A marker-free co-selection strategy for high efficiency homology-driven and NHEJ-based gene editing in human cells

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Abstract

Genome editing using designer nucleases has revolutionized molecular biology by allowing DNA to be inserted, deleted or replaced in the genome of several model organisms. This protocol describes a marker-free co-selection strategy for high efficiency homology-driven and NHEJ-based gene editing in human cells by generating dominant cellular resistance to ouabain, a highly potent plant-derived inhibitor of the Na^+,K^+-ATPase (1, 2, 3). This scarless co-conversion strategy is highly efficient and can yield a stable population of modified cells in 14 days. Techniques relative to sgRNA cloning, cell nucleofection, selection using ouabain and validation of gene disruption/insertion are described.

Reagents

General reagents

- Agarose A (Biobasic, cat. no. D0012)
- TAE buffer (Bioshop, cat. no. TAE222.4)
- Red safe (Froggabio, cat no. 21141)
- Gel loading dye Orange G, 6X (NEB, cat. no. B7022)
- 1 Kb plus DNA Ladder (Invitrogen, cat. no.10787-026)

CD34+ isolation

- Ficoll-Paque Plus (GE Healthcare, cat. no. 17-1440-03)
- EasySep^{TM} Human CD34 positive selection kit (StemCell Technologies, cat. no. 18056)
- Cryostor CS10 (StemCell Technologies, cat. no. 7930)

Cloning reagents

- pY036_ATP1A1_G3_Array (Addgene ID: 86619)
- pY036_ATP1A1_G5_Array (Addgene ID: 86620)
- eSpCas9(1.1)_No_FLAG_ATP1A1_G2_Dual_sgRNA (Addgene ID: 86612)
- eSpCas9(1.1)_No_FLAG_ATP1A1_G3_Dual_sgRNA (Addgene ID : 86613)
- LB growth media (10 g/L tryptone, 10 g/L NaCl, 5g/L Yeast extract)
- Ampicillin salt (Biobasic, cat. no. AB0028)
- BbsI (NEB, cat. no. R0539)
- Buffer 2.1 (NEB, cat. no. B7202S)
- T4 DNA ligase and buffer (NEB, cat. no. M0202)
- DH5 alpha competent cells
- gRNA custom DNA oligonucleotides with appropriate overhangs (Integrated DNA Technologies)
- Tris HCl 10mM pH 7.4
- Annealing Solution (10 mM Tris HCl pH 7.4, 1 mM EDTA, 50 mM NaCl)
- LB agar + ampicillin plates
- Midi prep plasmid purification kit (OMEGA bio-tek, cat. No. D6905-04)
- Gel extraction purification kit (Biobasic, cat. no. BS654)
- DNA clean and concentrator kit (Zymo research, cat. no. D4006)

Cell culture reagents

- 293 LTV cells (Cell Biolabs, cat. no. LTV 100)
- K562 cells (ATCC, cat. no. CCL-243)
- hTERT RPE-1 cells (ATCC, cat. no. CRL-4000)
- U-2 OS cells (ATCC, cat. no. HTB-96)
- RPMI 1640 with GlutaMAX supplement (Life Technologies, cat. no. 61870-036)

- DMEM high glucose with GlutaMAX supplement (Life Technologies, cat no.10566016)
- IMDM (Life Technologies, cat no. 12440-046)
- Mccoy's 5A (Modified) Medium (Life Technologies, cat. no.16600108)
- Tryple Express with phenol red (Life Technologies, cat. no.12605028)
- Fetal bovine serum of Canadian origin (Life Technologies, cat. no. 12483020)
- Human serum albumin (HSA) (CSL Behring, cat. no. 100340)
- Penicillin streptomycin solution (Life Technologies, cat. no.15140122)
- DNase I (Sigma, cat. no. D4513)
- PBS pH 7.4 (Life Technologies, cat. no. 10010049)
- Ouabain Octahydrate (Sigma Aldrich Canada, cat. no. O3125)
- SF cell line 4D-Nucl X Kit S (VWR international, cat. no. CA10064-156)
- SE cell line 4D-Nucl X Kit S (VWR international, cat. no. CA10064-150)
- P3 prim cell 4D-Nucl X Kit S (ESBE Scientific, cat. no. AMA-V4XP3032)
- Alt-R^{TM} CRISPR-Cas9 custom crRNA (Integrated DNA Technologies)
- Alt-R^{TM} CRISPR-Cas9 tracrRNA (Integrated DNA Technologies, cat. no. 1072534)
- Nuclease-Free IDTE Buffer (Integrated DNA Technologies, cat. no. 11-05-01-15)
- Alt-R^{TM} S.p. Cas9 Nuclease 3NLS (Integrated DNA Technologies, cat. no. 1074182)
- StemSpan ACF (StemCell Technologies, cat. no. 9855)
- SCF (Feldan, cat. no. A012)
- FLT3-L (Peprotech, cat. no. 30019)
- TPO (StemCell Technologies, cat. no. 78070)
- LDL (StemCell Technologies, cat. no. 2698)
- UM171 (StemCell Technologies, cat. no. 72912)
- StemSpan^{TM} CD34+ Expansion supplement (StemCell Technologies, cat. no. 02691)
- Solution 18-AO-DAPI (Chemometec, cat. no. 910-3018)
- Anti-human CD34-FITC (Immunotech, cat. no. IM1870)

Surveyor and restriction fragment length polymorphism assay reagents

- Quick Extract (Illumina, cat. no. QE09050)
- Phusion high fidelity DNA polymerase (Fisher Scientific, cat. no. F530L)
- Q5 high fidelity PCR Kit (NEB, cat. no. E0555)
- Deoxynucleotide solution mix, 40 µmol each (NEB, cat. no. N0447)
- Cel1 PCR primers, see Supplementary table 6 (3) (Integrated DNA Technologies)
- PCR product purification kit (Biobasic, cat. no. BS664-250)
- BmgBI (NEB, cat. no. R0628)
- ClaI (NEB, cat. no. R0197)
- Hpy188I (NEB, cat. no. R0617)
- Cutsmart buffer (NEB, cat. no. B7204S)

- Buffer 3.1 (NEB, cat. no. B7203S)
- Surveyor assay kit (Integrated DNA Technologies, cat. no. 706020)
- Acrylamid/bisacrylamid 29:1 30% (Biobasic, cat. no. A0010)
- Glycerol (Biobasic, cat. no. GB0232)
- Ultrapure water
- Ammonium persulfate (Sigma, cat. no. A3678)
- Tetramethylethylenediamine (Sigma, T9281)
- 5x DNA loading dye (75% glycerol, 10mM Tris-HCl pH7.4, 10 mM NaCl, 10 mM EDTA, 0.1% SDS, Orange G)
- 100 bp ladder (Invitrogen, cat. no. 15628-050)
- Cel1 buffer 2X (20 mM Tris pH 8.8, 3 mM $MgCl_2$, 100 mM KCl)
- AccuPrime Taq DNA polymerase with 10x AccuPrime PCR Buffer II (ThermoFisher Scientific, cat. no. 12339-016)
- Mini-PROTEAN TBE 10% precast gels (Bio-Rad, cat. no.161-1182)
- UltraPure TBE buffer, 10X (Life Technologies, cat. no. 15581-028)
- SYBR Gold nucleic acid gel stain, 10 000X (ThermoFisher Scientific, cat. no. S11494)
- Illustra GFX purification kit (GE Healthcare, cat. no. 28-9034-70)
- FastDigest PshAI (BoxI) (ThermoFisher Scientific, cat. no. FD1434)
- FastDigest PstI (ThermoFisher Scientific, cat. no. FD0615)
- FastDigest Buffer 10X (ThermoFisher Scientific, cat. no. B64)
- 50 bp ladder (Life Technologies, cat. no. 10416-014)

Equipment

- Table top centrifuge (Thermo scientific)
- Microcentrifuge (Thermo scientific)
- Filtered sterile pipette tips (VWR international)
- Axygen microcentrifuge tubes, 1.5 mL (Corning, cat. no. MCT-150-C)
- Falcon tubes polypropylene, 15 mL (Sarstedt)
- Falcon tubes polypropylene, 50 mL (Sarstedt)
- Vortex
- Micro-mini centrifuge (Fisher)
- Thermal cycler with programmable temperature stepping functionality (Bio-Rad)
- Horizontal electrophoresis system (Labnet)
- UV transilluminator (Alpha Innotech)
- LeucoSep tubes, 50 mL (VWR International, cat. no.89048-936)
- Nucleocounter NC-250 (Chemometec)
- Mini-PROTEAN Tetra Cell vertical gel electrophoresis apparatus (Bio-Rad)
- Power supply (Bio-Rad)
- Flow cytometry cell analyzer (BD Biosciences)

Cloning equipment

- Water bath 37°C and 42°C
- Scalpel blade
- 250 mL sterile erlenmeyer
- 37°C incubator shaker (250 rpm)
- Nanodrop (Thermo scientific)
- Microwave

Cell culture equipment

- Tissue culture flask, 75 cm^2 (T75 flask), filter cap (Sarstedt)
- Tissue culture flask, 25 cm^2 (T25 flask), filter cap (Sarstedt)
- Tissue culture plate, 96 wells (Sarstedt)
- Tissue culture plate, 48 wells (Sarstedt)
- Tissue culture plate, 24 wells (Sarstedt)
- Tissue culture plate, 12 wells (Sarstedt)
- Tissue culture plate, 6 wells (Sarstedt)
- Inverted microscope (Motic)
- Hemacytometer
- CO_2 incubator set at 37°C, 5% CO_2
- Amaxa 4D nucleofector, X unit (Lonza)

Surveyor and restriction fragment length polymorphism equipment

- 8-strip PCR tubes
- Heating block
- ChemiDoc imaging system (Bio-Rad)

Procedure

1- Design of sgRNA target sequence

(a) Screen genomic regions of interest using GPP Web Portal (http://portals.broadinstitute.org/gpp/public/), the MIT web-based CRISPR design tool (http://crispr.mit.edu/) or Benchling (https://benchling.com/academic) for potential PAM and gRNA sequences.

(b) If applicable, when using eSpCas9 dual vectors, change the first base pair to a guanine (G) as it is the preferred transcription start site for human U6 promoter.

(c) Add appropriate overhangs to the gRNA sequence.

eSpCas9 dual (Addgene #86612 and #86613) and AsCpf1 array vectors (Addgene #86619 and #86620) were designed with a BbsI cloning cassette. Hence, the custom oligonucleotides should be ordered with the following overhangs:

eSpCas9 dual vectors:

Top Oligo 5' **CACC**- (20nt gRNA target sequence) 3'
Bot Oligo 5' **AAAC-** (20nt gRNA reverse complement to the target sequence) 3'

AsCpf1 array vectors

Top Oligo 5' **AGAT**- (23nt gRNA target sequence) 3'
Bot Oligo 5' **AATT-** (23nt gRNA reverse complement to the target sequence) 3'

Further information on cloning in Cas9 single gRNA vectors was published previously (4).

2- sgRNA preparation

(a) Using filtered sterilized pipette tips, resuspend oligonucleotides at a concentration of 1µg/µL in Tris HCL 10 mM pH 7.4 (Eg. for 0.20 mg of oligonucleotide, add 200 µL of Tris HCL 10 mM pH 7.4).

(b) Vortex and spin down.

(c) Mix together top and bottom oligonucleotides for the same target:

2 µL of each oligonucleotide at 1 µg/µL
50 µL annealing solution (see cloning reagents and equipment)

(d) Anneal oligonucleotides in thermocycler using the following program:

95 °C	10 mins	
95 °C to 85 °C		-2 °C/s
85 °C	1 min	
85 °C to 75 °C		-0.3 °C/s
75 °C	1 min	
75 °C to 65 °C		-0.3 °C/s
65 °C	1 min	
65 °C to 55 °C		-0.3 °C/s
55 °C	1 min	
55 °C to 45 °C		-0.3 °C/s
45 °C	1 min	
45 °C to 35 °C		-0.3 °C/s
35 °C	1 min	
35 °C to 25 °C		-0.3 °C/s
25 °C	1 min	
4 °C	hold	

Always keep annealed oligonucleotides on ice.

(e) Dilute 1/50 for downstream applications.

3-Preparation of AsCpf1 and eSpCas9 dual vectors for gRNA cloning

(a) Digest eSpCas9 and Cpf1 plasmids using BbsI endonuclease:

2 µg of plasmid
2 µL BbsI
6 µL NEBuffer 2.1
__ µL Ultrapure water
60 µL

(b) Incubate 90 minutes at 37°C

(c) Load digested plasmid on a 0.8-1% agarose TAE 1x gel.

(d) Run at 135 volts for 60 minutes and visualise digested plasmid using a UV transilluminator.

(e) Excise digested plasmid band using a razor blade.

(f) Gel purify using gel purification kit. Elute in 30 µL elution buffer.

(g) Ligate digested plasmid and annealed oligonucleotides

2 µL plasmid
2 µL annealed oligonucleotides (1/50 dilution)
2 µL T4 DNA ligase buffer
1 µL T4 DNA ligase
___ Ultrapure water
20 uL

(h) Incubate 1 hour at room temperature.

(i) Thaw 100 µL of competent DH5 alpha cells on ice.

(j) Add 2 µL of ligation to competent cells.

(k) Incubate 30 minutes on ice.

(l) Heat shock 30 seconds at 42°C.

(m) Incubate 3 minutes on ice.

(n) Add 900 µL of LB media.

(o) Incubate at 37°C with agitation for 1 hour.

(p) Spin 5 minutes at 2000 rpm.

(q) Remove 800 µL of the supernatant.

(r) Plate on LB agar + ampicillin plates.

(s) Incubate 37°C O/N.

(t) Prepare midi preps of the cloned plasmids and sequence gRNAs using the following primers:

eSpCas9 dual vectors:

ATP1A1 gRNA sequencing primer GCAGACAAATGGCTCTAGCTG

Target sequencing primer CAGCTAGAGCCATTTGTCTGC

AsCpf1 array vectors:

U6 primer

4- Cell culture and Nucleofection

4.1 Cell maintenance

(a) Maintain HEK 293 LTV and RPE-1 cells at 37 °C under 5% CO_2 in DMEM high glucose + glutaMAX medium supplemented with 10% FBS and 1% penicillin-streptomycin.

(b) Maintain K562 cells at 37 °C under 5% CO_2 in RPMI 1640 + glutaMAX medium supplemented with 10% FBS and 1% penicillin-streptomycin.

(c) Maintain U-2 OS at 37 °C under 5% CO_2 in Mccoy's 5A (Modified) Medium with 10% FBS and 1% penicillin-streptomycin.

(d) Split HEK 293 LTV and U-2 OS every 2-3 days and RPE-1 every 4-5 days using tryple express with phenol red.

(e) Split K562 1/20 every 2-3 days by transferring 0.5 mL of suspension in a new flask containing 9.5 mL of fresh pre-warmed media.

(f) Split adherent cells 2 days before nucleofection in order to reach 80% confluence for nucleofection.

(g) Split K562 2 days before nucleofection in order to reach a cell count between 200 000 - 500 000 cells/mL for nucleofection.

4.2 Nucleofection

(a) Determine the amount of plasmid to nucleofect for each condition. Refer to Supplementary table 2 (3) for general guidelines. This quantity will vary depending on the efficacy of the sgRNA and downstream application.

- To co-select for NHEJ, use the following plasmids:

  eSpCas9(1.1)_No_FLAG_ATP1A1_G2_Dual_sgRNA (Addgene ID:86612)
  or
  pY036_ATP1A1_G3_Array (Addgene ID: 86619)

- To co-select for HDR, use the following plasmids:

  eSpCas9(1.1)_No_FLAG_ATP1A1_G3_Dual_sgRNA (Addgene ID :86613)
  or
  pY036_ATP1A1_G5_Array (Addgene ID:86620)

(b) Culture cells in T75 flasks until reaching the recommended confluence for nucleofection. **Cells should not be passage more than 10 times before nucleofection**.

(c) For each condition, add 2 mL of cell culture media to a well of a 12-wells plate. Place the plates in the incubator to equilibrate the media.

(d) Wash adherent cells with 5 mL of warmed PBS and trypsinize using tryple express.

(e) Count cells using a hemacytometer.

(f) Centrifuge $2x10^5$ cells per nucleofection condition.

(g) Discard the supernatant.

(h) Prepare nucleofection mix by combining nucleofector solution and supplement according to the manufacturer's instructions.

(i) Resuspend the cell pellet in nucleofection mix.

(j) In sterile 1.5 mL tubes, distribute 20 µL of cell suspension per condition.

(k) Add the volume of plasmids needed for each condition into the 1.5 mL tube containing the cells. Maximum total plasmid volume should not be higher than 10% of final volume

(l) Mix very gently, then transfer 20 µL of the mix to the nucleofection strip provided.

(m) Transfer the transfection strip into the Amaxa 4D-Nucleofector X unit.

(n) Choose the transfection protocol according to the recommendations for the selected cell line.

(o) Start nucleofection.

(p) Incubate K562 cells 10 minutes at room temperature following nucleofection. For adherent cell line proceed to (q).

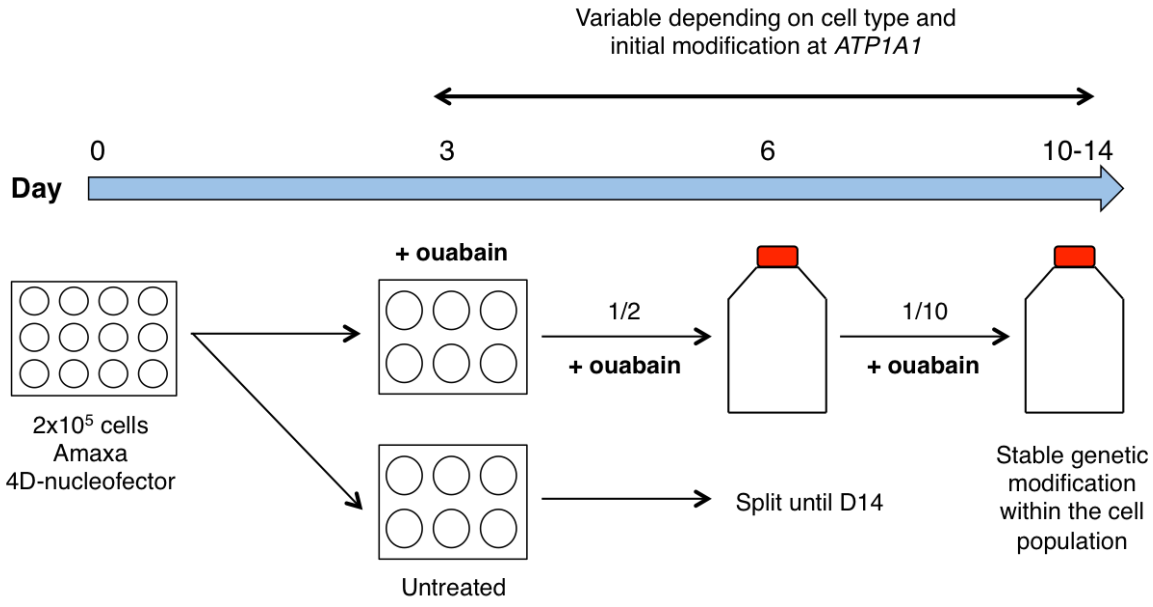
(q) Pipet 80 µL of prewarmed medium from the 12 wells plate prepared in (c) into the nucleofection well and mix gently by doing ups and downs.

(r) Transfer 80 µL from the nucleofection well to a well of the prewarmed plate prepared in (c). Transfer only 60 µL when nucleofecting 293T cells.

(s) Move plate backward and forward, then right to left to right, repeat same motions x 5 to ensure even distribution of cells.

(s) Incubate the 12-well plates at 37°C, 5% CO_2 for 72 hours, then start ouabain selection.

4.3 Ouabain selection



(a) Prepare ouabain solution.

- To prepare stock solution (68.6 mM), dissolve 50 mg of ouabain octahydrate in 1 mL of sterile water. At this concentration, ouabain is only soluble in hot water. To dissolve, heat the solution at 90 °C for 5-10 minutes. Vortex the solution a few times in order to reach complete dissolution.

- Prepare 1/10, 1/100 and 1/1000 (68.6 µM) solutions by serial dilution. All dilution steps should be carried out in warm water, heated and vortexed to ensure the homogeneity of the solution. From dilution 1/1000, use 7.3 µL for each milliliter of media to reach a concentration of 0.5 µM.

(b) Three days after nucleofection, split cells from each transfection into two 6 well plates.

In a first plate, add 500 µL of the cells for each condition and complete to 5 mL with warmed media. These non-treated cells will be used to determine the initial targeting efficacy in the population.

In a second plate, add 1.5 mL of the cells and complete to 5 mL with warmed media. Treat each well with 0.5 µM ouabain (36.5 µL of ouabain 1/1000 dilution).

- Trypsinize adherent cells before transferring to 6-well plate. Treatment with ouabain can be done simultaneously to cell plating. It is not necessary to wait until the cells have attached before adding ouabain.

- At confluence, split untreated cells 1/10 until selection of treated cells is complete.

- Selection with ouabain is robust. Within 48 hours, the majority of cells should die and only the cells that have gained ouabain resistance will proliferate. Nonetheless, subsequent split of the cells is of foremost importance to avoid interference from dead cells genomic DNA with subsequent analyses.

(c) After 3-6 days of selection, split cells ½ without wasting any live cell as follow:

- K562: Transfer 5 mL from the 6 well plate to a T75 flask and add 5 mL fresh media + 36.5 µL ouabain 1/1000 dilution*. Split K562 cells 3 days after treatment. Live cells should be at least 20-30% confluent before split.

- Adherent cells: Split ½ according to surface area. Trypisinize cells from a 6 well plate and transfer to a T25 flask in 5 mL fresh media + 36.5 µL ouabain 1/1000 dilution*. Some dead cells will attach to living cells and possibly cover them. It is important to let cells grow before eliminating dead cells. We recommend to wait at least 4 days after ouabain treatment to wash or split cells.

For all cell lines, timing should be adapted according to cell growth.

*Ouabain concentration must be maintained at 0.5 µM. Since ouabain is a very stable molecule, it is important to consider that the volume of cells taken for the split already contains 0.5µM ouabain. Thus, the amount of ouabain to be added depends only on the amount of fresh media added during the split.

(d) When treated cells reach about 70 % confluence, split the cells 1/10 in a T75 flask.

K562: Transfer 1 mL from the T75 flask to a new T75 flask and add 9 mL fresh media + 65,7 µL ouabain 1/1000.

Adherent cells: Split 1/10 according to surface area. Trypisinize cells from the T25 flask and transfer to a T75 flask in 10 mL fresh media + 73 µL ouabain 1/1000.

(e) At confluence, cell population is considered stable. Analyse gene targeting efficacy.

5. Surveyor Assay

(a) Extract genomic DNA from $2.5x10^5$ cells with 250 µL of QuickExtract DNA extraction solution per manufacturer's recommendations.

(b) Amplify loci of interest by PCR. Note that PCR primers should be designed to be eccentric from the predicted break site in order to yield fragments of different size following Surveyor digestion. PCR product should be between 400-650 bp.

An example is shown here for *ATP1A1*. Refer to Supplementary table 6 (3) for PCR primers used for other targets.

ATP1A1 Forward: GGATTAACATCTGCTCGTGCAGC
ATP1A1 Reverse: CACTTGTAAGAGCATCTACAACG

PCR mix per sample

1.0 µL DNA
0.5 µL dNTP 10 mM
0.5 µL ATP1A1 For 50 µM
0.5 µL ATP1A1 Rev 50 µM
5.0 µL Phusion HF buffer 5X
0.25 µL Phusion
17.25 µL Ultrapure water
25 µL

Use the following PCR program:

98 °C / 3 mins	
98 °C / 20 secs	30 cycles
65 °C / 20 secs	
72 °C / 20 secs	
72 °C / 5 minutes	
4 °C / Hold	

(c) Purify PCR using silica gel-based columns and elute in 30 µL of ultrapure water.

(d) Run 5 µL of purified PCR on 1% agarose TAE 1x gel at 130 volts for 20 minutes.

(e) Visualize gel using a UV transilluminator and determine the volume of purified PCR to be used for heterodimerization. As a rule of thumb, use 5 µL of purified PCR for conditions with a density similar to the 1,650 bp band of the 1kb plus DNA ladder, adjust other conditions accordingly.

(f) Proceed to heteroduplex formation using the following conditions:

5* µL purified PCR product
5* µL ultrapure water
10 µL Cel1 buffer 2X
20 µL

* The volume of PCR product used in the reaction was determined in (e) and varies between samples in order to normalize for DNA concentration. Adjust ultrapure water volumes accordingly.

If PCR products are faint, it is possible to increase the volume of sample to up to 16 µL by using 5X Cel1 buffer.

(g) Incubate samples in a thermocycler using the program previously described for sgRNA annealing (2d). Always keep samples on ice following heteroduplex formation.

(h) Prepare surveyor nuclease mix (per sample)

2 µL $MgCl_2$
1 µL Surveyor enhancer
1 µL Surveyor nuclease

(i) Distribute 4 µL per sample.

(j) Incubate at 42°C for 1 hour.
(k) Add 5 µL of 5x DNA loading dye, vortex, spin down.

(l) Cast a 10 % acrylamide gel or use a precast gel.

2.0 mL Acrylamide/bisacrylamide 29:1 30 %
0.6 mL TBE 10 x
0.6 mL Glycerol 80%
2.8 mL Ultrapure water
50 µL Ammonium persulfate 10%
5 µL Tetramethylethylenediamine

(m) Load 15 µL of the reaction and 5 µL of 100 bp ladder on the gel.

(n) Run gel in TBE 1x buffer at 60 volts for 10 minutes and at 120 volts for 120 minutes.

(o) Stain in 40 mL TBE 1 x + 2 µL Red safe for 20 minutes while rocking.

(p) Destain in deionized water for 20 minutes while rocking.

(q) Image using chemiDoc (Gel Red program).

(r) Perform quantification using the image lab software. Indels quantification can be done using the following formula:

$$\text{Indel (\%)} = (1-\text{SQRT}(1-(b+c)/(a+b+c))) \times 100$$

Where *a* is the integrated intensity of the undigested PCR product and *b* and *c* are the integrated intensity of the surveyor cleavage products.

6. Restriction fragment length polymorphism assay

(a) Extract genomic DNA from $2.5x10^5$ cells with 250 µL of QuickExtract DNA extraction solution per manufacturer's recommendations.

(b) Amplify loci of interest by PCR. (See 6b for *ATP1A1*)

(c) Purify PCR using silica gel-based columns and elute in 30 µL of ultrapure water.

(d) Run 5 µL of purified PCR on 1% agarose TAE 1x gel at 135 volts for 20 minutes.

(e) Visualize gel using a UV transilluminator and determine the volume of purified PCR to be used for digestion. As a rule of thumb, use 5 µL of PCR for conditions with a density similar to the 1,650 bp band of the 1kb plus DNA ladder. Adjust volumes for other conditions accordingly.

(f) Digest samples with appropriate enzyme for 2 hours at 37^oC in a final volume of 20 µL.

RD ssODN: Digest using ClaI (226 + 193 bp), BmgBI (217 + 202 bp) or Hpy188I (77 bp)

DR ssODN: Digest using PvuI (221 bp + 198 bp) or ClaI* (193 + 166 + 60 bp).

*As mentioned in the original publication, donor integration is made close to the target site. This results in a preferential integration of the second ClaI site, which causes an additional band at 226 bp to appear.

(g) Add 5 µL of 5x DNA loading dye, vortex, spin down.

(h) Cast a 10 % acrylamide gel or use a precast gel.

2.0 mL Acrylamide/bisacrylamide 29:1 30 %
0.6 mL TBE 10 x
0.6 mL Glycerol 80%
2.8 mL Ultrapure water
50 µL Ammonium persulfate 10%
5 µL Tetramethylethylenediamine

(i) Load 25 µL of the reaction and 5 µL of 100 bp ladder on the gel.

(j) Run gel in TBE 1X buffer at 120 volts for 120 minutes.

(k) Stain in 40 mL TBE 1X + 2 μL Red safe for 20 minutes while rocking.

(l) Destain in deionized water for 20 minutes while rocking.

(m) Image using chemiDoc (Gel Red program).

(n) Perform quantification using the image lab software. HDR can be quantified using the following formula:

$$HDR\ (\%) = ((b+c)/(a+b+c)) \times 100$$

Where *a* is the integrated intensity of the undigested PCR product and *b* and *c* are the integrated intensity of the RFLP cleavage products.

7. Human cord blood (CB) processing and mononuclear cell (MNC) isolation

(a) Transfer cord blood into 50 mL tubes and spin at 400 g for 15 minutes without brake. Discard plasma.

(b) Prepare LeucoSep tubes with 15 mL Ficoll-Paque Plus in each and spin at 1000g for 1 minute.

(c) Dilute cord blood with a volume of D-PBS (heated at 37^{o}C) to obtain a multiple of 30 mL.

(d) Add 30 mL of blood/D-PBS into LeucoSep tubes containing Ficoll and spin at 400 g for 30 minutes without brake.

(e) Gently collect mononuclear cells layer and transfer into new 50 mL tubes.

(f) Wash cells with D-PBS-2% Human Serum Albumin-1mM EDTA, spin at 300 g for 10 minutes without brake.

(g) Repeat the wash step, take a sample to count the cells with Solution 18 on the NC-250 and spin the rest at 120 g for 10 minutes without brake.

(h) Cryopreserve purified MNCs in Cryostor CS10.

8. CD34+ cell purification

(a) Thaw and pool MNCs from 5-6 different donors.

(b) Add 40 mL ice-cold IMDM/20% HSA.

(c) Spin at 300 g, 10 minutes with low brake.

(d) Resuspend the cell pellet in 40 mL IMDM/20% HSA.

(e) Add 2 mg DNase I.

(f) Incubate at RT, 15 minutes.

(g) Spin the cells at 300 g, 10 minutes with low brake.

(h) Proceed with isolation of $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) using CD34 positive selection according to manufacturer's instructions (EasySep™, StemCell Technologies).

(i) Take a sample from purified CD34+ cell suspension and add 5 µL of anti-human CD34-FITC antibodies (Immunotech).

(j) Incubate at RT, 20 minutes in the dark.

(k) Wash cells with 5 mL of PBS/ 1% FBS.

(l) Spin at 1000 g, 5 minutes.

(m) Resuspend cells in 1mL of PBS/ 1% FBS.

(n) Determine the CD34+ cell purity by flow cytometry.

9. Human CB $CD34^+$ cell culture

(a) Thaw and culture 500 000 cells/mL CD34+ HSPCs at 37 °C under 5% CO_2 in StemSpan ACF supplemented with 100 ng/mL SCF, 100 ng/mL FLT3-L, 50 ng/mL TPO, 10 µg/mL LDL and 35 nM UM171.

(b) Culture 16-24 hours before nucleofection.

10. CD34+ cell nucleofection

(a) Resuspend crRNA and tracrRNA to 200 µM stock solutions in Nuclease-Free IDTE Buffer.

(b) Freshly prepare crRNA:tracrRNA complexes by mixing equimolar amounts of the two RNA oligos (1.25 µL complex/transfection reaction).

crRNA guide sequence for HBB* : CTTGCCCCACAGGGCAGTAA

crRNA guide sequence for ATP1A1 G4: GTTCCTCTTCTGTAGCAGCT

*crRNA sequence for HBB has been previously published (5)

(c) Heat the obtained 100 µM crRNA:tracrRNA working solution from step (a) at 95°C for 5 minutes and transfer to ice immediately.

(d) Count cells with the Nucleocounter NC-250 using Solution 18 as per manufacturer's instructions.

(e) Collect cells by spinning at 300 g, 10 minutes.

(f) Wash cells with D-PBS.

(g) Spin at 300 g, 10 minutes.

(h) During the last spin, freshly prepare the Cas9 protein:gRNA ribonucleoprotein (RNP) complex by mixing 50 pmol of Cas9 protein with 125 pmol of crRNA:tracrRNA complexes from step (b) and incubate at RT for 10 minutes.

(i) Resuspend cells from step (f) in freshly supplemented P3 Nucleofector buffer (200 000 cells/20 µL P3 buffer) provided in P3 Primary Cell 4D-Nucleofector kit.

(j) Add ssODNs (100 pmol) and RNPs from step (g) in cell suspension and transfer into nucleocuvettes.

(k) Nucleofect CD34+ cells using the Amaxa 4D Nucleofector X unit and the E0-100 program according to manufacturer's recommendation.

(l) Following nucleofection, incubate cells at 30°C for 16h and then transfer to 37°C for two more days in AR medium.

(m) Take a sample from cells at D4 and extract DNA with QuickExtract (~1000 cells/μL) according to manufacturer's recommendations to evaluate gene targeting before ouabain selection.

11. Ouabain selection process for CD34+ cells

(a) From day 4 post-thaw, change the culture medium to StemSpan ACF containing 1X StemSpan CD34+ Expansion supplement and 35 nM UM171. During culture medium change of CD34+ cells, carefully remove ½ (for 96-well plates) or ¾ (for 48 or 24-well plates) volume of the well without disturbing the cells and replace with fresh medium.

(b) Starting day 6, add 0.5 μM ouabain to the cells during culture media change. Keep control cells without ouabain selection. It is critical to have an optimal amount of CD34+ cells before ouabain addition. Note that the portion of cells lacking editing in ATP1A1 locus will die in the first 72 hours post selection. Plating not enough cells before ouabain addition may result in no recovery.

(c) At D9 and D12, change culture medium. By five days of ouabain selection, obvious cell growth of resistant cells should be observed.

(d) At D14 post-thaw (8 days after selection start), collect and wash cells with D-PBS. Washing the cell pellet before DNA extraction of CD34+ cells is suitable to remove cell debris and dead cells.

(e) Extract DNA with QuickExtract and proceed with PCR amplification of regions of interest, PCR product purification, followed by Surveyor and RFLP assays.

12. PCR amplification

(a) Amplify loci of interest. An example is shown here for *ATP1A1* and *HBB*. Refer to Supplementary table 6 (3) for PCR primers used for other targets.

ATP1A1 Forward: GGATTAACATCTGCTCGTGCAGC
ATP1A1 Reverse: CACTTGTAAGAGCATCTACAACG

HBB Forward*: GACAGGTACGGCTGTCATCA
HBB Reverse: CAGCCTAAGGGTGGGAAAAT

*PCR primers for *HBB* have been previously published (6)

PCR mix/tube:

2.5 µL Accuprime Taq buffer II
1.25 µL Forward primer (10 µM)
1.25 µL Reverse primer (10 µM)
18.9 µL Nuclease-free H_2O
0.1 µL Accuprime Taq
1.0 µL DNA
25 µL

PCR program:

94°C / 5min
94°C / 30sec
60°C / 30sec x30
68°C / 30sec
68°C / 5min
4°C / ∞

(b) Purify *HBB* and *ATP1A1* PCR products with Illustra GFX Purification kit according to manufacturer's recommendations.

(c) Elute purified PCR product in 30 µL nuclease-free water.

14. Surveyor assay

(a) Refer to section 6 (c) to 6 (r) for Surveyor assay.

15. RFLP assay

(a) Perform BmgBI (RD donor) or ClaI (DR donor) digestions (incubate at 37°C for 90 minutes) on *ATP1A1* PCR products and PstI and PshA1 digestions (incubate at 37°C for 30 minutes) on *HBB* PCR products.

30-40 ng Purified PCR
2.0 µL Buffer 10X
1.0 µL Enzyme
___ µL Nuclease-free H_2O
20 µL

Buffers recommended by the manufacturer were used for each digestion reaction.

(c) Add 4 µL 6X Orange G DNA loading dye to the digestion reactions and load samples on 10% polyacrylamide gels.

(d) Run 15 µL of digestion reaction in a vertical electrophoresis system at 120V for 120 minutes.

(e) Stain the gels in 1X SYBR Gold for 30 minutes while rocking.

(f) Destain the gel in ultrapure water for 30 minutes while rocking.

(g) Image and quantify cleavage products using a quantitative imaging system without overexposing the bands.

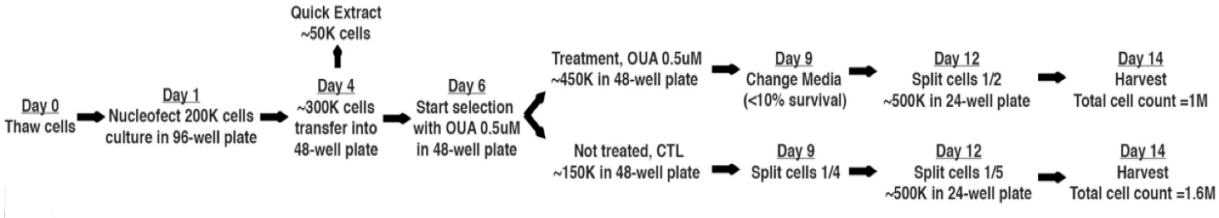
(h) HDR efficiency is estimated by using the following formula: $(b + c)/(a + b + c)$, where a is the integrated intensity for the undigested HDR PCR product, and b and c are the integrated intensities of cleavage fragments.

Time taken

(a) Cell lines

4-5 days for construction of sgRNA expression plasmid.
3 days for nucleofection and initial gene targeting.
7-15 days for ouabain selection.
2 days for DNA extraction, PCR and Surveyor and RFLP assays.

(b) Representative timeline for gene-editing and ouabain selection process of CD34+ cells.



Troubleshooting

1. No cell death following ouabain addition

   Wrong ouabain concentration or poor dilution. It is important to heat ouabain solution thoroughly to allow complete dissolution before using it. Thus, vortex cycles make it easier to obtain a homogeneous solution. If using a different cell line than the ones used here, we recommend testing different ouabain concentrations (ex: 0.5 µM to 2 µM).

2. Very low cell density 48-72h after ouabain selection

   Genome editing efficacy can vary depending on nucleofection and gRNA efficacy. It is therefore important to optimize the transfection conditions used. In addition, it is preferable to use cells that have not been passage more than 10 times. Also, it is important that cells are at least 70 % confluent before ouabain addition to avoid a slow recovery following selection.

3. Dead cells cover living adherent cells

   After ouabain treatment a majority of cells will die and some dead cells will remain attached to living cells and cover them. Although it's tempting to wash or split cells in order to eliminate dead cells, we recommend to wait at least 4 days to make sure living cells have grown sufficiently. This time will also allow for more dead cells to detach from living cells.

4. No positive selection of treated cells

   a) Disruption at *ATP1A1* locus is too high. In this case we recommend reducing the quantity of plasmid targeting *ATP1A1* in order to increased the fold enrichment between treated and non treated cells, thus fold enrichment in locus of interest will also increase.

   b) gRNA targeting locus of interest is highly efficient and cell selection isn't necessary.

5. Clonal enrichment of specific indels

   We have observed that using very low quantities of plasmid (ie. 5 ng) resulted in clonal enrichment of cells with a limited number of indels. If this problem arises, we suggest increasing the quantity of plasmid nucleofected.

6. Presence of heteroduplexes in Surveyor Assay.

   During Surveyor assay, bands corresponding to heteroduplexes can be observed above the parental strand. If so, we suggest repeating the experiment using less PCR product or increasing the amount of surveyor enzyme used. If heteroduplexes remain, these bands should be included in indels quantification.

7. Saturation of Surveyor signal.

   If high indels percentage (> 50 %) is observed during surveyor assay, signal may be saturated. If so, purified PCR product should be sequenced and analysed using TIDE software in order to obtain a more accurate indels frequency.

Anticipated results

1. Nucleofection efficacy of at least 80% as assessed by visualisation of GFP control using a fluorescence microscope 24-48 hours following nucleofection.

2. Cell death between 70-90% 48 hours after ouabain selection.

3. Stable genetic modification within the cell population 10-14 days after nucleofection.

4. % gene targeting at *ATP1A1* locus around 50% after complete selection. This number can be lower in K562 since they possess 3 copies of chromosome 1.

5. % gene targeting at locus of interest increased by 4-20 fold following selection

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