeting site should be 150 bp – 400 bp distant from the sequencing primer binding site. For fragment analysis, amplicon sizes ranging from 200-650 bp can be used to ensure a precise resolution up to a single base. However, the smaller the amplicons are the higher the resolution will be. For the RFLP assay the PCR should be designed in a way that product length should be in a range of 400-1200 bp and the sgRNA target site is off-set of the amplicon center.

3.5.2 PCR reaction and purification

Genomic DNA (gDNA) can be extracted from hiPS cells of either a semi-confluent well of a 6-well plate or a confluent well of a 12-well plate with the Wizard (Promega) or other DNA purification kits. PCR reactions are carried out using 100 ng gDNA in 50 µl with Herculase II according to manufacturer's instructions. For Sanger sequencing or fragment analysis the

PCR products are gel purified using e.g. the Wizard SV Gel and PCR Clean-Up System (Promega).

3.5.3 Sanger sequencing

Purified PCR products are prepared for sequencing using 20 pMol of sequencing primer and 60 -150 ng amplicon. The sequencing can be conducted by a commercial provider, e.g. LGC Genomics.

3.5.4 TIDE analysis

Tracking of Indels by DEcomposition (TIDE) is a method for quantitative assessment of genome editing events (https://tide.nki.nl/) [48]. By aligning the sgRNA sequence to the control sequence TIDE first identifies the expected Cas9 induced double-strand break site. Based on the peak heights TIDE then analyzes the abundance of aberrant nucleotides over the length of the sequence. The sequences are then evaluated with a decomposition algorithm to identify the insertion and deletion mutations in the subjected sequencing file. Clear sequencing traces are an essential requirement for solid indel detection and quantification. For the TIDE analysis the sequencing files of mutated and control samples can be uploaded to the TIDE web tool as .ab1 or .scf files and analyzed with the preset parameters, except for the indel size range which is reset to 20.

3.5.5 Fragment analysis

Fragment analysis is a useful tool to evaluate indel formation in single cell derived clones as well as in cell populations. When loading fluorescent labeled PCR products of the edited sgRNA target site on a capillary sequencer, amplicons differing in size due to small insertions or deletions can be separated with accuracy in resolution, depending on the size of the PCR product. PCR products with a length of up to 650 bp can be separated with a resolution of a single base. For this approach, fluorescently labeled forward primers (HEX, TET or 6-FAM) must be used to amplify the region of interest. Subsequently the PCR products are loaded on an agarose gel, purified using a PCR Clean-Up System (Promega) and 60 - 150 ng of the purified samples are subjected to analysis (SMB Service GmbH, Berlin, Germany) using LIZ-500 size standard.

3.5.6 RFLP assay

RFLP assays can be applied to assess indel formation when a restriction enzyme recognition site is located at the sgRNA targeting sequence, adjacent to the site of DSB, or to screen for

HDR events when additional restriction sites are inserted in the donor template. The amplicon size for this assay type should range from 400 bp to 1200 bp whereas the restriction site should be off-set to the center to facilitate the detection of both restriction fragments. The PCR products can be digested without further purification steps when choosing restriction enzymes that are active in the PCR buffer. For the digestion 20 µl PCR product are combined with 5 units of the selected enzyme, incubated at 37 °C for 1 h and analyzed on a 1.5 % agarose gel. RFLP analysis can also be combined with fragment analysis, when using fluorescent labeled primers for amplification. We recommend RFLP assays to determine the abundance of mutagenesis events in a bulk population, to estimate the efficacy of a CRISPR/Cas9 experiment and decide how many clones to analyze.

3.5.7 T7E1 Assay

The T7E1 and Surveyor assays can be used to assess indels and heterozygous point mutations in single cell derived clones as well as in bulk populations. Furthermore these assays are useful tools to test the efficacy of sgRNAs in cell lines like 293 cells. Both assays are used to detect DNA hetero-duplex formations of PCR amplicons by hybridization of mutant and wild type sequences or two different mutant sequences. These mutation-sensitive methods fail to detect homo-duplexes e.g. formed by two identical mutant sequences. Similar to RFLP the amplicon size for T7E1 assay should range from 400 bp to 1200 bp with the CRISPR/Cas9 targeting site off-set to the center of the amplicon. After the PCR reaction a clean-up step was performed and subsequently the purified fragments were denatured for 5 min at 98 °C in a heat block and re-annealed by switching of the heat block for 1-2 h. 200 ng PCR product was digested with 5 units T7E1 (NEB) for 15 min at 37 °C. After incubation the samples were immediately transferred to ice and supplemented with gel-loading-buffer. The digested fragments were separated on a 1.5 % agarose gel. In our experience the T7E1 assay often results in nonspecific cleavage products when applied to iPS cell derived gDNA, hence often needs further optimization steps for different loci. Therefore we recommend using RFLP (section 3.5.6), Fragment analysis (section 3.5.5) or TIDE analysis (section 3.5.4).

3.5.8 Off-target analysis

Off-target sites of sgRNA are predicted using CRISPOR or CRISPR DESIGNER (http://crispr.mit.edu/). In general off-target sites with an off-target hit-score > 0.9 are analyzed by PCR amplification and Sanger sequencing followed by TIDE analysis. In our

experiments we have observed no off-target effects when designing sgRNAs with the web tool CRISPOR (http://crispor.tefor.net/) and choosing sgRNAs with specificity scores > 60 and efficacy scores (Fusi score) > 45. Although CRISPR/Cas9 induced off-target effects are rare in hiPSCs, especially since safer and more accurate sgRNA designing web tools are available, we encourage the analysis of such potential effects in genome edited cells. In particular undesired mutations may distort the results when the edited cells are subjected to a phenotypic comparison with control cells.

4. Results

Since expansion and analyzing of genome edited iPSC clones is laborious it is important to pick and analyze a feasible number of clones. In the following section we summarize the experience we gathered in our lab to provide guidance in regard to the number of clones to be analysed. The following data are based on three independent sgRNAs each targeting an individual native locus. The results we present in section 4.1 are a summary of experiments where a single guide of each three was used at a time (Workflow A). In section 4.2 we summarize experiments that implement all three guides at once (Workflow B). At this point we would like to note that targeting efficacies are, among others, dependent on the locus of interest, cell lines and the performance of the selected sgRNA. Therefore, we consider our presented data as a reference point and not as a universal rule.

4.1 Results obtained with Workflow A

In the course of optimizing gene targeting with transient transfection of Cas9 and sgRNA (Workflow A) we started with an approach using a plasmid vector carrying Cas9 together with a fluorescent reporter and a separate vector encoding only for a single sgRNA without any selection gene. The hiPSCs were enriched by FACS for the fluorescent reporter 48h after transfection. With this approach we could enrich only the fraction of cells transfected with the nuclease but not the guide RNA (Table 2; separate vectors). We analyzed 176 sorted, single cell derived clones and obtained only two mutagenized clones (1 %) where one clone was targeted on both alleles and the second clone on one allele. In our next approach we transfected hiPSCs with combined vectors, coding for Cas9, the sgRNA cassette as well as a fluorescent reporter (pU6-(*BbsI*)sgRNA_CAG-Cas9-venus-bpA or pU6-(*BbsI*)sgRNA_CAG-Cas9_EF1-TagRFP). Using this strategy, we could enrich for cells carrying Cas9 and the guide RNA. By this method we achieved a mutagenesis rate of 17 % in 116 analyzed clones. 70 % of the mutagenized clones were targeted on both alleles

and 30% only on one (Table 2; combined vector).

Based on these results, we conclude that when it is intended to introduce frameshift indels in one single gene using transient transfection of Cas9 and sgRNA carrying fluorescent reporter vectors (Workflow A) followed by FACS enrichment, 24 cell clones is a reasonable number to obtain 2 to 4 clones with mutations on both alleles.

- Table 2-

4.2 Results obtained with Workflow B

In experiments where we used the doxycycline inducible Cas9 system with transient transfection of fluorescent reporter vectors that express three different sgRNAs (Workflow B) followed by FACS enrichment, we observed indel mutations in all three loci (Parkin, Dj1, Pink1) in two out of 24 single cell derived hiPS cell clones (8 %). In one clone we detected indels in two loci (4 %). None of the analyzed clones were mutated in only one locus. 71 % of targeted loci were mutated in a bi-allelic manner (Table 3; Single round).

Additionally, we conducted an experiment where we followed Workflow B as described above, but co-transfected the plasmid pCAG-Trex2-bpA to co-express the end-processing enzyme Trex2. This experiment resulted in a targeting efficacy of 15 % for triple and 15 % for double gene mutagenesis (20 clones analyzed in total). None of the analyzed clones were mutated in a single locus only. Analogous to the previous experiments the majority of the mutated loci were targeted on both alleles (Table 3; Single round +Trex2).

Finally we could improve the targeting efficacy by repeating the transfection and FACS sorting procedure a second time (Table 3; Second round). In 15 of in total 57 single cell derived human iPS cell clones we could detect triple gene mutagenesis (26 %). 7 clones were targeted on two loci (12 %) and 4 clones carried indels in only one of the targeted loci (7 %). In accordance with our previous observation the majority of mutations occurred on both alleles of a locus.

-Table 3-

Thus we recommend based on our experience, when it is intended to introduce frameshift indels in multiple loci, we suggest using the doxycycline inducible Cas9 system with transient transfection of sgRNA carrying fluorescent reporter vectors (Workflow B) followed by FACS enrichment. In this case we recommend selecting 36 to 48 clones to have a feasible number of multiple edited cell clones. To minimize the number of clones to analyze during multiplex gene editing we suggest: a) co-transfection with the plasmid pCAG-Trex2-

bpA for expression of the end-processing enzyme Trex2 and b) repeating the transfection and FACS enriching procedure a second time (sections 3.3 and 3.4). In this regard analyzing 16 to 24 clones should be adequate to obtain various clones with mutations on both alleles for the desired loci (Table 2 and Table 3). If precise genome editing is desired (HDR events) we suggest to first determine the efficacy of mutagenesis events in a bulk population e.g. by RFLP analysis and depending on that estimate the number of clones to be picked and analyzed.

4.3 Quality of targeted human iPSCs

To monitor, if the CRISPR/Cas9 based gene targeting strategies we describe here have an influence on the genetic stability of the targeted cells, we performed SNP analysis based virtual karyotyping on edited human iPS cells. The karyotyping was performed with the Infinium OmniExpressExome-8 v1.2 BeadChip system that covers over 960,000 common SNPs over the human genome. We analyzed two hiPSC clones that were successively targeted at the AAVS1 locus to initially create doxycycline inducible Cas9 expressing hiPSCs and afterwards at three native loci by two rounds of transfection followed by FACS enrichment. Both characterized clones were successfully targeted on both alleles of all three loci.

The SNP based karyotyping revealed no chromosomal aberrations. We detected eight SNPs that were absent in an early passage of the parental cell line (data not shown). It is unclear if these SNPs emerged during cultivation or are an artifact of the editing process. However, pluripotent stem cells are generally considered as genetically unstable and we could demonstrate that our editing method does not exacerbate this phenomenon.

Additionally we analyzed the edited clones for potential mutations at predicted CRISPR/Cas9 off-target sites for the individual sgRNAs used here (section 3.5.8). We analyzed the most relevant loci with a hit-score > 0.9 regarding to CRISPOR and CRISPR DESIGNER. We could not detect any mutation at these sites.

-Figure 7-

We subjected targeted cell clones mentioned above to immunostainings for the pluripotency markers OCT4, NANOG, SOX2 and SSEA4 (Figure 7A). We confirmed that targeted hiPSCs express common pluripotency markers.

Furthermore we examined the differentiation potential of hiPS cells, targeted with our workflow, to the ectodermal lineage. We performed a neuronal differentiation assay with

gene targeted hiPSCs and could demonstrate that these cells are capable to differentiate to TUJ1 and TH expressing neuronal cells (Figure 7B).

5. Conclusion

Two groundbreaking discoveries of the past decade, human iPS cell technology and gene editing using CRISPR/Cas9, provide new opportunities for researchers. Combining both allows us to study the function of a gene in a human system in the context of specific diseases or even gene therapeutically applications by correcting gene defects in patient cells. Using CRISPR/Cas9 in immortalized, fast-cycling cell lines is well established. However, using this system in hiPS cells is more challenging due to the sensitive requirements of hiPS cells to maintain culture pluripotency. Although generating precise knockins via HDR is relatively inefficient, the possibility to use selection markers or introducing recognition sites for restriction enzymes in the repair template facilitates screening for successfully targeted cells. CRISPR/Cas9 mediated gene disruption by introducing indel mutations on the other hand occurs more frequently but screening of clonal derived cells is more laborious.

We describe here a simple and efficient workflow for clonal isolation and screening of genome engineered human iPS cells without prior drug selection. Applying this workflow to hiPS cell lines equipped with an inducible Cas9 under a strong promoter even enables the generation of multiple targeted mutant alleles in a single experiment.

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Tables

Table 1 Summary of analysis methods for genome editing events

Assay	Advantage	Disadvantage	Application
Sanger/Tide	Quick and simple; High accuracy for single cell derived clones	Low accuracy for quantification in bulk population	Indel detection in clonal derived cells
Cloning/Sanger	High accuracy	Very laborious	For verification of knockins or indels in single cell derived clones
Fragment Analysis	Quick and simple; High accuracy for single cell derived clones	Requires special equipment/service	Indel detection in clonal derived cells or bulk populations
RFLP	Quick and simple; High accuracy for single cell derived clones	Low accuracy for quantification on bulk populations	Detection of knockins or indels in single cell derived clones or bulk populations
Deep Seq	High accuracy	Requires special equipment/service	Detection of KI or indels in bulk populations and single cell derived clones
T7E1/Surveyor	Quick and simple	Need thorough optimization	To pre-test sgRNAs in cell lines

Table 2 Results of gene editing in hiPSC using Workflow A

Approach: Workflow A	Analyzed clones	Single gene mutagenesis	Mono allelic events	Bi alleleic events
Separate vectors: Cas9-fluorescent reporter and sgRNA	176	1%	50%	50%
Combined vector: Cas9-sgRNA-fluorescent reporter	116	17%	30%	70%

Table 2 Results of gene editing in hiPSC using Workflow B

Approach: Workflow B	Analyzed clones	Single gene mutagenesis	Double gene mutagenesis	Triple gene mutagenesis	Mono allelic events	Bi alleleic events
Single round: inducible Cas9 with transient sgRNA-fluorescent reporter	24	0%	4%	8%	29%	71%
Single round +Trex2: inducible Cas9 with transient sgRNA-fluorescent reporter and mTrex2	20	0%	15%	15%	33%	67%
Second round: inducible Cas9 with transient sgRNA-fluorescent reporter and Trex2	57	7%	12%	26%	16%	84%

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Figure Legends

Figure 1 The CRISPR/Cas9 nuclease system

A: Cas9 and sgRNA recognize 20 nt target sequences located upstream of the invariant 3 bp NGG PAM sequence (red letters). B: If a target sequence is found upstream of the PAM site, each of the RuvC and HNH nuclease domains of Cas9 cut one strand of the target DNA, creating a DSB (triangles). C: DSBs in target genes can be either repaired by NHEJ, leading to small sequence deletions and gene knockout alleles. Precise sequence modifications (Knockin alleles) are introduced by HDR with the homology regions of DNA repair templates, either an ssODN or plasmid vector.

Figure 2 Strategies for intracellular delivery of components required for CRISPR/Cas9-mediated gene editing

A Transfection of an expression vector carrying both a sgRNA cassette and the Cas9 gene enables gene editing via DSBs in wildtype cells. **B** For transgenic cell lines that express Cas9 under a doxycycline-inducible promoter, transfection of a vector encoding for sgRNA(s) only is sufficient. Successive administration of doxycycline activates Cas9 expression. This approach achieves improved efficacy at targeting more than one locus. A reporter gene included in either of these strategies allows for the identification of transfected cells. **C** Doxycycline (DOX) dependent expression of Cas9 is provided by a modified AAVS1 locus. While the DOX-inducible promoter TREtight and the Cas9 gene are knocked in between the first two exons of the PPP1R12C gene (AAVS1 locus) on one allele, the other allele harbors the Transactivator gene rtTA3 expressed under the constitutively active CAGGS promoter. Presence of DOX mediates binding of rtTA to TREtight, thus activation of Cas9 expression.

Figure 3 HDR efficacy using ssODNs of various architectures

Cells of the hiPSC line BCRT#1 that heterozygously express EGFP driven by a CAG promoter from the AAVS1 locus were co-transfected with an expression vector for both Cas9 and sgRNA targeting EGFP and various ssODN donor templates.
A Donor templates were designed to convert EGFP to BFP in case of HDR events, in symmetric (sym) [44] or asymmetric (asm) [42] fashion, with and without phosphorothioate modification (PTO) [43]. **B, C** Positively transfected cells were isolated by FACS, based on

an RFP fluorophore included in the expression vector. 7 days post transfection HDR/ indel frequency was assessed by FACS analysis. Absent fluorescent signal corresponds to indels due to disrupted EGFP, BFP^+ cells to successful HDR and $EGFP^+$ cells indicate cells not undergoing any event. Occurrence of no event was generally low (<4%). HDR frequency of both PTO-modified symmetric (6.1%) and asymmetric (4.0) ssODNs were below the efficacy of unmodified asymmetric donor templates (12.8%). The highest HDR efficacy was obtained using unmodified symmetric ssODNs (20.8%). Experiments were conducted in three independent replicates; error bars represent standard error of the mean; significance as per Student's t-test: *<0.05, **<0.005, ***<0.0005.

Figure 4 Overview on the workflow for CRISPR/Cas9-mediated generation of a transgenic hiPS cell line without drug selection

Initially, vectors expressing components necessary for CRISPR/Cas9-mediated gene editing, i.e. locus specific sgRNA, a fluorescent reporter and optionally Cas9, are required. Available combined plasmids provide convenient cloning strategies to include custom sgRNA sequences. After transfection of wildtype hiPS cells with these vectors, the included fluorescent reporter enables enrichment of successfully transfected cells utilizing FACS. Isolated transfected cells are sparsely seeded and expanded for subsequent growth of single cell-derived colonies. Appropriate means for analyses allow for the selection of a favored colony, which is then established as cell line.

Figure 5 Transfection efficacy and cell viability applying various transfection methods using vectors of different size and diverse cell lines

The efficacy of iPS cells transfected with a vector was compared using a small guide-only (4 kb) and a large combined plasmid (12 kb) applying three methods: Gene Pulser Xcell, Neon Transfection System and Lipofectamine 3000. By Gene Pulser Xcell 20 µg of DNA were transfected, 5 µg by Neon System and 1.5 µg by Lipofectamine 3000. Efficacy was assessed by FACS analysis, counting positively transfected single cells detected by a fluorescent reporter included in the vector. $5x10^5$ cells were subjected to Gene Pulser Xcell and Neon Transfection System of which $1x10^5$ were subsequently cultured. $1x10^5$ cells were subjected to Lipofectamine 3000 transfection. Viability was determined after 48h by FACS based quantification of remaining single cells. **A** Cells of the BCRT#1 iPS cell line were transfected at differing efficacy when subjected to Gene Pulser Xcell, Neon Transfection System and

Lipofectamine 3000. Transfection efficacy delivering the small guide-only vector amounts to 41% of all FACS identified single cells using Gene Pulser Xcell, 65% with the Neon System and 82% after Lipofectamine 3000 transfection. Delivering the large combined vector yields a decreased efficacy regardless the method used. Transfection by Gene Pulser Xcell results in 10% of successfully transfected single cells, by Neon Transfection System 27% and applying Lipofectamine 3000 achieve 59%. **B** Viability of transfected BCRT#1 iPS cells varies depending on the transfection method used as well as on the size of the vector delivered. After transfection of the 4 kb vector by Gene Pulser Xcell, Neon System and Lipofectamine 3000, 6900, 5400 and 6800 remaining single cells were detected, respectively. Subjecting the 12 kb vector to transfection yielded 4000, 400 and 2500 live cells. **C** Efficacy of transfecting a 4 kb vector varied between the hiPS cell lines BCRT#1, XM001 and MIRJT7i-mND2-0. Treatment with Gene Pulser Xcell resulted in 41%, 50% and 25% efficacy, with Neon Transfection System 65%, 90% and 38%, with Lipofectamine 3000 82%, 96% and 90%, respectively. **D** Cell viability was evaluated after applying different transfection methods to BCRT#1, XM001 and MIRJT7i-mND2-0 iPS cell lines. After transfection by Gene Pulser Xcell 6800, 5800 and 4200 live cells of the respective cell lines were detected, after Neon Transfection System application 5400, 5700 and 2600 remaining single cells were quantified, and after Lipofectamine 3000, 6900, 5100 and 100 cells. Experiments were performed in triplicates; error bars represent standard error of the mean; significance as per Student's t-test: *<0.05, **<0.005, ***<0.0005, n.s.=not significant.

Figure 6 Scheme of the enrichment of hiPS cells transfected for CRISPR/Cas9-mediated gene editing

A After transfection of hiPS cells with an appropriate reporter expressing vector, transfected cells are identified by FACS based on their fluorescent signal. To increase the yield of successfully targeted cells, isolated cells can be expanded and resubjected to transfection and FACS enrichment. Representative image of a FACS plot. **B1** Single FACS-isolated transfected hiPS cells are cultivated sparsely. **B2-B5** During the next 5 days following seeding a small colony arises from a single cell. **B6** After 5 days of culturing a colony forms large enough for manual picking and further expansion. Scale bar represents 100 µm.

Figure 7 Targeted hiPSCs express pluripotency markers and can differentiate to the ectodermal lineage

A Edited hiPS cells (BCRT#1; edited) and the parental cell line (BCRT#1; parental) were subjected to immuno-cytochemistry using antibodies against the pluripotency markers SSEA4 (NEB #4755S; 1:500) and NANOG (NEB #4903S; 1:500), OCT4 (NEB #2750S; 1:500) or SOX2 (NEB #3579S; 1:500), respectively. Nuclei were visualized by DAPI. The edited cell line was targeted in two successive steps. Firstly, the *AAVS1* locus was targeted to express doxycycline inducible Cas9. Subsequently, three native loci were biallelically mutated following Workflow B, including two rounds of transfection (and co-transfection of Trex2) and FACS enrichment. **B** The edited cell line (BCRT#1; edited) and a control cell line which was targeted at the *AAVS1* locus to express Cas9 under a doxycycline inducible promotor (BCRT#1; control) were differentiated using the Dopaminergic Neuron Differentiation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. At Day 38 cells were fixed and subjected to immuno-cytochemistry for the pan-neuronal marker TUJ1 (BioLegend #801202; 1:1000) and the dopaminergic neuronal marker TH (Pel-Freez #P40101; 1:1000). Nuclei were visualized by DAPI. **A and B** Cells were cultivated on glass cover slips coated with VTN-T (**A**) or Laminin/Poly-D-Lysin (**B**). Prior to staining cells were fixed for 7 min at RT with 4 % PFA in PBS. Cells were washed thrice with PBS and subsequently permeabilized and blocked for 30 min at RT with PBS containing 3 % BSA and 0.2 % TritonX-100. The cells were washed three more times with PBS and incubated with the respective primary antibodies O/N at 4 °C. The next day, cells were again washed thrice with PBS and incubated for 1 h at RT with secondary antibodies (goat-anti rabbit-AF488 and goat anti-mouse-AF546, both Thermo Fisher Scientific) and DAPI in PBS supplemented with 3 % BSA. After final three washes in PBS cells were mounted on glass slides with Fluoromount-G (SouthernBiotech). Imaging was performed on a LSM700 confocal microscope (Zeiss) with a 20x objective.

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Figure 1

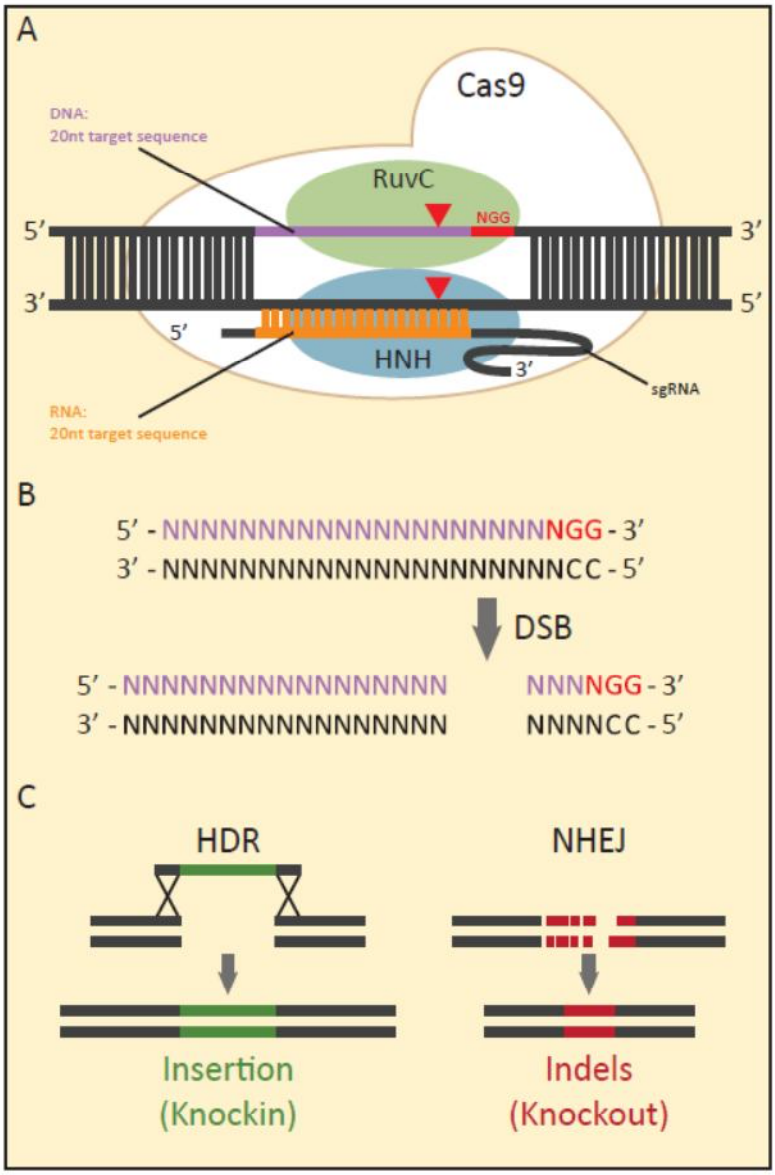
A
Cas9
DNA:
20nt target sequence
RuvC
NGG
5′
3′
3′
5′
5′
HNH
3′
sgRNA
RNA:
20nt target sequence
B
5′ - NNNNNNNNNNNNNNNNNNNNNGG - 3′
3′ - NNNNNNNNNNNNNNNNNNNNNCC - 5′
DSB
5′ - NNNNNNNNNNNNNNNNN
NNNNGG - 3′
3′ - NNNNNNNNNNNNNNNNN
NNNNCC - 5′
C
HDR
NHEJ
Insertion
(Knockin)
Indels
(Knockout)

Figure 2

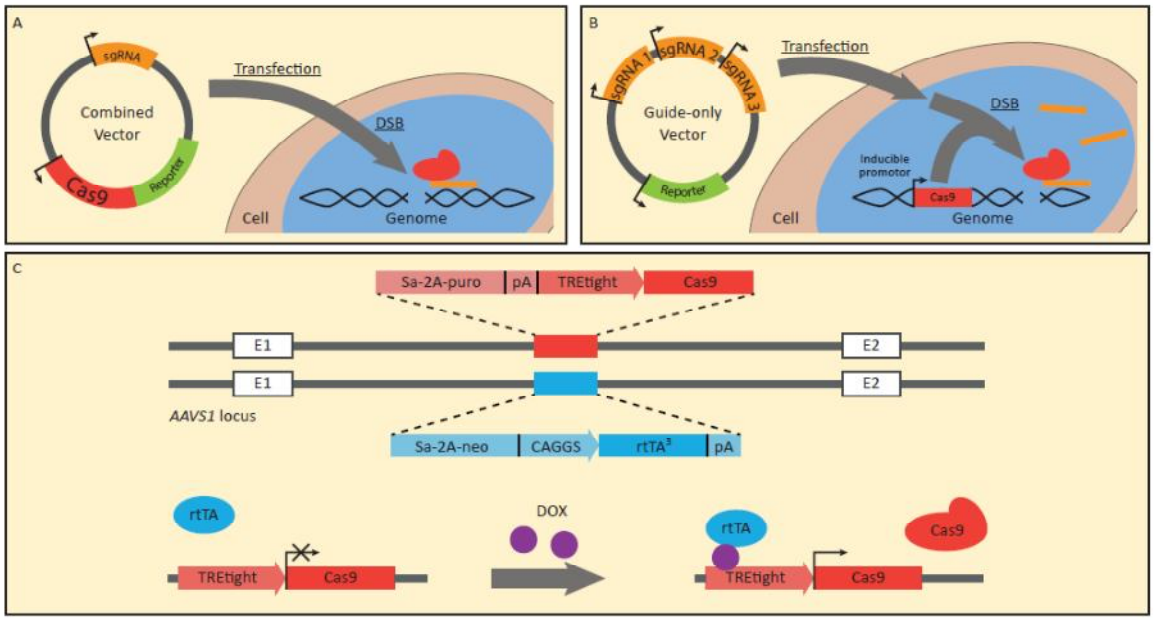

Figure 3

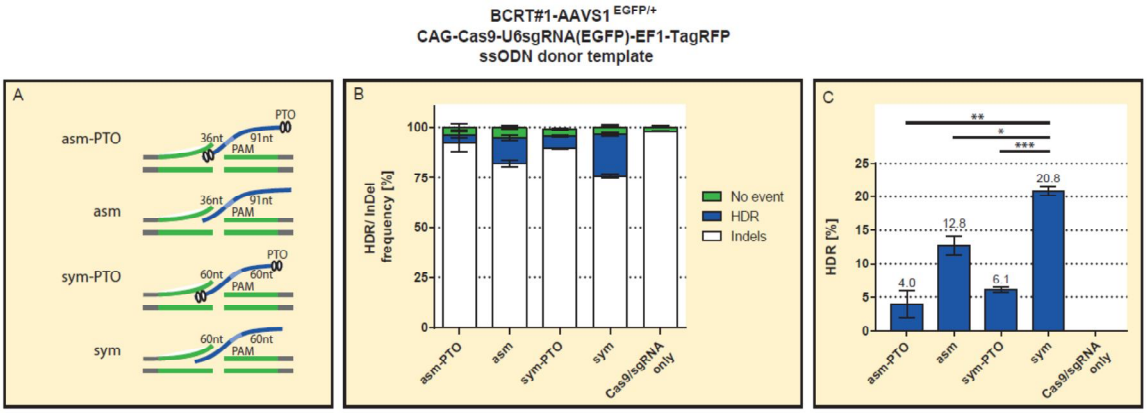

Figure 4

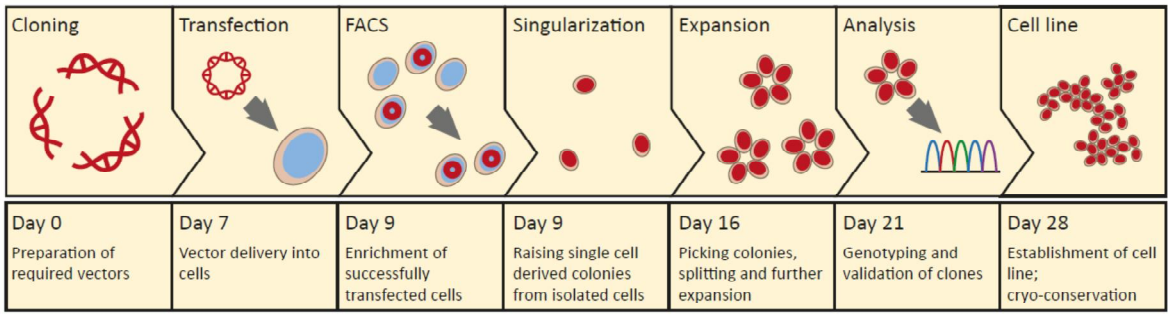

Figure 5

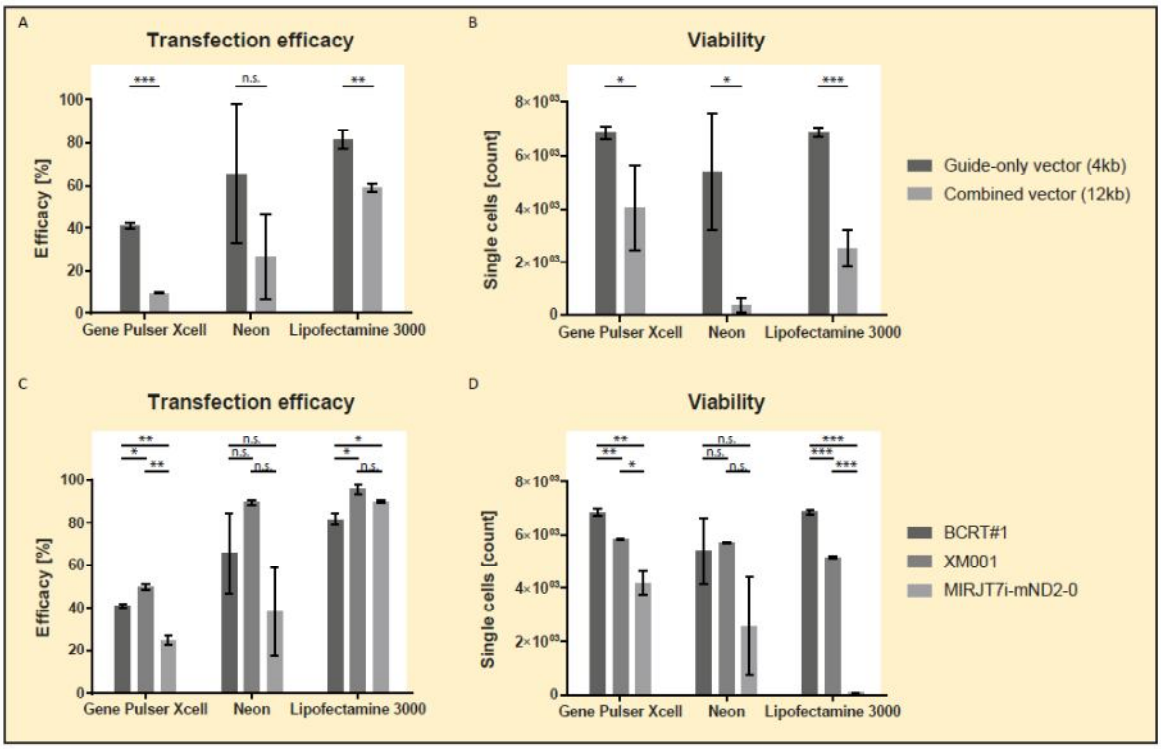

Figure 6

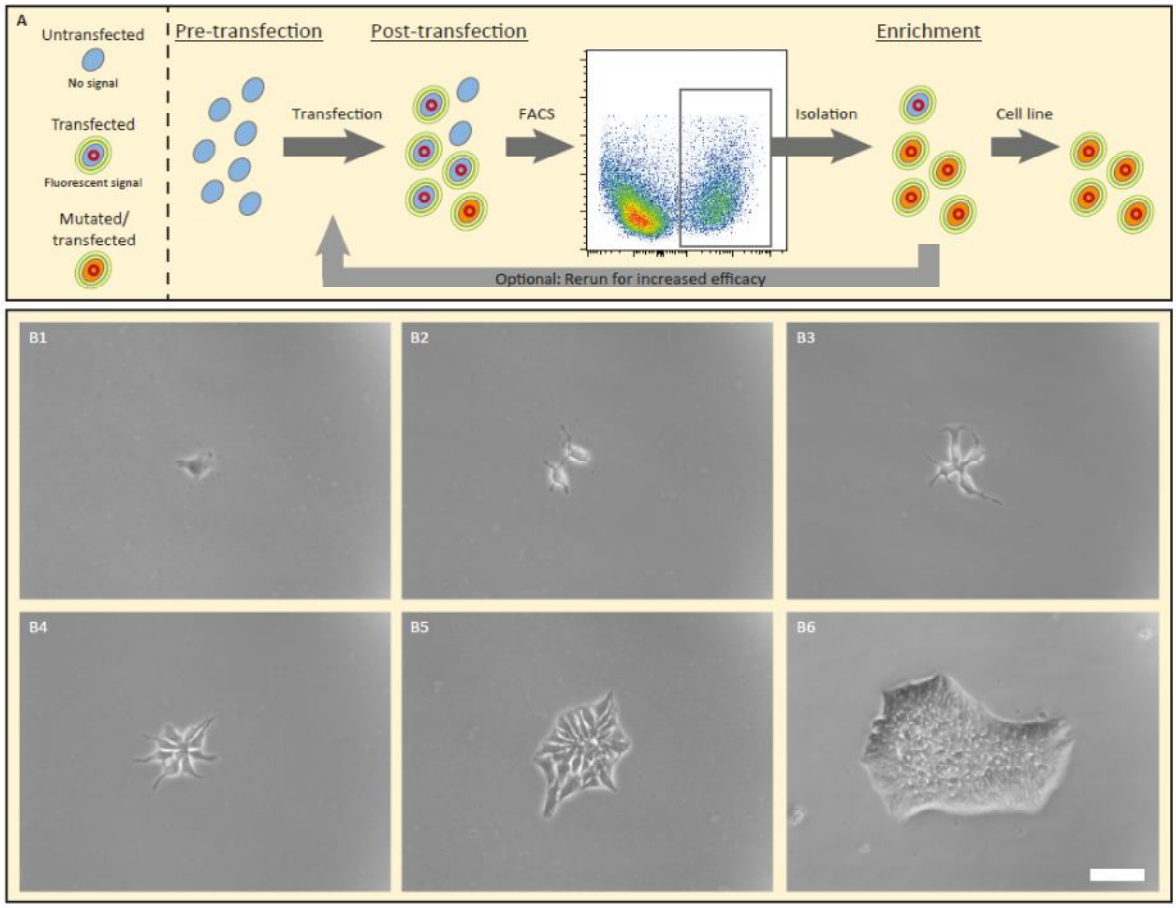

Figure 7