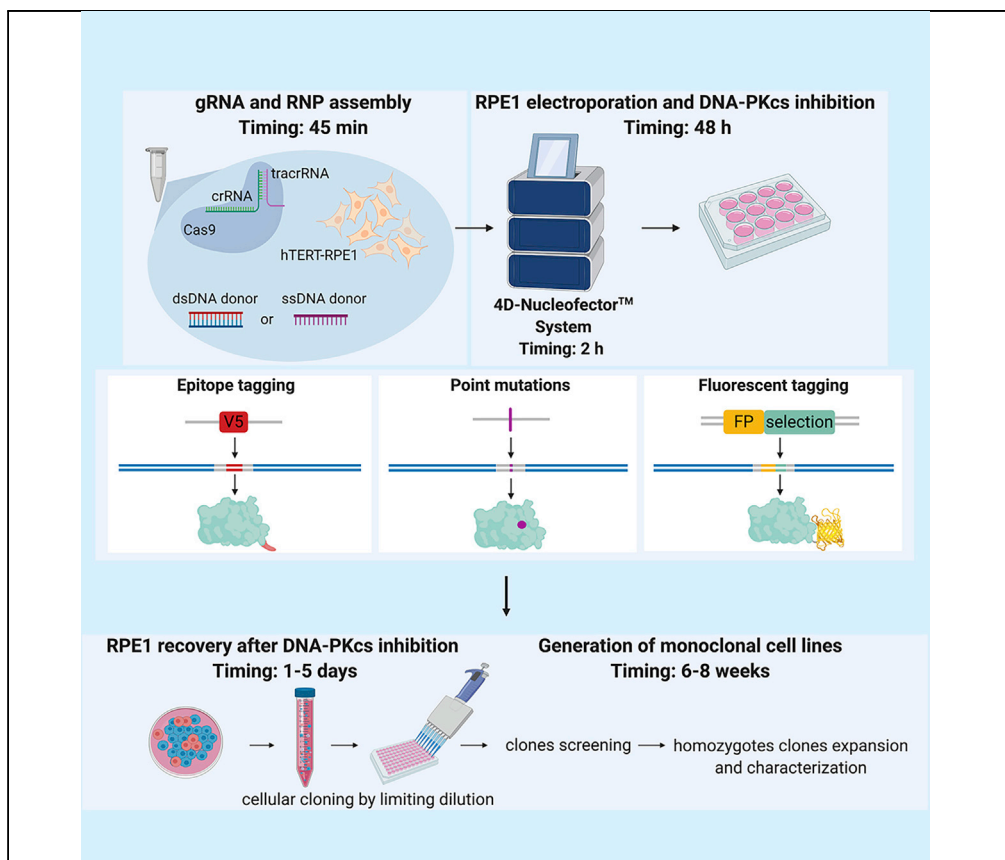


Protocol

CRISPR/Cas9 ribonucleoprotein-mediated knockin generation in hTERT-RPE1 cells



hTERT-RPE1 cells are genetically stable near diploid cells widely used to model cell division, DNA repair, or ciliogenesis in a non-transformed context. However, poor transfectability and limited homology-directed repair capacity hamper their amenability to gene editing. Here, we describe a protocol for rapid and efficient generation of diverse homozygous knockins. In contrast to other approaches, this strategy bypasses the need for molecular cloning. Our approach can also be applied to a variety of cell types including cancer and induced pluripotent stem cells (iPSCs).

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Highlights

Rapid and efficient generation of knockins in hTERT-RPE1 cells

Step-by-step guide for RNP-mediated editing bypassing the need for molecular cloning

Enhanced knockin efficiency by transient inhibition of NHEJ

Suitable for epitope tagging, point mutations, or fluorescent tagging

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Protocol

CRISPR/Cas9 ribonucleoprotein-mediated knockin generation in hTERT-RPE1 cells

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SUMMARY

hTERT-RPE1 cells are genetically stable near diploid cells widely used to model cell division, DNA repair, or ciliogenesis in a non-transformed context. However, poor transfectability and limited homology-directed repair capacity hamper their amenability to gene editing. Here, we describe a protocol for rapid and efficient generation of diverse homozygous knockins. In contrast to other approaches, this strategy bypasses the need for molecular cloning. Our approach can also be applied to a variety of cell types including cancer and induced pluripotent stem cells (iPSCs).

BEFORE YOU BEGIN

crRNA design

crRNAs are designed using simultaneously three online tools, providing as search base an 80–100 bp genomic sequence retrieved from Ensembl Genome Browser and centered around the preferred editing site (see [key resources table](#)):

1. IDT™ Custom Alt-R® CRISPR-Cas9 guide RNA
2. CRISPOR
3. CRISPick - Broad Institute

Possible editing sites can be the START/STOP codon of the isoform of interest in case of N/C-terminal protein tagging, or the site to be subjected to point mutation. One crRNA is usually selected, privileging the vicinity of the Cas9 cut site to the editing target site: max 5–10 bp when possible. In fact, it has been demonstrated that the homology-directed repair (HDR) rate decreases dramatically when the template insertion occurs more than 5–10 bp away from the cut site ([Inui et al., 2014](#)). When multiple crRNA options exist, a) highest predicted on-target cut efficiency and b) lowest predicted off-target cut efficiency are also taken into consideration.

HDR donor DNA design

When designing an HDR donor DNA, it is important to assess whether the desired edit results in disruption of the Cas9 cleavage site (i.e., by PAM or crRNA recognition sequence abrogation). If the chosen editing strategy does not by itself prevent Cas9 nuclease activity towards the genomic locus after incorporation of the desired edit, silent mutations in the PAM sequence and/or seed



region of the crRNA should be introduced in the donor sequence in order to augment the editing efficiency.

This protocol is optimized for three different types of gene edits, each of which foresees the employment of a dedicated type of HDR donor DNA:

1. to obtain the knockin of small epitopes (i.e., 10–15 amino acids), Ultramer® DNA Oligonucleotides (IDT™) of about 150 bp are designed as shown in Figure 1A. The single stranded donor sequence includes two symmetric homologous flanking regions (50 bp each), identical to sequences adjacent to the insertion site which does not necessarily correspond to the gRNA cut site. In between the two homologous flanking regions, the oligonucleotide includes the sequence to be inserted. In our example, the latter consists of the small V5-epitope (aa: GKPIP NPLLGLDST), preceded by a short flexible linker (the dipeptide GS; note that the example

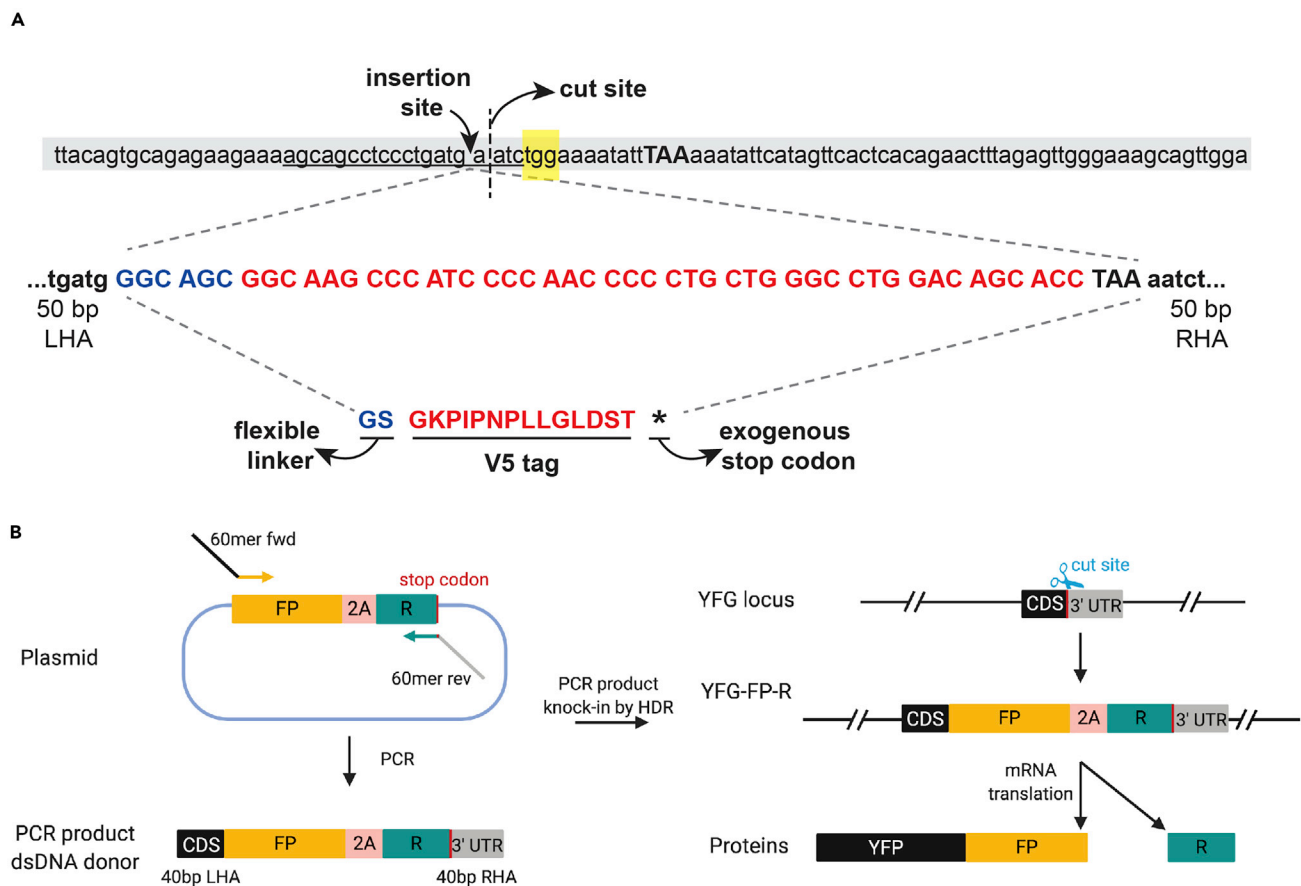


Figure 1. Examples of strategies for protein tagging using ssDNA or PCR products as HDR templates

(A) Upper part: the genomic sequence coding for the C-terminus of the centriolar distal appendage protein SCLT1 is reported. The underlined sequence represents the crRNA recognition site, with the PAM sequence in yellow and the cut site indicated by the vertical dashed line. Given that the cut site is 14 bp away from the endogenous STOP codon (**TAA** in bold uppercase letters), the insertion site was arbitrarily positioned 1 bp away from the cut site, i.e., at the closest possible junction between codons for SCLT1. In the lower part, codons (above) and the corresponding amino acidic residues (below) composing the insert are reported: blue capital letters refer to the flexible linker followed by V5-tag (in red) and an additional exogenous stop codon (in black). 50 bp LHA or RHA = 50 base pairs Left Homology Arm or Right Homology Arm.

(B) Schematic for the generation of a C-terminal tagging of a protein (Your Favorite Protein, YFP) with a fluorescent protein (FP) using a PCR product as donor DNA. The PCR template is constituted by a standard plasmid (left side) carrying the FP, a 2A element and a resistance cassette (R). The PCR reaction is performed using a pair of 60mer primers. On the right side the editing at the targeted locus (Your Favorite Gene, YFG) is represented.

- reports tagging near the C-terminal end of the protein). The percentage of cells carrying at least heterozygote V5-tagging using this strategy spans from 35% to more than 60%.
- to introduce point mutations, Ultramer® DNA Oligonucleotides (IDT™) of about 85 bp carrying the base to be substituted in the center are used. We have observed editing efficiencies spanning from 10% to over 30% when using this strategy.
 - to fuse endogenous proteins with larger tags (e.g., fluorescent proteins), dsPCR products up to ca. 2 kbp are utilized and generated following the eFlut strategy (Stewart-Ornstein and Lahav, 2016). A pair of 60mer oligonucleotides, serving as PCR primers, is designed to incorporate 40 overhanging bases of homology to the insertion site at the 5'-end; 20 bases of annealing sequence, complementary to a standard plasmid, are incorporated at their 3'-end. The plasmid, serving as template for the PCR reaction, carries the fluorescent protein and an antibiotic resistance, separated by a 2A peptide (Figure 1B). In the absence of antibiotic selection, knockin efficiency of larger tags is significantly less efficient than in 1. and 2. Yet, when combined with antibiotic selection as detailed below, this approach yields over 80% of positively tagged cells on at least one allele.

Note: for the strategies 1. and 2., phosphorothioate bond modifications are introduced at the outermost two nucleotide bonds at both the 5'- and 3'-end of the Ultramer® sequence. This modification increases the internucleotide linkage resistance to nuclease degradation (Stein et al., 1988).

PCR primer design

PCR reactions performed on genomic DNA extracted from cells after electroporation can serve as a diagnostic tool to rapidly visualize, on agarose gel (1%-2%), the presence of upshifted bands corresponding to the size of the edited sequence, including either a small epitope or a larger marker. PCR primers are designed to produce a ca. 200 bp amplicon on the parental genome, allowing to clearly discriminate even the presence of a small upshift (i.e., an increment of ca. 50 bp in the case of small epitope tagging). When the desired edit does not influence the size of possible PCR products (i.e., when inserting point mutations), PCR primers for amplicons of 400–700 bp are designed, having the predicted cut site 200–300 bp downstream of the annealing site of the forward primer. This allows to assess whether the efficiency of the RNP-mediated gene editing has been successful by subjecting PCR products to Sanger sequencing and analyzing the resulting chromatograms with tools such as ICE (Synthego) (Hsiau et al., 2018) or TIDE / TIDER (Brinkman et al., 2014; Brinkman and van Steensel, 2019), see key resources table.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals and recombinant proteins		
Cas9	Purified in house according to (Anders and Jinek, 2014)	From the plasmid "pET-28b-NLS-Cas9-2NLS-His"
Cas9	IDT™	Cat# 1081058
NU7441	Selleck Chemicals	Cat# S2638
Experimental models: cell lines		
hTERT-RPE1	(Burigotto et al., 2020)	N/A
Oligonucleotides		
Alt-R® CRISPR-Cas9 crRNA	IDT™	N/A
Alt-R® CRISPR-Cas9 tracrRNA	IDT™	Cat# 1072532
Alt-R® Cas9 Electroporation Enhancer	IDT™	Cat# 1075915
Ultramer® DNA Oligonucleotides	IDT™	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HPRT1 crRNA: 5' GCATTTCTCAGTCCTAAACA 3'	(Yu et al., 2016)	N/A
HPRT1 Fwd Primer: 5' CAGCA GCTGTTCTGAGTACTT 3'	This protocol	N/A
HPRT1 Rev Primer: 5' CCCTTGACCCAGAAATTCCAC 3'	This protocol	N/A
Recombinant DNA		
pET-28b-Cas9-His	(Gagnon et al., 2014)	Addgene plasmid #47327
pET-28b-NLS-Cas9-2NLS-His	This study, modifying the pET-28b-Cas9-His with two additional NLS sequences fused to Cas9	N/A
Software and algorithms		
ICE	(Hsiau et al., 2018)	https://ice.synthego.com/
TIDE	(Brinkman et al., 2014)	https://tide.nki.nl
TIDER	(Brinkman and van Steensel, 2019)	https://tide.nki.nl
IDT™ Custom Alt-R® CRISPR-Cas9 guide RNA	N/A	https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM
CRISPOR	(Concordet and Haeussler, 2018)	http://crispor.tefor.net/
CRISPick - Broad Institute	(Doench et al., 2016)	https://portals.broadinstitute.org/gppx/crispick/public
Other		
4D-Nucleofector™ X Unit	Lonza	Cat# AAF-1002X
4D-Nucleofector™ Core Unit	Lonza	Cat# AAF-1002B
P3 Primary Cell 4D-Nucleofector™ X KitS	Lonza	Cat# V4XP-3032

STEP-BY-STEP METHOD DETAILS

gRNA preparation and RNP assembly

⌚ **Timing:** 45 min

⚠ **CRITICAL:** Work in sterile conditions under a biological hood.

1. Resuspend Alt-R® CRISPR-Cas9 crRNA (IDT™) and Alt-R® CRISPR-Cas9 tracrRNA (IDT™) in Nuclease Free Duplex Buffer (IDT™) to a final concentration of 100 μM and keep on ice.
2. Prepare gRNAs by mixing 1:1 crRNA and tracrRNA.
 - a. 5 μL of 100 μM crRNA
 - b. 5 μL of 100 μM tracrRNA
3. Heat the mixture at 95°C for 5 min.
4. Cool down samples to 18°C–22°C on the benchtop for 5 min.

⏸ **Pause point:** crRNAs, tracrRNA and gRNAs can be stored at –20°C for 1 year. Homemade Cas9 (Anders and Jinek, 2014) instead can be stored at –80°C in 20% v/v glycerol.

5. Mix the following components to form the ribonucleoprotein (RNP).
 - a. 3.0 μL of gRNA
 - b. 120 pmol of Cas9 (see [key resources table](#). Typically 1.8 μL at a 70 μM concentration)
 - c. If needed, add Nuclease Free Duplex Buffer (IDT™) to a final volume of 5 μL per reaction
6. Incubate for 15–20 min at 18°C–22°C.

⏸ **Pause point:** RNPs can be stored at 4°C for 1 month and at –20°C for 2 years.

hTERT-RPE1 electroporation

⌚ Timing: 2 h

RPE1 cells are electroporated in the 4D-Nucleofector™ System (Lonza) using the P3 Primary Cell 4D-Nucleofector™ X Kit S (Lonza).

7. Resuspend Ultramer® DNA Oligonucleotides (IDT™) in Nuclease Free Duplex Buffer (IDT™) to a final concentration of 100 μM and keep on ice. Resuspended Ultramer® DNA Oligonucleotides (IDT™) can be stored at –20°C for 2 years.
8. Prepare P3 Primary Cell Full Electroporation Buffer (Lonza) to a final volume of 20 μL per reaction and keep on ice.
 - a. 16.4 μL of P3 Primary Cell Nucleofector™ Solution
 - b. 3.6 μL of Supplement
9. Set up the 4D-Nucleofector™ System (Lonza) program in advance.

Note: Lonza (<https://knowledge.lonza.com/>) provides a cell line-specific 4D-Nucleofector™ System electroporation program and kit. EA-104 program and P3 Primary Cell kit are used as the optimal setup for RPE1 cells. The same kit is used for the electroporation of human iPSCs in combination with program CM-113, while for example the SE Cell Line kit and CH-125 program can be used for MDA-MB-231 breast cancer cells.

10. Starting from a 10 cm culture dish, detach cells by trypsinization and transfer the cell suspension in a 15 mL tube.

Note: Consider that contact inhibition displayed by RPE1 cells might, when present, negatively impact on the fraction of actively cycling cells, hindering thereby the HDR efficiency. Cells for electroporation should be harvested when growing exponentially, i.e., at less than 80% confluency.

11. Centrifuge cell suspension at 400 × g for 5 min and discard the supernatant.
12. Resuspend the pellet with 5 mL PBS (pre-warmed at 37°C).
13. Count (e.g., using the LUNA Automated Cell Counter, Logos Biosystems) the required number of cells (2×10^5 cells/reaction) and transfer the corresponding volume in a 1.5 mL tube.
14. Centrifuge the cell suspension at 400 × g for 5 min.
15. Discard the supernatant and resuspend the pellet in 20 μL P3 Primary Cell Full Electroporation Buffer (or an alternative buffer if a different cell line is used).
16. Electroporation mix is obtained combining:
 - a. 20 μL of cell suspension in P3 Primary Cell Full Electroporation Buffer
 - b. 1.2 μL of Alt-R® Cas9 Electroporation Enhancer (IDT™)
 - c. 1.2 μL of Ultramer® DNA Oligonucleotide (100 μM, IDT™), corresponding to 1.2 nmol
 - d. 5 μL of RNP complex
 - e. PBS to a final reaction volume of 30 μL.

Alternatives: If using PCR amplicons (ca.2 kbp) as donor DNAs add 200 ng of purified PCR product (NucleoSpin® Gel and PCR Clean-up columns, Macherey-Nagel) to the reaction instead of the Ultramer® DNA Oligonucleotide. Note that using 50 ng of PCR product as HDR donor significantly reduced the knockin efficiency.

17. Mix gently each sample and transfer 25 μL in a free well of the 16-well electroporation strip (Lonza).
18. Place the strip in the proper 4D-Nucleofector™ System (Lonza).
19. Electroporate the cells, recover the strip and turn off the instrument.

Note: The unused wells from the strip can be used for another experiment.

△ **CRITICAL:** Work in sterile conditions under a biological hood.

DNA-PKcs inhibition and recovery of the cells

⌚ **Timing:** 3–7 days

The electroporation reaction is followed by a 48-hour treatment with the DNA-PKcs inhibitor NU7441 with the ultimate aim to favor the occurrence of homology-directed repair (HDR) at the Cas9-generated double strand break.

20. Keep the cells into the strip for 10 min.
21. Meanwhile prepare the post-electroporation culture medium by adding NU7441 DNA-PKcs inhibitor to pre-warmed DMEM-F12 to a final concentration of 1 μ M and fill a 12-well plate (1–2 mL/well).

Note: The administration of the DNA-PKcs inhibitor NU7441 resulted in an evident improvement of tagging efficiency for all the editing strategies implemented with the present protocol (see [Figure 2](#) as an example). The observed favorable NU7441 effect was confirmed also in breast adenocarcinoma MDA-MB-231 cells or in human iPSCs, suggesting that various cell types might display the same trend. See [problem 3](#) for a discussion of the poor tolerability of NU7441 displayed by some cell types.

22. Add 75 μ L of NU7441 DMEM-F12 medium to each well of the electroporation strip and resuspend the cells.
23. Use a capillary tip (e.g., a gel-saver tip) to collect the whole volume (100 μ L) and seed the cell suspension in the 12-well plate.
24. Incubate the cells at 37°C with 5% CO₂ and change the culture medium 18–24 h after electroporation, maintaining NU7441 treatment.
25. 48 h after electroporation change the medium with fresh DMEM-F12 complete medium without NU7441.
26. Keep cells in culture up to when they are fully recovered.

Note: RPE1 cells may require from 3 to 7 days to completely recover after electroporation.

Alternatives: Ultramer® DNA Oligonucleotides-based protocols do not require antibiotic selection (up to 60% of cells after electroporation are positive for the desired edit). Contrarily, tagging with larger markers is less efficient: donor DNAs are designed so that they contain a Neomycin resistance cassette enabling to enrich for the population of edited cells, [Figure 1B \(Stewart-Ornstein and Lahav, 2016\)](#) upon antibiotic selection. In this case, fully recovered RPE1 cells after electroporation are kept under antibiotic selection with G418 (400 μ g/mL, InvivoGen) for 10 days.

Editing efficiency analysis via PCR, followed either by analytical electrophoresis or Sanger sequencing and Inference of CRISPR Edits (ICE)

⌚ **Timing:** 3 days

27. Harvest part of the fully recovered cells after electroporation, while keep the rest in culture, and extract genomic DNA using the NucleoSpin® Tissue kit (Macherey-Nagel) following manufacturer's instructions for cultured cells.

Note: 50% of a confluent 12-well plate well is sufficient to successfully perform genomic DNA extraction resulting in ca. 1.5 μ g of genomic DNA.

Cycle Steps	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	35
Annealing	-	10 s	
Extension	72°C	-	
Final extension	72°C	2 min	1
	4°C	∞	

Note: Derive the annealing temperature using the Thermo Fisher T_m Calculator for Phusion Polymerase (available on the manufacturer's web page).

Note: Calculate the extension time considering 30 s per 1 kb following manufacturer's instructions for high complexity genomic DNA.

Alternatives: For point mutations application of this protocol, skip step 30 and proceed with steps 31 and 32.

30. If the desired edit induces a change in size of the designed PCR amplicon (as in the case of epitope tagging of a protein), run the PCR product on an analytical agarose gel. Evaluate the editing efficiency based on the observed electrophoretic pattern in the heterogenous cell population stemming from the electroporation (Figure 3).
31. Purify PCR products at the expected molecular weight using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), then quantify the eluate.
32. Samples are subjected to Sanger sequencing at a commercial provider (e.g., Eurofins) utilizing the forward PCR primer as sequencing primer and the resulting chromatograms are analyzed using the ICE online tool by Synthego (Hsiao et al., 2018), or TIDE / TIDER (Brinkman et al., 2014; Brinkman and van Steensel, 2019).

Generation of monoclonal cell lines by limiting dilution

⌚ Timing: 6–8 weeks

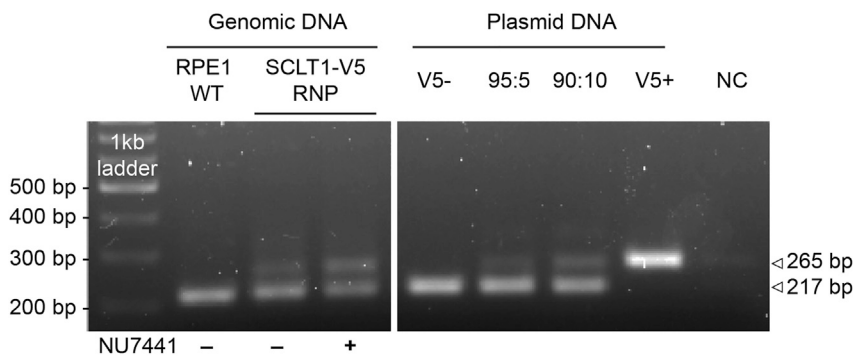


Figure 3. Analyses of RNP-mediated editing efficiency using agarose gel electrophoresis

Small PCR products were run in 2% agarose gel to separate upper and lower band corresponding to edited and parental templates, respectively.

Synthetic pIDNA containing either the parental sequence (V5-) or the expected sequence after integration of V5 (V5+), were generated and used as reference for the expected electrophoretic behavior. Synthetic controls were used alone or mixed in the indicated fractions of parental:edited DNA (right part of the gel). The PCR strategy established V5-tag insertion at the desired site after RNP electroporation (left part of the gel). Moreover, the augmented editing efficiency upon DNA-PKcs inhibition (NU7441 +) is confirmed. NC = negative control. Both gel parts in this figure stem from the same image.

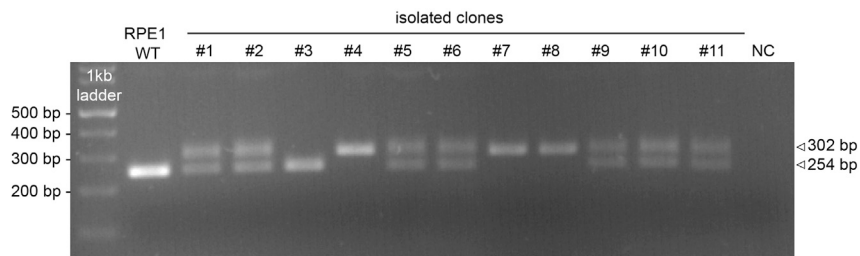


Figure 4. Genotyping of candidate V5+ monoclonal RPE1-derivatives

Small-amplicon PCRs spanning the V5 insertion genomic site were designed to yield either a 254 bp (non-edited parental DNA) or 302 bp (V5+ DNA) PCR product. PCR reactions were run on genomic DNA isolates obtained from 11 independent clones generated by limiting dilution, thereby revealing homo- vs heterozygosity of the tagging.

Note: The number of clones to be screened in order to successfully isolate the edited RPE1 derivative varies in dependence of the editing efficiency of the strategy. While the work carried out in the previous section can influence the size of the screening pool, we estimate that the percentage of cells carrying at least one edited allele when utilizing an Ultramer® as HDR template varies between 35% and 62%. When using the eFlut strategy, we observed that on average 84% of the cells surviving antibiotic selection was carrying at least one allele characterized by the desired edit.

33. Fill the appropriate number of 96-well plates (usually 6–10) with 200 μL /well of pre-warmed DMEM-F12.
34. Once electroporated cells are fully recovered detach them by trypsinization and count them using the LUNA Automated Cell Counter (Logos Biosystems).
35. Add 0.5 or 1 cell/well contained in 10 μL of diluted cell suspension to the 96-well plates.
36. Incubate cells for at least 2 weeks before proceeding with expansion and characterization (i.e., immunoblotting, immunofluorescence, PCR, Sanger sequencing, etc.), see Figure 4 as example.

Note: The clonogenic potential of RPE1 cells handled as described is approximately 15%, i.e., each 96 well plate will give rise to clones in ca. 15 wells when seeding 1 cell/well.

Note: When the tagging confers an increase in fluorescence of the cells, FACS-sorting can greatly improve the percentage of productively tagged cells within a heterogeneous population and/or it can be exploited to seed individual fluorescent cells in a microtiter plate for cloning (Collin et al., 2013). If productive HDR confers an antibiotic resistance as suggested here however, considering that over 80% of the cells surviving antibiotic selection bear the desired integration, FACS-sorting might provide little/no advantage.

Note: Inspect all the wells during the first two weeks of incubation and exclude those which are not suitable for further analyses (i.e., polyclonal or empty wells).

Note: RPE1 cells effectively undergo contact inhibition when reaching confluency. This phenomenon can be exploited to defer the screening of clones stemming from the same 96-well plate seeding iteration. For instance, once all suitable candidates are transferred from a 96- to a 24-well plate, a fraction of clones can be kept at confluency while expanding the rest to a format compatible with genetic/functional characterization.

EXPECTED OUTCOMES

This genetic engineering methodology presents several key advantages which allow to enhance the overall efficiency of the process. First, as opposed to other well established knockin protocols in the literature (Bak, Dever and Porteus, 2018), with our approach molecular cloning can be readily

bypassed, resulting into a major shortening of the execution time. Second, the electroporation of pre-formed RNP complexes enables to obtain higher editing efficiency and, equally important, it allows to yield fewer off-target effects and nonspecific tag integrations than methods relying on other delivery systems (Gaj et al., 2017). In fact, RNPs combine a fast action and a short half-life once in the intracellular environment, contrary to what happens with lentiviral vectors expressing Cas9 that integrate permanently in the cellular genome. Additionally, our results using RPE1 cells show that the transient pharmacological inhibition of DNA-PKcs activity can be harnessed to enhance HDR in the presence of double strand breaks (DSBs) induced by Cas9 nuclease. Despite the fact that we never addressed whether pharmacological enhancement of HDR-mediated events promotes per se homozygous knockins (disfavoring heterozygous edits), it is evident that the administration of the DNA-PKcs inhibitor NU7441 results in an overall improvement of tagging efficiency for all our target proteins, conceivably facilitating the isolation of clones carrying both homozygous and heterozygous edits. Our results strongly corroborate the notion that it is possible to favor HDR-mediated repair events by precisely controlling the balance existing between this mechanism and non-homologous end joining (NHEJ), thus concomitantly reducing the subsequent workload necessary to isolate monoclonal cell lines carrying the desired HDR-mediated edits in homozygosis.

LIMITATIONS

The main limitation of this protocol is that it relies on a specific device for Cas9 RNP electroporation, which might hinder the wide applicability of this approach. To tackle this issue, we have assessed the ability of the same Cas9 RNP, directed towards an intronic region of the *HPRT1* gene by a specific crRNA, to promote edits when delivered with two distinct commercially available electroporators, namely 4D-Nucleofector™ System (Lonza) and Neon™ Transfection System (Thermo). While the 4D device yielded a higher editing efficiency, likely due to the most efficient delivery, the edits obtained with the Neon appeared qualitatively comparable, i.e., composed predominantly by +1, –1 and –10 INDELS (Figure 5). Thus, we speculate that the delivery method of Cas9 RNP might impact on the overall efficiency of the protocol, without however significantly altering the quality of the obtained edits. Moreover, we suggest that the *HPRT1* crRNA described here, used in conjunction with sequencing and ICE/TIDE analyses- of the *HPRT1* locus, might serve as a reference for estimating the efficiency of the chosen Cas9 RNP delivery method, regardless of it relying on electroporation or lipofection.

Moreover, as for previously published gene editing methods, this approach suffers from a series of limitations which are inherent to the concept of protein tagging. First of all, editing efficiency strongly depends on the activity of the crRNA used to drive the Cas9-mediated cleavage. Each crRNA sequence in fact behaves differently depending on several factors, such as the accessibility of the DNA target site. Another limitation is constituted by the fact that the introduction of the insert, be it an epitope or a larger marker, may perturb target protein function.

TROUBLESHOOTING

Problem 1

Low levels of editing efficiency

As mentioned above, a variety of factors may impact on the final editing efficiency for a specific target sequence: no or low editing efficiency may occur.

Potential solution

There are different potential solutions for this issue. First of all, a second round of crRNA design may be necessary to obtain sufficient Cas9 nuclease activity at the site of interest. An additional variable influencing Cas9 activity is that gene editing in eukaryotic cells requires the translocation of Cas9 in the nucleus and this depends on the presence of one or more NLS (Nuclear Localization Signal) in the nuclease sequence, therefore the selection of the Cas9 nuclease has to be carefully evaluated as

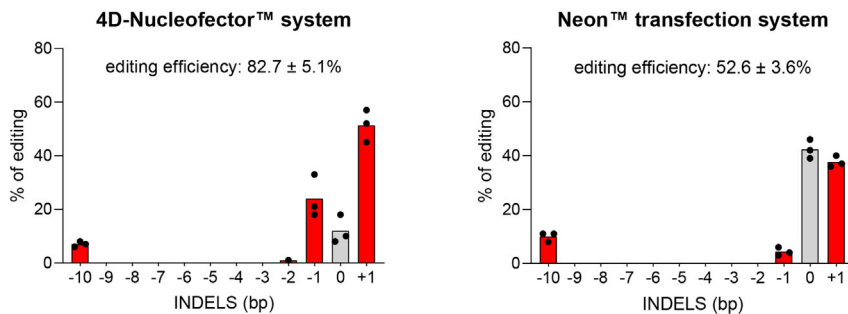


Figure 5. Comparison between the edits obtained using different electroporation devices

RPE1 cells were electroporated with an RNP targeting an intronic region in the *HPRT1* locus using the indicated commercial device. 5 days later genomic DNA were isolated, and the edits were characterized by Sanger sequencing followed by ICE analysis. Individual values of biological replicates and their mean are displayed for every possible INDEL. Editing efficiencies \pm standard deviations. Neon transfection was performed utilizing 200.000 cells and the following settings: 1350 V, 20 msec, 2 pulses and 5 μ L of RNP.

well. The use of alternative Cas endonucleases, e.g., Cas12a (TTTV PAM sequence) widening the range of crRNAs and therefore of targetable sequences to A/T rich loci should be also considered. Moreover, the target site itself has to be considered as a factor that is potentially influencing the final outcome. For instance, manipulation of a specific locus may disrupt the endogenous functionality of the target by means of either the introduction of random INDELS or the knockin of the desired insert, thus causing the loss of the majority of the edited cells (in the case of an essential gene for example) and resulting in an underestimated rate of editing efficiency.

Problem 2

Low quality chromatograms

Potential solution

Try to optimize the PCR reaction by adjusting the reaction mix, i.e., reaction buffers, DMSO, etc., and/or on the PCR protocol, i.e., annealing temperature, extension time, number of cycles, etc., following manufacturer's instruction. In some cases, running gradient- as well as touchdown-PCRs may be useful to define the optimal annealing temperature.

Alternatively, consider the possibility to design new couples of primers for the same target site.

Problem 3

Insufficient NU7441 effect or NU7441 toxicity

Potential solution

Adjust the NU7441 concentration/treatment duration or employ other NHEJ inhibitors, targeting either DNA-PKcs itself or other proteins relevant for NHEJ such as DNA Ligase IV (Maruyama et al., 2015) or 53BP1 (Canny et al., 2018).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Luca L Fava (luca.fava@unitn.it).

Materials availability

Reagents generated within this study are available upon request.

Data and code availability

This study did not generate any unique datasets or codes.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.G., A. Casini, A. Cereseto, and L.L.F. conceived and designed experiments; S.G., M.B., A.M., G.M., and A.B. performed experiments, S.G., M.B., A.M., and L.L.F. analyzed the data, S.G. and L.L.F. wrote the manuscript; all authors edited the manuscript; L.L.F. conceived and supervised the study.

DECLARATION OF INTERESTS

A. Cereseto is a founder and part of the Board of Directors of Alia Therapeutics Srl. A. Casini is a founder and an employee of Alia Therapeutics Srl.

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