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METHODS Issue: CRISPR-Cas systems for genome engineering and investigation

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

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## 1 **Abstract**

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

14

## 15 **1. Introduction**

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

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

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

42

### 43 **1.1 Gene editing in hiPSCs**

44 Due to its efficiency and simplicity, the CRISPR/Cas9 system derived from *Streptococcus*  
45 *pyogenes* [10] has become the preferred method for gene editing in mammalian cells [11–  
46 13]. CRISPR/Cas9 gene editing requires two basic components to achieve a DNA double-  
47 strand break (DSB) and subsequent gene editing: the Cas9 nuclease protein and a single  
48 guide (sg)RNA to home the Cas9 nuclease. A DNA repair template is often additionally  
49 provided to allow for homology directed repair (HDR). The first twenty nucleotides of sgRNAs  
50 direct Cas9 to a specified complementary DNA target sequence via RNA-DNA hybridization.  
51 The target sequence is always located upstream of an invariant protospacer adjacent motif  
52 (PAM) sequence (NGG for SpCas9 from *Streptococcus pyogenes*). Upon correct  
53 hybridization the Cas9 nuclease undergoes a conformational change and creates a DSB  
54 located 3 bp upstream of the PAM site (Fig.1A, B) [14]. An advantage of the CRISPR/Cas9  
55 system is that the Cas9 protein component is of universal use and new target sites can be  
56 easily targeted by addition of a new sgRNA sequence. The gene editing process follows  
57 induction of DSBs where DNA repair pathways result in deletion, replacement or insertion of  
58 sequences.

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

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

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

94         Frequent gene editing applications include the correction of disease-related mutations  
95 in patient-derived hiPSCs. The resulting engineered hiPSCs differ from the parental cells  
96 exclusively at the edited loci and are otherwise isogenic. Parallel differentiation of these  
97 isogenic cell lines into disease-relevant cell-types provides the basis for the phenotypic  
98 analysis of cellular pathologies that are directly attributable to the genetic specific mutations  
99 [2,20,21]. This reversion of disease-related phenotypes in cells derived from corrected  
100 hiPSCs can serve as a paradigm for the feasibility of future cell based therapies. The  
101 CRISPR/Cas9 system can be used for modification of both copies of a target gene, or  
102 restricted to induce changes only in alleles of interest by selective gene editing based on  
103 haplotype-specific target sites [22–25].

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

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

124

- Figure 1 -

125

126

## 127 **1.2 Human induced pluripotent stem cell lines**

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

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

### 142 **1.3 Gene editing approach**

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

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

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

170 The first approach, enables editing of single genes by transfection of a vector  
171 encoding Cas9 and sgRNA (cloned into pU6-(BbsI)sgRNA\_CAG-Cas9-bpA\_EF1-TagRFP or  
172 pU6-(BbsI)sgRNA\_CAG-Cas9-venus-bpA, section 3.1.1) into unmodified hiPSC lines



173 (Workflow A, Fig. 2A). Included fluorescent Venus or RFP reporters enable subsequent  
174 FACS isolation of transfected cells. The second approach focuses on multiplex gene editing  
175 (Workflow B, Fig. 2B, 2C). This multiplexing protocol uses hiPSC lines with a doxycycline  
176 inducible Cas9 expression system integrated in the AAVS1 locus. We currently use a  
177 modified version of the iCRISPR system [19] which is available upon request. As described  
178 in section 3.3, these iCRISPR lines show a strong Cas9 expression when induced with  
179 doxycycline. These cells are transfected with a small vector for the expression a fluorescent  
180 Venus reporter and of multiple sgRNAs (cloned into plasmid pU6-(BbsI)sgRNA\_CAG-Venus-  
181 bpA; section 3.1.1). This allows the fraction of successfully transfected hiPSCs expressing  
182 sgRNAs and the reporter to be isolated by FACS. The sorted cells are then plated for the  
183 establishment of single cell derived colonies (section 3.4).  
184 Further improvements of this technology may be possible. We have found Cas9 to be  
185 heterogeneously expressed in iCRISPR cell lines after doxycycline induction. Therefore, we  
186 are further testing vectors for the induction of Cas9 and a second fluorescent reporter,  
187 enabling the two color FACS enrichment for cells expressing high levels of Cas9 and  
188 sgRNAs.

189

190

## - Figure 2 -

191

### 192 **1.4 Guide RNA selection and project planning**

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

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

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

242           Recent studies suggest that choice of the target strand and an asymmetric design of  
243 ssODNs increase the knockin efficiency [42] and that phosphorothioate (PTO) modifications  
244 improve stability and HDR efficacy [43]. However, it was not shown if these rules apply to  
245 hiPSCs as well. We have performed a side by side evaluation of ssODNs with symmetric or  
246 asymmetric structure with or without PTO modifications (Fig. 3). Analogously to Glaser et al.  
247 [44], we used human iPS cells heterozygously expressing a CAG promoter driven EGFP  
248 from the AAVS1 locus as a reporter system for HDR. The EGFP gene was targeted with a  
249 sgRNA to induce a DSB. By homologous recombination of donor templates that substitute 4  
250 bp, the original green fluorescent EGFP is converted to blue fluorescent BFP and the PAM  
251 sequence rendered non-functional, as previously reported [44]. Based on this assay, we  
252 observed that in hiPSCs unmodified ssODNs without PTO modifications yield higher HDR  
253 rates compared to modified ones (Fig. 3). Furthermore, in contrast to HEK293 cells as  
254 reported by Richardson et al. [42], a symmetric architecture of ssODNs resulted in higher  
255 HDR efficiencies than an asymmetrical in hiPSCs (Fig. 3). However, the efficacy of  
256 CRISPR/Cas9 mediated targeting of specific loci depends on the cell line subjected, the  
257 localization of the locus within the chromatin architecture and the sequence itself. Therefore,  
258 these observations cannot be generalized, but may vary in respect to the individual purpose.

259

### - Figure 3-

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

273

## 274 **1.5 Workflow of CRISPR/Cas9 gene editing in hiPSCs**

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

296  
297

298 **- Figure 4-**

## 299 **2. Materials**

### 300 **2.1 Vectors**

#### 301 Workflow A

302 - pU6-(*BbsI*)sgRNA\_CAG-Cas9-bpA\_EF1-TagRFP (Addgene ID 86987,

303 [www.addgene.org](http://www.addgene.org)) or

304 -pU6-(*BbsI*)sgRNA\_CAG-Cas9-venus-bpA (Addgene ID 86986)

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

307

#### 308 Workflow B

309 - pU6-(*BbsI*)sgRNA\_CAG-venus-bpA (Addgene ID 86985)  
310 Plasmid for the cloning of single or multiple sgRNAs into the *BbsI* site and the  
311 expression of Venus.

312

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

315

## 316 **2.2 Cloning sgRNAs**

- 317 1. Empty sgRNA cloning vectors (section 2.1)
- 318 2. A pair of target specific sgRNA oligos with *BbsI* overhangs, e.g. MWG-Biotech
- 319 3. *BbsI* restriction enzyme, NEB, #R0539S
- 320 4. *AscI* restriction enzyme, NEB, #R0558S
- 321 5. T4 Ligase, NEB, #M0202S
- 322 6. PureYield™ Plasmid Miniprep System, Promega, #A1222
- 323 7. Chemical competent *E. coli* DH5 $\alpha$ , Thermo Fisher, #18265-017
- 324 8. Carbenicillin, Sigma, #C3416
- 325 9. LB medium and LB agar plates
- 326 10. Agarose gel electrophoresis (setup and consumables)

327

## 328 **2.3 General hiPS cell culture**

- 329 1. hiPS cell lines (e.g. HPSI0114i-kolf\_2, [www.hipsci.org](http://www.hipsci.org) or MIRJT7i-mND2-0,  
330 [www.wicell.org](http://www.wicell.org))
- 331 2. Essential 8 medium, Thermo Fisher Scientific, # A1517001
- 332 3. Y-27632 (dihydrochloride), Biomol, # Cay10005583-5
- 333 4. DPBS -/- (-Ca<sup>2+</sup>, -Mg<sup>2+</sup>), Life Technologies, # 14190169
- 334 5. Vitronectin (VTN-N) Recombinant Human, Life Technologies, # A14700
- 335 6. Matrigel Matrix, Fisher Scientific, # 10162371
- 336 7. DMEM/F-12 HEPES, Life Technologies, # 31330038
- 337 8. Cell culture plates (e.g. Corning Costar 6-well plates, # CLS3516-50EA)
- 338 9. Accutase solution, Sigma-Aldrich, # A6964
- 339 10. Bambanker Serum Free Cell Freezing Medium, BioCat, # BB01-NP
- 340 11. DMSO, Sigma-Aldrich, # D5879
- 341 12. Doxycycline Hydrochloride, Sigma-Aldrich, # D9891
- 342 13. Dissociation buffer: 0.18 % NaCl, 0.5 mM EDTA in DPBS (to 491.92 ml DPBS add 5  
343 ml of 0.5M EDTA pH 8.0 and 3.8 ml of 5M NaCl)

344 14. ReLeSR, Stemcell Technologies, # 05872

345

## 346 **2.4 Lipofection of hiPS cells**

347 1. Lipofectamine 3000 transfection kit, Life Technologies, # L3000001

348 2. Opti-MEM I reduced serum medium, Gibco, # 31985062

349

## 350 **2.5 Electroporation of hiPS cells with the Neon system**

351 1. Neon Transfection system, Thermo Fisher Scientific, # MPK5000

352 2. Neon transfection kit 100µl, Thermo Fisher Scientific, # MPK10025

353

## 354 **2.6 Electroporation of hiPS cells with the Gene Pulser system**

355 1. Gene Pulser Xcell Electroporation Systems, Bio-Rad, # 1652660

356 2. Gene Pulser electroporation cuvette 0.4 cm, Bio-Rad, # 1652081

357

## 358 **2.7 FACS enrichment of transfected hiPS cells**

359 1. 0.45 µm PTFE membrane filter, Millipore, # SLCR025NS

360 2. FACS tubes with 35 µm strainer cap, Falcon, # 352235

361 3. Penicillin/Streptomycin (10,000 U/ml), Life Technologies, # 15140122

362 4. Gentamicin (10 mg/mL), Life Technologies, # 15710049

363 5. RevitaCell Supplement (100X), Life Technologies, # A26445-01

364 6. Fluorescence activated cell sorter (e.g. BD FACSAria III, BD Biosciences)

365

## 366 **2.8 Analyzing modifications**

367 1. Wizard Genomic DNA Purification Kit, Promega, # A1125

368 2. Wizard DNA Clean-Up System, Promega, # A9282

369 3. Herculase II Fusion DNA Polymerase Kit, Agilent, # 600679

370 4. Q5 High-Fidelity DNA Polymerase, NEB, # M0491S

371 5. dNTP Set, Thermo Fisher Scientific, # R0181

372 6. Gene specific primers, e.g. MWG-Biotech

373 7. Fluorescence labeled gene specific primers, e.g. MWG-Biotech

374 8. Restriction enzymes, NEB

375 9. Thermocycler, e.g. Eppendorf

376 10. Agarose gel electrophoresis (setup and consumables)

377

378

379 **3. Methods**

380

381 **3.1 Cloning of sgRNAs**

382 This protocol recommends the usage of either plasmid pU6-(*BbsI*)sgRNA\_CAG-Cas9-  
383 venus-bpA or pU6-(*BbsI*)sgRNA\_CAG-Cas9\_EF1-TagRFP (Workflow A), encoding for  
384 sgRNA and Cas9 and a fluorescent Venus or RFP reporter, or usage of pU6-  
385 (*BbsI*)sgRNA\_CAG-venus-bpA, carrying a sgRNA-cassette and a fluorescent reporter only  
386 (Workflow B). These vectors allow expression of the sgRNA by the human U6-promoter. This  
387 promoter requires a “G” base at the transcription start site. Hence, it is recommended using  
388 CRISPR/Cas9 target sites starting with a “G”. Otherwise an additional “G” should be added  
389 at the start of the sgRNA sequence. It should be noted that wildtype Cas9 is amenable to  
390 the inclusion of an extra “G” but other RNA guided nucleases are not (Cas9-HF/eCas9). All  
391 three above mentioned plasmids together with their maps and sequences are deposited at  
392 Addgene ([www.addgene.org](http://www.addgene.org)) which also provides a wide range of other vector systems for  
393 Cas9 expression and sgRNA cloning.

394

395

396 **3.1.1 Cloning of a single sgRNA**

397 **Annealing of sgRNA oligos**

398 The sgRNA oligos can be cloned into the above mentioned vectors by *BbsI* restriction  
399 enzyme overhangs, with N<sub>1</sub>-N<sub>20</sub> as the selected Cas9 target sequence:

400

401 sgRNA-oligo-F 5' –CACC(G)N<sub>1</sub>NNNNNNNNNNNNNNNNNNNNN<sub>20</sub> –3'

402 sgRNA-oligo-R 5' –AAACNNNNNNNNNNNNNNNNNNNNNNN(C) –3'

403

- 404 1. Resuspend oligos at 1 µg/µl in 1x TE-buffer.
- 405 2. Combine in a microcentrifuge tube
  - 406 - 1 µl oligo F (1 µg/µl)
  - 407 - 1 µl oligo R (1 µg/µl)
  - 408 - 98 µl 1x TE-buffer
- 409 3. Incubate 5 min at 98 °C in a heat block.
- 410 4. Switch off the heat block and cool down slowly to RT for 1-2 h.
- 411 5. Put the annealed oligos on ice or store at -20 °C.

412

413 **Digestion of sgRNA expression vector**

- 414 1. Set up digestion reaction as following:  
415 - X  $\mu$ l of the above mentioned vectors (5  $\mu$ g)  
416 - 2.5  $\mu$ l *BbsI* (store at -80 °C)  
417 - 10  $\mu$ l NEB2 buffer  
418 - Fill up to 100  $\mu$ l with nuclease free water  
419 2. Incubate at 37°C for 1 h.  
420 3. Inactivate the restriction enzyme for 20 min at 65 °C.  
421 4. Load the digested vector on a 0.9% agarose gel and extract the linearized vector  
422 using a DNA gel extraction kit. Expected fragment sizes are: pU6-  
423 (BbsI)sgRNA\_CAG-Cas9-venus-bpA – 10.1 kb; pU6-(BbsI)sgRNA\_CAG-Cas9-  
424 bpA\_EF1-RFP – 11.6 kb; pU6-(BbsI)sgRNA\_CAG-venus-bpA – 6.0 kb.

425

### 426 **Ligation of sgRNA oligos**

- 427 1. Set up ligation in a microcentrifuge tube  
428 - X  $\mu$ l linearized vectors (100 ng)  
429 - 1.5  $\mu$ l annealed oligos (1  $\mu$ g/ $\mu$ l each)  
430 - 1.5  $\mu$ l fresh ligase buffer  
431 - 1  $\mu$ l T4 DNA Ligase  
432 - Fill up to 15  $\mu$ l with nuclease free water and incubate O/N at 16 °C.

### 433 **Transformation**

- 434 1. Thaw chemically competent *E. coli* on ice slowly.  
435 2. Pipet the transformation reaction on ice  
436 - 50  $\mu$ l chemically competent DH5 alpha *E. coli*  
437 - 5  $\mu$ l of the ligation mix.  
438 3. Incubate 30 min on ice.  
439 4. Perform the heat shock in a water bath at 42 °C for 90 sec.  
440 5. Incubate 3 min on ice.  
441 6. Add 1 ml LB medium w/o antibiotics and incubate 30 min at 37°C at 200 rpm.  
442 7. Plate the transformation on LB-Agar plates 50  $\mu$ g/ml carbenicillin or ampicillin and  
443 incubate O/N at 37°C.

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

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



449 efficacy.

450

451

### 452 **3.1.2 Cloning of multiple sgRNAs**

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

461

462 Gibson-pU6-A\_F CAGGAAACAGCTATGACCATGAGGGCCCCCTTCACCGAGGGCCTATTTTC

463 Gibson-pU6-A\_R CCGATGGCCAGGCCGATGCTGTGATCAAAAAAAGCACCGACTCGG

464

465 Gibson-pU6-B\_F ACAGCATCGGCCTGGCCATCGGGCCCCCTTCACCGAGGGCCTATTTTC

466 Gibson-pU6-B\_R CTTGGCCATCTCGTTGCTGAAGATCAAAAAAAGCACCGACTCGG

467

468 Gibson-pU6-C\_F TTCAGCAACGAGATGGCCAAGGCCCCCTTCACCGAGGGCCTATTTTC

469 Gibson-pU6-C\_R GTCAATAATCAATGTGGAATCCGGGATCAAAAAAAGCACCGACTCGG

470

471 Use primer combinations A\_F/A\_R; B\_F/B\_R and C\_F/C\_R if three cassettes are desired to  
472 be cloned and combinations A\_F/A\_R and B\_F/C\_R if it is desired to clone two sgRNA  
473 cassettes.

474

### 475 **Amplification of guide RNA cassettes with overhangs for assembly**

476 1. Set up PCR reaction in 50 µl reaction volume:

477 - 5 – 20 ng plasmid DNA

478 - 0.25 µM primer (each)

479 - 250 µM dNTPs (each)

480 - 0.5 µl Herculase II

481 - Fill up to 50 µl with nuclease free water.

482 2. Carry out the PCR reaction with following conditions: 98 °C, 2 min; [98 °C, 30 sec;  
483 55°C, 30 sec; 72 °C, 30 sec] x30 cycles; 72 °C 2 min.

484 3. Load the PCR products on a 1% agarose gel and purify the 412 bp fragment using a

485 DNA gel extraction kit.

486

### 487 **Digestion of sgRNA expression vector**

488 1. Set up digestion reaction as following:

- 489 - X µl plasmid DNA, pU6-(*BbsI*)sgRNA\_CAG-venus-bpA (5 µg)
- 490 - 2.5 µl *AscI* restriction enzyme
- 491 - 10 µl CutSmart buffer
- 492 - Fill up to 100 µl with nuclease-free water

493 2. Incubate at 37°C for 1 h.

494 3. Inactivate the restriction enzyme for 20 min at 65 °C.

495 4. Load the digested vector on a 0.9% agarose gel and extract the linearized vector  
496 backbone using a DNA gel extraction kit. Expected fragment sizes are: 5586 bp for  
497 the vector backbone and 383 bp for the empty sgRNA cassette.

498

### 499 **Gibson assembly reaction**

500 1. Set up assembly reaction as following:

- 501 - 200 ng *AscI* linearized plasmid DNA, pU6-(*BbsI*)sgRNA\_CAG-venus-bpA
- 502 - 30 ng PCR product (each)
- 503 - 10 µl Gibson assembly master mix (2x)
- 504 - Fill up to 20 µl with nuclease-free water

505 2. Incubate at 50 °C for 1 h.

506 3. Perform transformation as described in section 3.1.1

507 4. Conduct test digests with *NdeI* on five to ten plasmid mini preparations. The expected  
508 fragment sizes are: for assembly of two sgRNA expression cassettes 5515 bp; 436  
509 bp and 391 bp and for three sgRNA expression cassettes 5515 bp; 436 bp, 391 bp  
510 and 390 bp. Subject two plasmids with the correct digestion pattern for Sanger  
511 sequencing using following primers:

512 pU6-(*BbsI*)sgRNAseqF: TTGTGTGGAATTGTGAGCGG

513 pU6-(*BbsI*)sgRNAseqR: GGCTATGAACTAATGACCCCG

514

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

517

518

### 519 **3.2 Feeder-free hiPSC culture conditions**

520 Cultivation of high-quality human iPS cells requires optimal and aseptic conditions.  
521 The cultures should be free of viral, mycoplasmic and bacterial contaminations and have a  
522 normal karyotype. It is recommended to cultivate human iPSCs in antibiotic-free media to  
523 avoid the overlooking of mycoplasma infections. Therefore, it is worth to emphasize the  
524 importance of aseptic culture conditions. The optimal growth conditions for human iPS cells  
525 are in a humidified, hypoxic incubator equipped with a HEPA-filter at 37°C with 1-5 % O<sub>2</sub> and  
526 5 % CO<sub>2</sub>. Feeder-free iPS cell culturing requires special culture media and culture plate  
527 coatings. Since different hiPSC lines may demand different conditions, it is recommended to  
528 test multiple commercially available hiPSC media and coatings. Pluripotent stem cells, such  
529 as hiPSCs have a doubling time of 18-20 h and should therefore be passaged every 3 to 4  
530 days. If the culture becomes over-confluent, iPS cells tend to grow as multi-layers and  
531 spontaneously differentiate. Induced pluripotent stem cells are sensitive to environmental  
532 changes, thus monitor the culture on a daily base. To keep the culture in high-quality  
533 condition, it is recommended to reduce the time of cells being outside the incubator to a  
534 minimum. When exposed to stress by e.g. dissociating to single cells or transfection, iPS  
535 cells induce apoptosis by activation of the ROCK (Rho-associated protein kinase) pathway.  
536 To avoid cell death a ROCK inhibitor (Y-27632) should be added to the culture medium  
537 overnight, when the cells are transfected or passaged. The addition of ROCK inhibitor  
538 changes the morphology of the iPSCs, cells will grow extensions and look fibroblast-like.  
539 These changes are reversible and will vanish when Y-27532 is removed.

540

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

544

### 545 **3.2.1 General feeder-free human iPSC culture**

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

555

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

561

### 562 **3.2.2 Passaging hiPSC using dissociation buffer**

- 563 1. Remove medium and wash the cells once with DPBS.
- 564 2. Add 0.5 ml dissociation buffer (see 2.3.13) per well of a 6-well plate and incubate for  
565 3-5 min at 37 °C.
- 566 3. When colonies begin to detach at the edges, remove the dissociation buffer and add  
567 0.5 ml of Essential 8 medium.
- 568 4. Detach cells by tapping the plate and gently flush them off with medium using a  
569 pipette.
- 570 5. Pipet carefully 2-3 times to break bigger cell clumps into small ones.
- 571 6. Resuspend the cells in appropriate volume of medium (optional: including 10 µM  
572 ROCK inhibitor) and plate the cells on cell culture plate with an appropriate coating.
- 573 7. Change medium the following day.

574

### 575 **3.2.3 Passaging hiPSC using Accutase**

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

585

### 586 **3.2.4 Cryopreservation of hiPSC**

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

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

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

597

### 598 **3.2.5 Thawing hiPSC**

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

618

### 619 **3.3 Transfection of CRISPR/Cas9 constructs into hiPS cells**

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

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

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

639

640

### - Figure 5 -

641

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

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

651

652

#### 653 **3.3.1 Reagent-based transient transfection using Lipofectamine 3000**

654

- 655 1. Prepare plasmid DNA at a concentration of 0.5-1 µg/µl in deionized water or TE  
656 buffer. The DNA used for transfection should be of high quality as poor quality of DNA  
657 might decrease the efficacy of transfection.
- 658 2. One day prior to transfection dissociate the cells using Accutase and seed  $5 \times 10^4$  –  
659  $1 \times 10^5$  cells per well of a pre-coated 6-well plate as single cells or in small clumps.

- 660 Cultivate the cells in fresh medium containing 10  $\mu\text{M}$  Y-27632 overnight.
- 661 3. Change the medium ahead of transfection.
- 662 4. Dilute depending on the size of the plasmid between 1  $\mu\text{g}$  and 2.5  $\mu\text{g}$  DNA in 125  $\mu\text{l}$
- 663 of Opti-MEM reduced serum medium in tube labeled as A. Add 3.75  $\mu\text{l}$  of p3000
- 664 reagent and mix well. Dilute 5  $\mu\text{l}$  Lipofectamine 3000 reagent in 125  $\mu\text{l}$  of Opti-MEM
- 665 reduced serum medium in tube B. Diluted Lipofectamine 3000 should be used within
- 666 15 minutes. Longer times can result in decreased transfection efficacy.
- 667 5. Add the content of tube A to tube B, mix well by pipetting and incubate at room
- 668 temperature for 5 minutes.
- 669 6. Add the DNA-lipid complex to one well of a 6-well plate in a dropwise manner and
- 670 gently rock the plate to ensure its distribution over the whole well. Some cell lines are
- 671 sensitive to Lipofectamine 3000.
- 672 Optional: The addition of 5  $\mu\text{M}$  Y-27632 to the culture medium for 24 h increases
- 673 viability.
- 674 7. Change medium on the following day. When iPS cells harboring a doxycycline
- 675 inducible Cas9 were used, feed the cells for 48 h with medium supplemented with 1
- 676  $\mu\text{g}/\text{ml}$  doxycycline.
- 677 8. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis
- 678 of bulk population (see section 3.5 for the method of choice for analysis).
- 679 9.

680

### 681 **3.3.2 Electroporation-based transient transfection using the Neon system**

682 The following protocol describes the electroporation procedure using the 100  $\mu\text{l}$  Neon

683 Transfection kit, aiming for a 6-well plate format. The DNA used should be of high quality to

684 achieve the optimal results.

685

- 686 1. Prepare highly concentrated plasmid DNA (1-5  $\mu\text{g}/\mu\text{l}$ ) in deionized water or TE buffer.
- 687 The amount of plasmid DNA should not exceed 10% of the total volume of
- 688 resuspension buffer.
- 689 2. Coat a 6-well plate with appropriate coating.
- 690 3. Pre-warm Essential 8 medium containing 10  $\mu\text{M}$  Y-27632.
- 691 4. Change the medium ahead of transfection.
- 692 5. Dissociate the cells using Accutase and spin down for 4 min at 300 g.
- 693 6. Wash the cell pellet once with PBS.
- 694 7. Dilute 5.5  $\mu\text{g}$  total plasmid DNA in 110  $\mu\text{l}$  buffer R (part of Neon transfection kit) in a

- 695 microcentrifuge tube.
- 696 8. Resuspend  $6 \times 10^5$  cells in 110  $\mu$ l buffer R containing the DNA. Care should be taken  
697 while resuspending for no air bubbles will be formed. Presence of air bubbles will lead  
698 to unsuccessful electroporation.
- 699 9. Fill 3 ml of electrolytic buffer E2 (provided with the Neon kit) into a Neon tube and  
700 insert it into the pipetting station. A single Neon tube can be used up to 10 times.
- 701 10. Insert a 100  $\mu$ l Neon tip into the Neon pipette. Make sure that the tip is adjusted  
702 correctly before pipetting the cell/ DNA mix.
- 703 11. Load the appropriate Neon program (it is advised to optimize settings for each cell  
704 line using a fluorescent plasmid). The electroporation parameters we generally use  
705 are 1 pulse at 1300 V and a duration of 30 ms.
- 706 12. Press start and wait until the procedure is complete. A single Neon tip can be used  
707 twice with the same plasmid/cell line.
- 708 13. Transfer the electroporated cells to a microcentrifuge tube containing 500  $\mu$ L medium  
709 supplemented with 10  $\mu$ M Y-27632 and pellet at 300 g for 4 min.
- 710 14. Resuspend the cells in 600  $\mu$ L fresh medium supplemented with Y-27632. Plate 100  
711  $\mu$ L cell suspension into one well of a pre-coated 6-well plate containing 2 ml medium  
712 supplemented with 10  $\mu$ M Y-27632.
- 713 15. Rock the plate gently and incubate the cells.
- 714 16. Change medium the following day. When iPS cells harboring a doxycycline inducible  
715 Cas9 were used, feed the cells for 48 h with medium supplement with 1  $\mu$ g/mL  
716 doxycycline.
- 717 Optional: The addition of 5  $\mu$ M Y-27632 to the culture medium for 24h on the day  
718 after transfection improves viability.
- 719 17. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis  
720 of bulk population (section 3.5).
- 721

### 722 **3.3.3 Electroporation-based transient transfection using the Gene Pulser system**

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



- 730 and chill on ice.
- 731 7. Dissociate the cells using Accutase and spin down for 4 min at 300 g.
- 732 8. Wash the cells once with DPBS, take an aliquot for counting and pellet again at 300 g  
733 for 4 min.
- 734 9. Resuspend at  $16 \times 10^6$  cells/ ml in ice-cold DNA/ PBS dilution and carefully transfer 800  
735  $\mu$ l of it to the electroporation cuvette containing the DNA, avoid air bubble formation.
- 736 10. Incubate on ice for 5 minutes and proceed with electroporation. We generally use  
737 following parameters: single pulse at 300 V and 500  $\mu$ F, the time constant should be  
738 between 10 ms and 15 ms. It is advised to optimize settings for each cell line using a  
739 fluorescent plasmid.
- 740 11. Immediately after the pulse add 500  $\mu$ l of pre-warmed culture medium supplemented  
741 with ROCK inhibitor Y-27632 to the cells.
- 742 12. Transfer the cells to a microcentrifuge tube and pellet them by centrifugation.
- 743 13. Resuspend the cells in 600  $\mu$ l fresh medium containing Y-27632 and plate 100  $\mu$ l cell  
744 suspension into one well of a pre-coated 6-well plate containing 2 ml medium  
745 supplemented with 10  $\mu$ M Y-27632.
- 746 14. Rock the plate gently and incubate the cells.
- 747 15. Change medium the following day. When hiPS cells harboring a doxycycline inducible  
748 Cas9 were used, feed the cells for 48 h with medium supplement with 1  $\mu$ g/ml  
749 doxycycline.
- 750 Optional: The addition of 5  $\mu$ M Y-27632 to the culture medium for 24 h improves  
751 viability.
- 752 16. Keep in culture until FACS sorting (section 3.4) or extraction of total DNA for analysis  
753 of bulk population (see section 3.5).
- 754

### 755 **3.4 FACS enrichment and clonal isolation hiPS cells**

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

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

767

768 **- Figure 6 -**

769

### 770 **3.4.1 FACS enrichment of transfected hiPSC**

- 771 1. Prepare post-FACS medium: 50 % fresh Essential 8 medium, 50 % conditioned  
772 Essential 8 medium (medium that was conditioned for 24 h on hiPS cells with 50-80  
773 % confluency of the same line and filtered through a 0.45 µm PTFE membrane filter)  
774 supplemented with 10 µM Rock inhibitor Y-27632 , RevitaCell Supplement (1X),  
775 Pen/Strep (1X), and Gentamicin (1X).
- 776 2. Coat a 6-well plate with appropriate coating.
- 777 3. For each FACS sample, prepare 1.5 ml microcentrifuge tube with 1 ml post-FACS  
778 medium and keep on ice.
- 779 4. Pre-warm Essential 8 medium containing 10 µM Y-27632, Pen/Strep (1X), and  
780 Gentamicin (1X).
- 781 5. Dissociate cells using Accutase and spin down for 4 min at 300 g.
- 782 6. Resuspend the cells thoroughly by pipetting the cells 5-10 times to achieve a single  
783 cell suspension. We suggest resuspension in 200 to 300 µl medium per well of a 6-  
784 well plate at 70 % - 80 % confluency
- 785 7. Strain through a strainer cap with a mesh size of 35 µm into a FACS tube.
- 786 8. Put cells on ice and proceed with FACS immediately.
- 787 9. Depending on the nature of your experiment and the chosen fluorophore, sort for your  
788 desired cell population directly into a cooled 1.5 ml microcentrifuge tube containing 1  
789 ml of post-FACS medium.
- 790 10. Seed 300-500 cells per well in 2 ml post-FACS medium in a 6-well format as quickly  
791 as possible after sorting. Prepare several wells for each sample.
- 792 11. Make sure single cells are evenly distributed throughout the well to ensure  
793 emergence of single cell derived colonies.
- 794 12. Additionally, seed the remaining cells at a higher density on a separate well. This well  
795 can be used after expansion for gDNA extraction from the sorted bulk population and  
796 subsequent PCR based genotyping to determine the rough gene targeting efficacy for  
797 example by RFLP. This can be very helpful to determine the number of single clones  
798 that need to be analyzed for the specific gene targeting event.

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

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

803

### 804 **3.4.2 Clonal isolation of hiPSCs**

805 1. Culture the sorted cells for 5 days in 50 % conditioned and 50 % fresh medium until  
806 small colonies emerge. Change medium daily.

807 Optional: The addition of 5  $\mu$ M Rock inhibitor Y-27632 for 1 to 2 days to the medium  
808 (50 % conditioned and 50 % fresh medium) improves viability of single cell derived  
809 clones.

810 2. At day 6 switch to regular Essential 8 medium and continue with daily feeding.

811 3. Closely monitor the growth of the single cell derived colonies to exclude the possibility  
812 that neighboring colonies fuse together. This can be done by circling neighboring  
813 colonies on the bottom side of the well with a marker pen.

814 4. After approximately 7 to 10 days colonies are large enough for manual picking.

815 5. Gently scratch the colonies into smaller, checkered patches either using a small  
816 needle or using a 10  $\mu$ l pipette tip utilizing a stereo microscope in a sterile  
817 environment.

818 6. Transfer the patches of one clone to one well of a pre-coated 12-well plate containing  
819 pre-warmed medium supplemented with 10  $\mu$ M Y-27632.

820 7. Gently resuspend the cell patches by pipetting up and down with a 1000  $\mu$ l tip. After a  
821 few days colonies may need to be dissociated with EDTA within the plate to break  
822 apart the large colony and avoid differentiation of the cells.

823 8. As soon as the cells build larger colonies, split each clone on 2 separate wells in  
824 either 12- or 6-well format in a 20 % to 80 % ratio. Use one plate (80 %) for further  
825 expansion and subsequent gDNA extraction, followed by PCR based genotyping to  
826 detect CRISPR/Cas9 edited clones.

827 9. Meanwhile, you can freeze the second plate as a back-up (20 %). It is recommended  
828 to use the BamBanker cryopreservation solution instead of 10 % DMSO when  
829 freezing iPSCs in a multiwell plate instead of cryovials.

830 10. Aspirate the medium and cover the wells with dissociation buffer. Incubate the plate  
831 for 3-5 min at 37 °C. Aspirate the Dissociation reagent and add 0.3-0.5 ml of  
832 BamBanker cryopreservation solution. Gently dissociate the colonies by repeated  
833 pipetting. Seal the plate with Parafilm and store the plate at -80 °C in a Styrofoam box  
834 until further use.

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

837

### 838 **3.5 Analysis techniques of CRISPR/Cas9 induced modifications**

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

849

850 **- Table 1 -**

851

#### 852 **3.5.1 PCR design**

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

864

#### 865 **3.5.2 PCR reaction and purification**

866 Genomic DNA (gDNA) can be extracted from hiPS cells of either a semi-confluent well of a  
867 6-well plate or a confluent well of a 12-well plate with the Wizard (Promega) or other DNA  
868 purification kits. PCR reactions are carried out using 100 ng gDNA in 50 µl with Herculase II  
869 according to manufacturer's instructions. For Sanger sequencing or fragment analysis the

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

872

### 873 **3.5.3 Sanger sequencing**

874 Purified PCR products are prepared for sequencing using 20 pMol of sequencing primer and  
875 60 -150 ng amplicon. The sequencing can be conducted by a commercial provider, e.g. LGC  
876 Genomics.

877

### 878 **3.5.4 TIDE analysis**

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

889

### 890 **3.5.5 Fragment analysis**

891 Fragment analysis is a useful tool to evaluate indel formation in single cell derived clones as  
892 well as in cell populations. When loading fluorescent labeled PCR products of the edited  
893 sgRNA target site on a capillary sequencer, amplicons differing in size due to small insertions  
894 or deletions can be separated with accuracy in resolution, depending on the size of the PCR  
895 product. PCR products with a length of up to 650 bp can be separated with a resolution of a  
896 single base. For this approach, fluorescently labeled forward primers (HEX, TET or 6-FAM)  
897 must be used to amplify the region of interest. Subsequently the PCR products are loaded on  
898 an agarose gel, purified using a PCR Clean-Up System (Promega) and 60 - 150 ng of the  
899 purified samples are subjected to analysis (SMB Service GmbH, Berlin, Germany) using LIZ-  
900 500 size standard.

901

### 902 **3.5.6 RFLP assay**

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

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

915

### 916 **3.5.7 T7E1 Assay**

917 The T7E1 and Surveyor assays can be used to assess indels and heterozygous point  
918 mutations in single cell derived clones as well as in bulk populations. Furthermore these  
919 assays are useful tools to test the efficacy of sgRNAs in cell lines like 293 cells. Both assays  
920 are used to detect DNA hetero-duplex formations of PCR amplicons by hybridization of  
921 mutant and wild type sequences or two different mutant sequences. These mutation-  
922 sensitive methods fail to detect homo-duplexes e.g. formed by two identical mutant  
923 sequences. Similar to RFLP the amplicon size for T7E1 assay should range from 400 bp to  
924 1200 bp with the CRISPR/Cas9 targeting site off-set to the center of the amplicon. After the  
925 PCR reaction a clean-up step was performed and subsequently the purified fragments were  
926 denatured for 5 min at 98 °C in a heat block and re-annealed by switching of the heat block  
927 for 1-2 h. 200 ng PCR product was digested with 5 units T7E1 (NEB) for 15 min at 37 °C.  
928 After incubation the samples were immediately transferred to ice and supplemented with gel-  
929 loading-buffer. The digested fragments were separated on a 1.5 % agarose gel. In our  
930 experience the T7E1 assay often results in nonspecific cleavage products when applied to  
931 iPS cell derived gDNA, hence often needs further optimization steps for different loci.  
932 Therefore we recommend using RFLP (section 3.5.6), Fragment analysis (section 3.5.5) or  
933 TIDE analysis (section 3.5.4).

934

935

### 936 **3.5.8 Off-target analysis**

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

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

947

948

#### 949 **4. Results**

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

960

#### 961 **4.1 Results obtained with Workflow A**

962 In the course of optimizing gene targeting with transient transfection of Cas9 and  
963 sgRNA (Workflow A) we started with an approach using a plasmid vector carrying Cas9  
964 together with a fluorescent reporter and a separate vector encoding only for a single sgRNA  
965 without any selection gene. The hiPSCs were enriched by FACS for the fluorescent reporter  
966 48h after transfection. With this approach we could enrich only the fraction of cells  
967 transfected with the nuclease but not the guide RNA (Table 2; separate vectors). We  
968 analyzed 176 sorted, single cell derived clones and obtained only two mutagenized clones  
969 (1 %) where one clone was targeted on both alleles and the second clone on one allele. In  
970 our next approach we transfected hiPSCs with combined vectors, coding for Cas9, the  
971 sgRNA cassette as well as a fluorescent reporter (pU6-(*BbsI*)sgRNA\_CAG-Cas9-venus-bpA  
972 or pU6-(*BbsI*)sgRNA\_CAG-Cas9\_EF1-TagRFP). Using this strategy, we could enrich for  
973 cells carrying Cas9 and the guide RNA. By this method we achieved a mutagenesis rate of  
974 17 % in 116 analyzed clones. 70 % of the mutagenized clones were targeted on both alleles

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

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

980

981 **- Table 2-**

982

#### 983 **4.2 Results obtained with Workflow B**

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

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

996 Finally we could improve the targeting efficacy by repeating the transfection and FACS  
997 sorting procedure a second time (Table 3; Second round). In 15 of in total 57 single cell  
998 derived human iPS cell clones we could detect triple gene mutagenesis (26 %). 7 clones  
999 were targeted on two loci (12 %) and 4 clones carried indels in only one of the targeted loci  
1000 (7 %). In accordance with our previous observation the majority of mutations occurred on  
1001 both alleles of a locus.

1002 **-Table 3-**

1003

1004 Thus we recommend based on our experience, when it is intended to introduce  
1005 frameshift indels in multiple loci, we suggest using the doxycycline inducible Cas9 system  
1006 with transient transfection of sgRNA carrying fluorescent reporter vectors (Workflow B)  
1007 followed by FACS enrichment. In this case we recommend selecting 36 to 48 clones to have  
1008 a feasible number of multiple edited cell clones. To minimize the number of clones to analyze  
1009 during multiplex gene editing we suggest: a) co-transfection with the plasmid pCAG-Trex2-



1010 bpA for expression of the end-processing enzyme Trex2 and b) repeating the transfection  
1011 and FACS enriching procedure a second time (sections 3.3 and 3.4). In this regard analyzing  
1012 16 to 24 clones should be adequate to obtain various clones with mutations on both alleles  
1013 for the desired loci (Table 2 and Table 3). If precise genome editing is desired (HDR events)  
1014 we suggest to first determine the efficacy of mutagenesis events in a bulk population e.g. by  
1015 RFLP analysis and depending on that estimate the number of clones to be picked and  
1016 analyzed.

1017

### 1018 **4.3 Quality of targeted human iPSCs**

1019 To monitor, if the CRISPR/Cas9 based gene targeting strategies we describe here have an  
1020 influence on the genetic stability of the targeted cells, we performed SNP analysis based  
1021 virtual karyotyping on edited human iPS cells. The karyotyping was performed with the  
1022 Infinium OmniExpressExome-8 v1.2 BeadChip system that covers over 960,000 common  
1023 SNPs over the human genome. We analyzed two hiPSC clones that were successively  
1024 targeted at the AAVS1 locus to initially create doxycycline inducible Cas9 expressing hiPSCs  
1025 and afterwards at three native loci by two rounds of transfection followed by FACS  
1026 enrichment. Both characterized clones were successfully targeted on both alleles of all three  
1027 loci.

1028 The SNP based karyotyping revealed no chromosomal aberrations. We detected eight SNPs  
1029 that were absent in an early passage of the parental cell line (data not shown). It is unclear if  
1030 these SNPs emerged during cultivation or are an artifact of the editing process. However,  
1031 pluripotent stem cells are generally considered as genetically unstable and we could  
1032 demonstrate that our editing method does not exacerbate this phenomenon.

1033 Additionally we analyzed the edited clones for potential mutations at predicted  
1034 CRISPR/Cas9 off-target sites for the individual sgRNAs used here (section 3.5.8). We  
1035 analyzed the most relevant loci with a hit-score > 0.9 regarding to CRISPOR and CRISPR  
1036 DESIGNER. We could not detect any mutation at these sites.

1037

### 1038 **-Figure 7-**

1039

1040 We subjected targeted cell clones mentioned above to immunostainings for the  
1041 pluripotency markers OCT4, NANOG, SOX2 and SSEA4 (Figure 7A). We confirmed that  
1042 targeted hiPSCs express common pluripotency markers.

1043 Furthermore we examined the differentiation potential of hiPS cells, targeted with our  
1044 workflow, to the ectodermal lineage. We performed a neuronal differentiation assay with

1045 gene targeted hiPSCs and could demonstrate that these cells are capable to differentiate to  
1046 TUJ1 and TH expressing neuronal cells (Figure 7B).

1047

1048

1049

## 1050 **5. Conclusion**

1051 Two groundbreaking discoveries of the past decade, human iPS cell technology and gene  
1052 editing using CRISPR/Cas9, provide new opportunities for researchers. Combining both  
1053 allows us to study the function of a gene in a human system in the context of specific  
1054 diseases or even gene therapeutically applications by correcting gene defects in patient  
1055 cells. Using CRISPR/Cas9 in immortalized, fast-cycling cell lines is well established.  
1056 However, using this system in hiPS cells is more challenging due to the sensitive  
1057 requirements of hiPS cells to maintain culture pluripotency. Although generating precise  
1058 knockins via HDR is relatively inefficient, the possibility to use selection markers or  
1059 introducing recognition sites for restriction enzymes in the repair template facilitates  
1060 screening for successfully targeted cells. CRISPR/Cas9 mediated gene disruption by  
1061 introducing indel mutations on the other hand occurs more frequently but screening of clonal  
1062 derived cells is more laborious.

1063 We describe here a simple and efficient workflow for clonal isolation and screening of  
1064 genome engineered human iPS cells without prior drug selection. Applying this workflow to  
1065 hiPS cell lines equipped with an inducible Cas9 under a strong promoter even enables the  
1066 generation of multiple targeted mutant alleles in a single experiment.

1067

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1072 performed karyotyping and SNP analysis (iPSC Core Facility, Max-Delbrück-Center, Berlin).  
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## 1079 **Tables**

1080 **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

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1090 **Table 2 Results of gene editing in hiPSC using Workflow A**

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

1091

1092 **Table 2 Results of gene editing in hiPSC using Workflow B**

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

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1099 **Figure Legends**

1100 **Figure 1 The CRISPR/Cas9 nuclease system**

1101 A: Cas9 and sgRNA recognize 20 nt target sequences located upstream of the invariant 3 bp  
1102 NGG PAM sequence (red letters). B: If a target sequence is found upstream of the PAM site,  
1103 each of the RuvC and HNH nuclease domains of Cas9 cut one strand of the target DNA,  
1104 creating a DSB (triangles). C: DSBs in target genes can be either repaired by NHEJ, leading  
1105 to small sequence deletions and gene knockout alleles. Precise sequence modifications  
1106 (Knockin alleles) are introduced by HDR with the homology regions of DNA repair templates,  
1107 either an ssODN or plasmid vector.

1108

1109

1110 **Figure 2 Strategies for intracellular delivery of components required for CRISPR/Cas9-**  
1111 **mediated gene editing**

1112 **A** Transfection of an expression vector carrying both a sgRNA cassette and the Cas9 gene  
1113 enables gene editing via DSBs in wildtype cells. **B** For transgenic cell lines that express  
1114 Cas9 under a doxycycline-inducible promoter, transfection of a vector encoding for sgRNA(s)  
1115 only is sufficient. Successive administration of doxycycline activates Cas9 expression. This  
1116 approach achieves improved efficacy at targeting more than one locus. A reporter gene  
1117 included in either of these strategies allows for the identification of transfected cells. **C**  
1118 Doxycycline (DOX) dependent expression of Cas9 is provided by a modified AAVS1 locus.  
1119 While the DOX-inducible promoter TREtight and the Cas9 gene are knocked in between the  
1120 first two exons of the PPP1R12C gene (AAVS1 locus) on one allele, the other allele harbors  
1121 the Transactivator gene rtTA3 expressed under the constitutively active CAGGS promoter.  
1122 Presence of DOX mediates binding of rtTA to TREtight, thus activation of Cas9 expression.

1123

1124 **Figure 3 HDR efficacy using ssODNs of various architectures**

1125 Cells of the hiPSC line BCRT#1 that heterozygously express EGFP driven by a CAG  
1126 promoter from the AAVS1 locus were co-transfected with an expression vector for both Cas9  
1127 and sgRNA targeting EGFP and various ssODN donor templates.  
1128 **A** Donor templates were designed to convert EGFP to BFP in case of HDR events, in  
1129 symmetric (sym) [44] or asymmetric (asm) [42] fashion, with and without phosphorothioate  
1130 modification (PTO) [43]. **B, C** Positively transfected cells were isolated by FACS, based on

1131 an RFP fluorophore included in the expression vector. 7 days post transfection HDR/ indel  
1132 frequency was assessed by FACS analysis. Absent fluorescent signal corresponds to indels  
1133 due to disrupted EGFP, BFP<sup>+</sup> cells to successful HDR and EGFP<sup>+</sup> cells indicate cells not  
1134 undergoing any event. Occurrence of no event was generally low (<4%). HDR frequency of  
1135 both PTO-modified symmetric (6.1%) and asymmetric (4.0) ssODNs were below the efficacy  
1136 of unmodified asymmetric donor templates (12.8%). The highest HDR efficacy was obtained  
1137 using unmodified symmetric ssODNs (20.8%). Experiments were conducted in three  
1138 independent replicates; error bars represent standard error of the mean; significance as per  
1139 Student's t-test: \*<0.05, \*\*<0.005, \*\*\*<0.0005.

1140

1141 **Figure 4 Overview on the workflow for CRISPR/Cas9-mediated generation of a**  
1142 **transgenic hiPS cell line without drug selection**

1143 Initially, vectors expressing components necessary for CRISPR/Cas9-mediated gene editing,  
1144 i.e. locus specific sgRNA, a fluorescent reporter and optionally Cas9, are required. Available  
1145 combined plasmids provide convenient cloning strategies to include custom sgRNA  
1146 sequences. After transfection of wildtype hiPS cells with these vectors, the included  
1147 fluorescent reporter enables enrichment of successfully transfected cells utilizing FACS.  
1148 Isolated transfected cells are sparsely seeded and expanded for subsequent growth of single  
1149 cell-derived colonies. Appropriate means for analyses allow for the selection of a favored  
1150 colony, which is then established as cell line.

1151

1152

1153 **Figure 5 Transfection efficacy and cell viability applying various transfection methods**  
1154 **using vectors of different size and diverse cell lines**

1155 The efficacy of iPS cells transfected with a vector was compared using a small guide-only (4  
1156 kb) and a large combined plasmid (12 kb) applying three methods: Gene Pulser Xcell, Neon  
1157 Transfection System and Lipofectamine 3000. By Gene Pulser Xcell 20 µg of DNA were  
1158 transfected, 5 µg by Neon System and 1.5 µg by Lipofectamine 3000. Efficacy was assessed  
1159 by FACS analysis, counting positively transfected single cells detected by a fluorescent  
1160 reporter included in the vector. 5x10<sup>5</sup> cells were subjected to Gene Pulser Xcell and Neon  
1161 Transfection System of which 1x10<sup>5</sup> were subsequently cultured. 1x10<sup>5</sup> cells were subjected  
1162 to Lipofectamine 3000 transfection. Viability was determined after 48h by FACS based  
1163 quantification of remaining single cells. **A** Cells of the BCRT#1 iPS cell line were transfected  
1164 at differing efficacy when subjected to Gene Pulser Xcell, Neon Transfection System and

1165 Lipofectamine 3000. Transfection efficacy delivering the small guide-only vector amounts to  
1166 41% of all FACS identified single cells using Gene Pulser Xcell, 65% with the Neon System  
1167 and 82% after Lipofectamine 3000 transfection. Delivering the large combined vector yields a  
1168 decreased efficacy regardless the method used. Transfection by Gene Pulser Xcell results in  
1169 10% of successfully transfected single cells, by Neon Transfection System 27% and applying  
1170 Lipofectamine 3000 achieve 59%. **B** Viability of transfected BCRT#1 iPS cells varies  
1171 depending on the transfection method used as well as on the size of the vector delivered.  
1172 After transfection of the 4 kb vector by Gene Pulser Xcell, Neon System and Lipofectamine  
1173 3000, 6900, 5400 and 6800 remaining single cells were detected, respectively. Subjecting  
1174 the 12 kb vector to transfection yielded 4000, 400 and 2500 live cells. **C** Efficacy of  
1175 transfecting a 4 kb vector varied between the hiPS cell lines BCRT#1, XM001 and MIRJT7i-  
1176 mND2-0. Treatment with Gene Pulser Xcell resulted in 41%, 50% and 25% efficacy, with  
1177 Neon Transfection System 65%, 90% and 38%, with Lipofectamine 3000 82%, 96% and  
1178 90%, respectively. **D** Cell viability was evaluated after applying different transfection methods  
1179 to BCRT#1, XM001 and MIRJT7i-mND2-0 iPS cell lines. After transfection by Gene Pulser  
1180 Xcell 6800, 5800 and 4200 live cells of the respective cell lines were detected, after Neon  
1181 Transfection System application 5400, 5700 and 2600 remaining single cells were quantified,  
1182 and after Lipofectamine 3000, 6900, 5100 and 100 cells. Experiments were performed in  
1183 triplicates; error bars represent standard error of the mean; significance as per Student's t-  
1184 test: \*<0.05, \*\*<0.005, \*\*\*<0.0005, n.s.=not significant.

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1188 **Figure 6 Scheme of the enrichment of hiPS cells transfected for CRISPR/Cas9-**  
1189 **mediated gene editing**

1190 **A** After transfection of hiPS cells with an appropriate reporter expressing vector, transfected  
1191 cells are identified by FACS based on their fluorescent signal. To increase the yield of  
1192 successfully targeted cells, isolated cells can be expanded and resubjected to transfection  
1193 and FACS enrichment. Representative image of a FACS plot. **B1** Single FACS-isolated  
1194 transfected hiPS cells are cultivated sparsely. **B2-B5** During the next 5 days following  
1195 seeding a small colony arises from a single cell. **B6** After 5 days of culturing a colony forms  
1196 large enough for manual picking and further expansion. Scale bar represents 100 µm.

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1200 **Figure 7 Targeted hiPSCs express pluripotency markers and can differentiate to the**  
1201 **ectodermal lineage**

1202 **A** Edited hiPS cells (BCRT#1; edited) and the parental cell line (BCRT#1; parental) were  
1203 subjected to immuno-cytochemistry using antibodies against the pluripotency markers  
1204 SSEA4 (NEB #4755S; 1:500) and NANOG (NEB #4903S; 1:500), OCT4 (NEB #2750S;  
1205 1:500) or SOX2 (NEB #3579S; 1:500), respectively. Nuclei were visualized by DAPI. The  
1206 edited cell line was targeted in two successive steps. Firstly, the *AAVS1* locus was targeted  
1207 to express doxycycline inducible Cas9. Subsequently, three native loci were biallelically  
1208 mutated following Workflow B, including two rounds of transfection (and co-transfection of  
1209 Trex2) and FACS enrichment. **B** The edited cell line (BCRT#1; edited) and a control cell line  
1210 which was targeted at the *AAVS1* locus to express Cas9 under a doxycycline inducible  
1211 promotor (BCRT#1; control) were differentiated using the Dopaminergic Neuron  
1212 Differentiation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. At Day  
1213 38 cells were fixed and subjected to immuno-cytochemistry for the pan-neuronal marker  
1214 TUJ1 (BioLegend #801202; 1:1000) and the dopaminergic neuronal marker TH (Pel-Freez  
1215 #P40101; 1:1000). Nuclei were visualized by DAPI. **A and B** Cells were cultivated on glass  
1216 cover slips coated with VTN-T (**A**) or Laminin/Poly-D-Lysin (**B**). Prior to staining cells were  
1217 fixed for 7 min at RT with 4 % PFA in PBS. Cells were washed thrice with PBS and  
1218 subsequently permeabilized and blocked for 30 min at RT with PBS containing 3 % BSA and  
1219 0.2 % TritonX-100. The cells were washed three more times with PBS and incubated with the  
1220 respective primary antibodies O/N at 4 °C. The next day, cells were again washed thrice  
1221 with PBS and incubated for 1 h at RT with secondary antibodies (goat-anti rabbit-AF488 and  
1222 goat anti-mouse-AF546, both Thermo Fisher Scientific) and DAPI in PBS supplemented with  
1223 3 % BSA. After final three washes in PBS cells were mounted on glass slides with  
1224 Fluoromount-G (SouthernBiotech). Imaging was performed on a LSM700 confocal  
1225 microscope (Zeiss) with a 20x objective.

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1233 **References**

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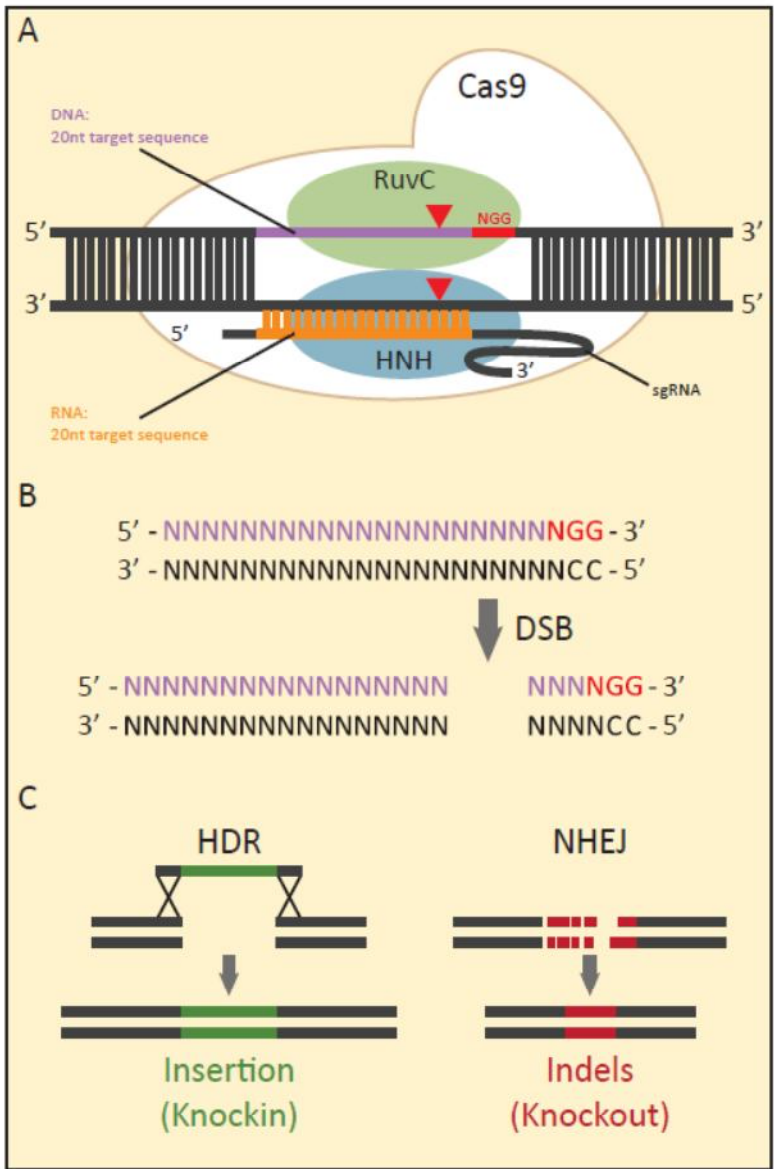
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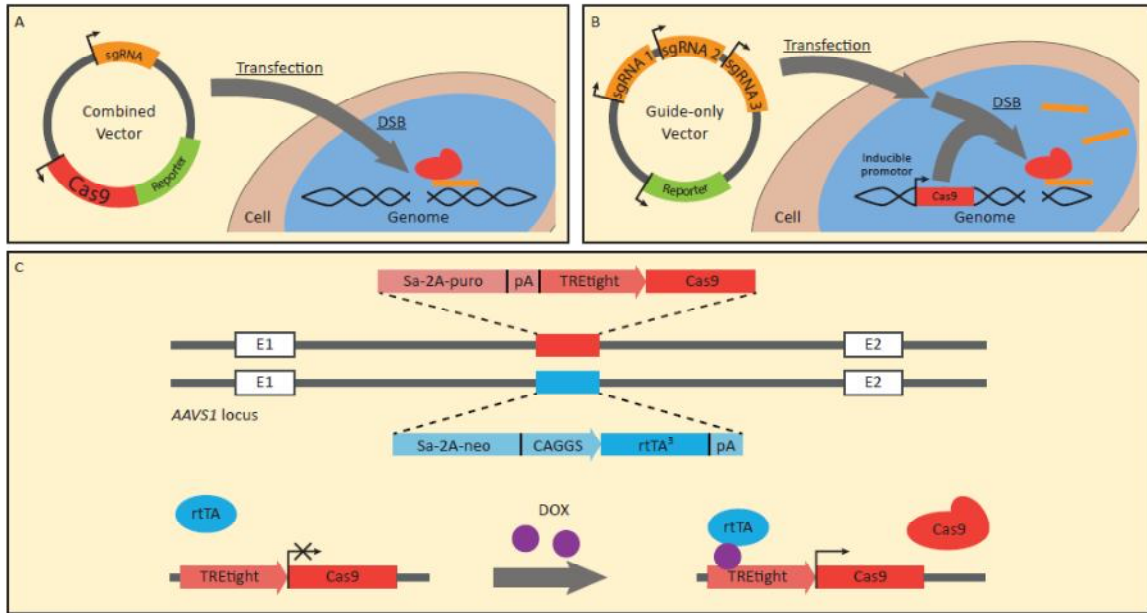
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1399 **Figure 1**



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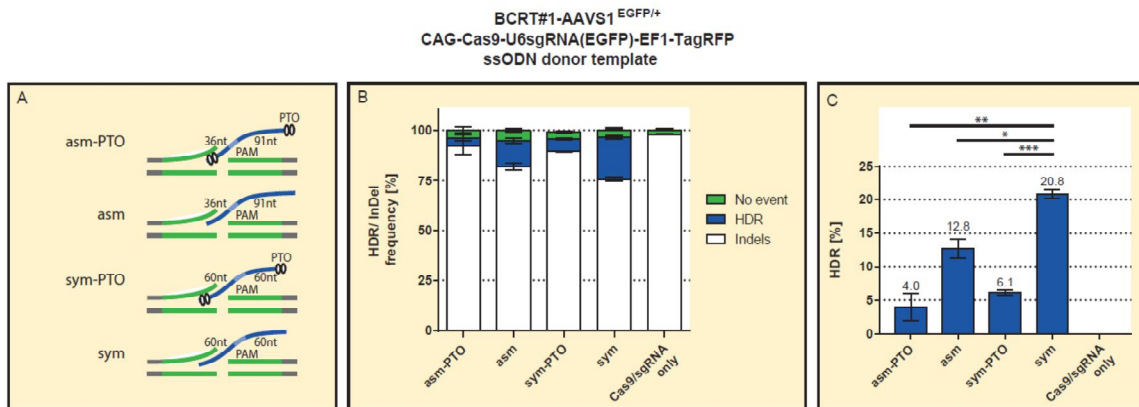
1409 **Figure 2**



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1412 **Figure 3**



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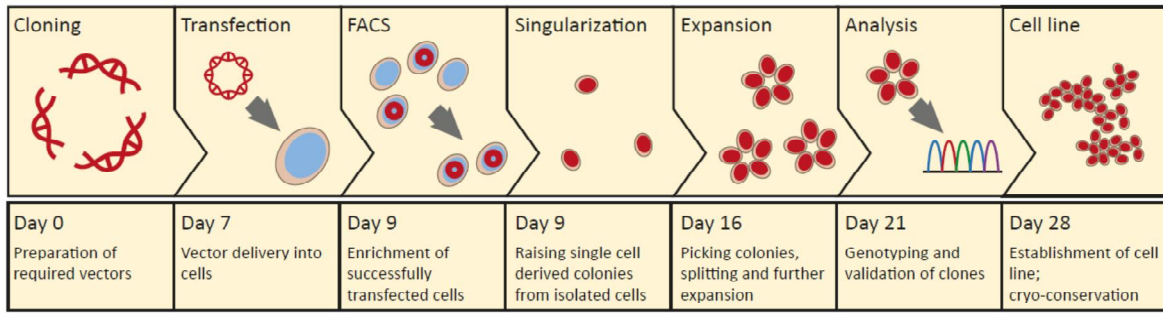
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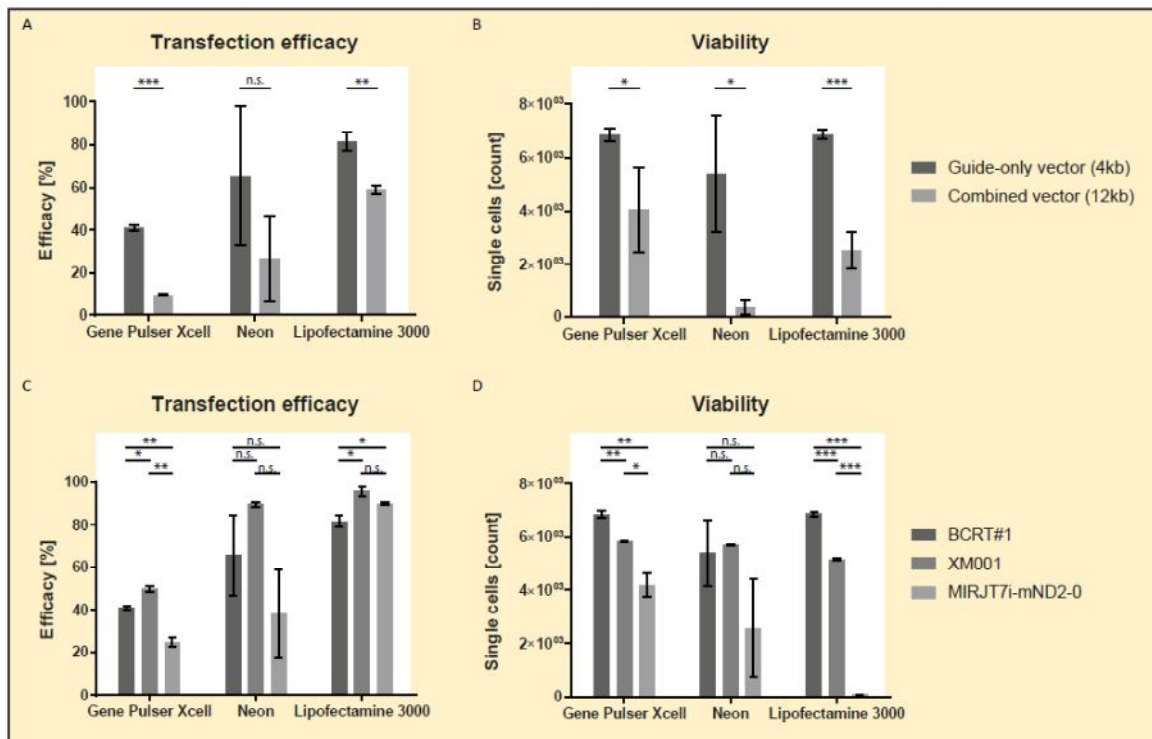
1421 **Figure 4**



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1423 **Figure 5**

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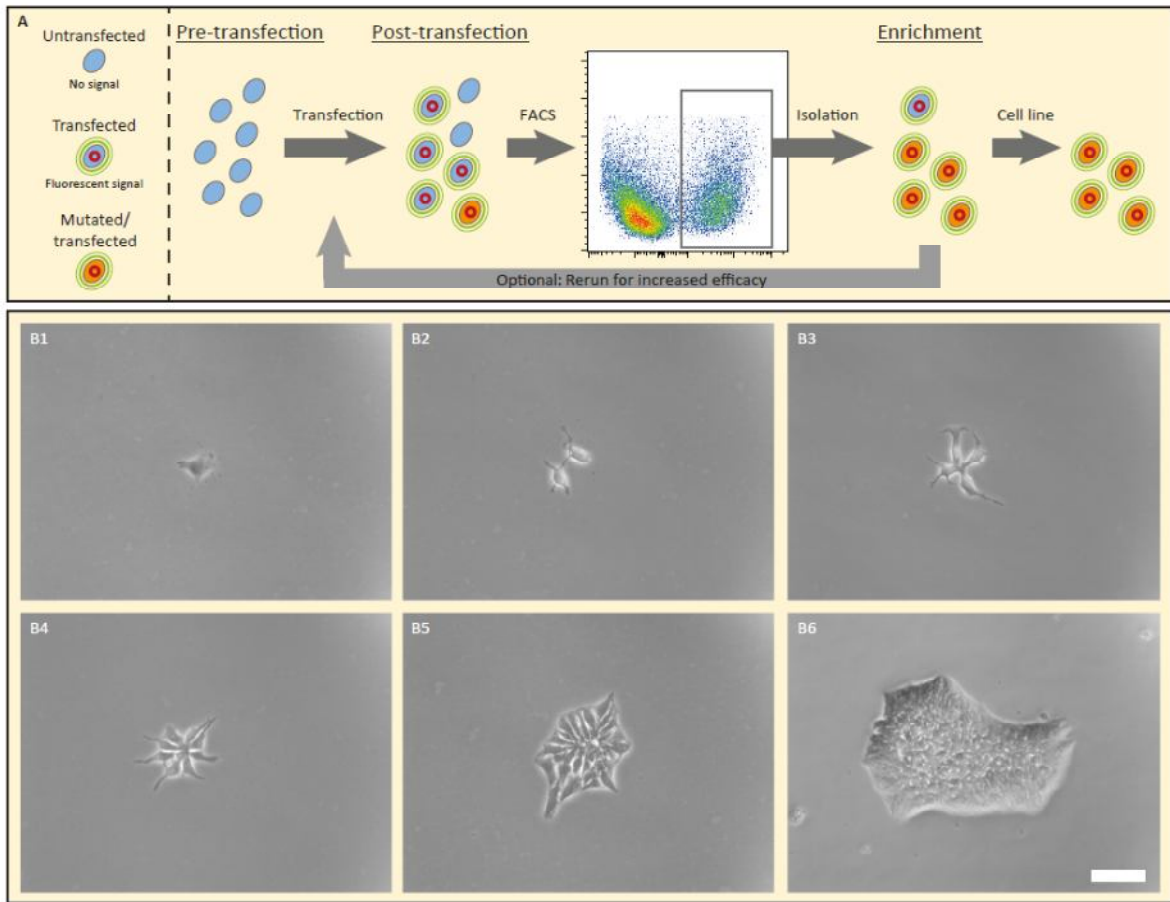
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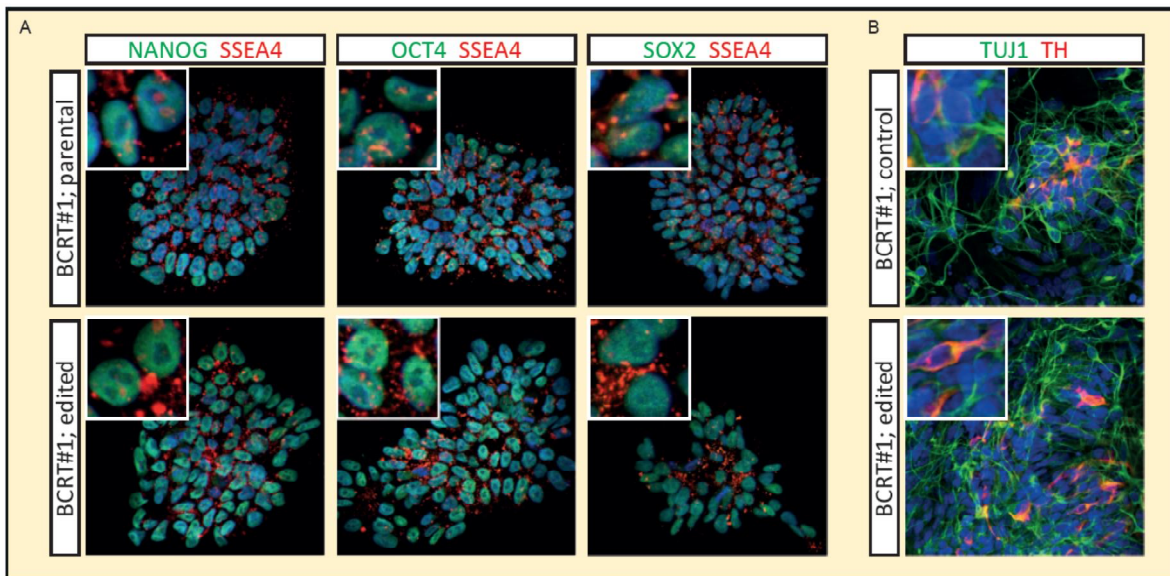
1433 **Figure 6**



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1436 **Figure 7**



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