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EDDU Protocols

Generation of Knockout Cell Lines Using CRISPR-Cas9 and ddPCR Technology

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1 Introduction

1.1 Objectives

This protocol describes how to generate a gene knockout cell line through a combination of techniques that includes:

- CRISPR-Cas9 editing;
- Droplet digital PCR (ddPCR) technology;
- Single cell sorting.

1.2 Protocol overview

Precise genome editing by homology-directed repair (HDR) is used in this protocol to incorporate a sequence of three stop codons (StopTag) into the target DNA to generate a gene knockout (KO). This strategy is more efficient and precise than creating a random deletion using CRISPR-Cas9 technology.

First, the cell line to knockout the gene of interest is chosen by confirming a high abundance of the protein expressed from this gene according to the values on the [PAXdb](#) website. After selecting the appropriate cell line, a CRISPR-Cas9 strategy is designed to insert the StopTag into a cell genome as follows:

- A guide RNA (gRNA) is designed to target the gene to be knocked out. This gRNA is dedicated to form a ribonucleoprotein (RNP) complex with the Cas9 endonuclease to allow the enzyme to cut the DNA at a specific site.
- A single-stranded oligodeoxynucleotide (ssODN) containing the StopTag and homology arms flanking this sequence is designed to be integrated into the DNA while serving as a template for HDR.

Next, in order to activate HDR and to facilitate integration of the StopTag into the specific region of the target gene, the RNP complex along with the ssODN are delivered by nucleofection into the cells in which the gene is to be knocked out (**Figure 1**). Since HDR occurs at low frequency, cells after nucleofection are cultured in media supplemented with HDR enhancer to promote recombination of the template into a precise location within the genome.

The polyclonal cell population is subsequently screened by droplet digital PCR (ddPCR) to confirm gene editing. Precise detection of edited alleles is achieved using specially designed probes with two different fluorophores: one specific to the StopTag sequence and the other one to the original allele. The use of these probes along with the droplet digital technology yields an accurate discrimination and a high-resolution quantification of edited alleles.

If gene editing is successful, cells are then separated using fluorescence-activated cell sorting (FACS) to isolate monoclonal KO cell clones. Clones are expanded and genotyped by ddPCR and Sanger sequencing to identify cells with a successful gene KO. The sequence-validated KO clones are expanded and frozen down to be used in future applications.

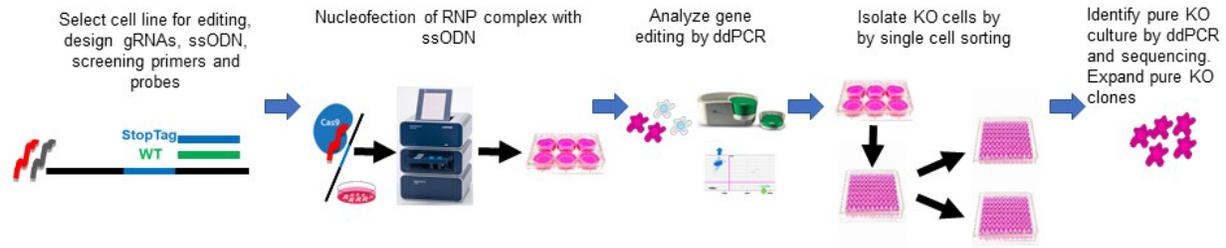


Figure 1. Workflow for the strategy of gene KO generation.

1.3 Technical and safety considerations

The following information should be read before starting the procedure:

- Ensure that the cells in culture are mycoplasma-free before starting the process.
- Use sterile tubes, plates, pipets and tips to avoid contamination.
- Use cells with low passage number as high passage number can have a negative effect on transfection efficiency and gene expression, and potential effects on overall cell growth and viability.
- For successful generation of a gene knockout, it is essential to choose a cell line that expresses the gene of interest. To verify the expression levels of a gene in various cell lines refer to the [PAXdb](#) website.
- Cell lines must be handled in a Class II biosafety laminar flow hood to create a sterile environment for cell culture experiments and to protect the user from possible biohazards. Appropriate institutional Environmental Health and Safety (EHS) office regulations must be followed.
- Personal protective equipment (PPE), including a lab coat, face mask and disposable gloves must be worn while handling cell lines.
- Avoid exposing the cells to prolonged periods outside the incubator (cells should be maintained at 37°C with 5% CO₂ unless stated otherwise).
- DNA and RNA quality can be significantly diminished with multiple freeze-thaw cycles and by contamination with human DNA and nucleases. Thus, avoid excess freeze-thaw cycles and aliquot reagents into smaller amounts.
- Before running a PCR or ddPCR reaction, thaw frozen reagent stocks on ice or at room temperature (do not heat). Enzymes such as DNA polymerase should always be kept on ice. Keep thawed materials on ice during use. Do not leave reagents at room temperature for extended periods. Right before use, mix each reagent thoroughly, and then centrifuge briefly to collect all the liquid at the bottom of each tube.
- Protect fluorescent DNA probes from light to avoid photobleaching. Cover tubes and plates containing probes with aluminum foil.
- Make aliquots of primers and gRNAs to avoid contamination and repeated thawing and freezing of these reagents.

1.4 Abbreviation list

Abbreviation	Full Name
Bp	Base pair
Cm	Centimeter
CRISPR	Clustered regularly interspaced short palindromic repeats
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxyribonucleotide triphosphate
ddPCR	Droplet digital polymerase chain reaction
EHS	Environmental Health and Safety
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
G	Gram
gRNA	Guide RNA
H	Hour
HDR	Homology directed repair
KO	Knockout
L	Litre
mL	Millilitre
mRNA	Messenger RNA
µL	Microliter
µM	Micromolar
Min	Minute
M	Molar
PAM	Protospacer adjacent motif
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PPE	Personal protective equipment
QE	QuickExtract™ DNA Extraction Solution
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
RT	Room temperature
S	Second
ssODN	Single-stranded oligodeoxynucleotide
TBE buffer	Tris/Borate/EDTA buffer
Tm	Melting temperature
WT	Wild type

2 Materials

Refer to the product datasheet from the supplier for further details on storage and preparation instructions. It is important to highlight that the lot number for some reagents can vary and the use of reagents coming from different lots might affect the efficiency of the protocol.

2.1 Labware

Item	Supplier	Catalogue #
Cell culture plate, 24 wells	ThermoFisher Scientific	087721
Cell culture dish, 10 cm	ThermoFisher Scientific	08772E
Cell culture plate, 6 wells	ThermoFisher Scientific	08-7721B
Cell culture plate, 96 wells	Eppendorf	0030730119
Cell culture plate, 96 wells	Corning	353072
Cell culture plate (ddPCR™), 96 wells	BioRad	12001925
Conical tube, 1.5 mL	ThermoFisher Scientific	MCT-175-L-C
Conical tube, 15 mL	ThermoFisher Scientific	14-959-70C
Conical tube, 50 mL	ThermoFisher Scientific	1495949A
Cryovials	Sarstedt	72.379
DG8 Cartridges and Gaskets	BioRad	1864007
DG8 Cartridge Holder	BioRad	1863051
Gilson Pipetman G 4-Pack Starter Kit. Includes: P2G, P20G, P200G and P1000G	Mandel	GF-F167360
Gilson Pipetman G, 8 x 200 Multichannel Pipette, 8-channel, 20-200ul.	Mandel	GF-F144072
Gilson Pipetman P10G	Mandel	GF-F144055
Ethanol spray bottle	VWR	470050-014
FACS tube, 1.5 mL	BD Falcon	352008
FACS sterile tube with cap, 5 mL	BD Falcon	352058
Luna cell counter slide	Logos Biosystems	#05181401
MicroAmp™ Clear Adhesive Film	ThermoFisher Scientific	4306311
MicroAmp® Optical 8-Tube Strip	ThermoFisher Scientific	4316567
MicroAmp™ Optical 96-Well Reaction Plate	ThermoFisher Scientific	8010560
Parafilm	Bemis	PM-999
PCR caps	Bio-Rad	TCS0803
Petri dish, 100 mm x 15 mm	ThermoFisher Scientific	FB0875712

Item	Supplier	Catalogue #
Pierceable Foil Heat Seal	BioRad	1814040
Pipet tips	VWR	89079-444
Pipet tips GP-LTS-A-250µL	RAININ Mettler Toledo	30389278
Pipet-Lite Multi Pipette L8- 50XLS+	RAININ Mettler Toledo	17013804
Plastic serological pipet, 5 mL	ThermoFisher Scientific	13-678-11D
Plastic serological pipet, 10 mL	ThermoFisher Scientific	13-678-11E
Plastic serological pipet, 25 mL	ThermoFisher Scientific	13-678-11
Sample filter, 30 µm	Miltenyi Biotec	130-041-407
Sterile petri dishes, 100mm x 15mm	VWR	25384-208
Sterile syringe filter, 0.2 µm	VWR	28-145-501
Sterile syringe, 10 mL	BD	302995

2.2 Reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Agarose	BioShop	AGA001.1	100%	0.5 – 2%	Stock: RT Working: RT
Alt-R® HDR Enhancer	IDT	1081073	N/A	N/A	-20°C
Alt-R® S.p. HiFi Cas9 Nuclease V3	IDT	1081061	N/A	N/A	-20°C
Amaxa™ SE Cell Line 4D-Nucleofector™ X Kit S	Lonza	V4XC-1032	N/A	N/A	4°C
Boric Acid	Bioshop	TRS001.5	N/A	N/A	RT
ddPCR Supermix for Probes (no dUTP)	BioRad	1863024	N/A	N/A	-20°C
Dimethyl sulfoxide (DMSO)	Sigma	D2650	100%	10%	Stock: RT Working: RT
DNA Ladder RTU, 100 bp	Froggabio	DM001-R500F	N/A	N/A	Stock: -20°C Working: RT
Droplet Generation Oil for Probes	BioRad	1864110	N/A	N/A	RT
Droplet Reader Oil	BioRad	1863004	N/A	N/A	RT

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/L glucose L-glutamine + pyruvate	Multicell	319-005-CL	1x	1x	Stock: 4°C Working: 37°C
EDTA	Multicell	625-060CG	N/A	N/A	RT
FACSFlow	BD	342003	N/A	N/A	Stock: RT Working: RT
FBS	Gibco	12484-028	1x	1x	Stock: -20°C Working: RT
Fluo-DNA loading buffer	ZmTech	LB-001B	6x	1x	Stock: -20°C Working: RT
Guide RNA (supplied with TE buffer)	Synthego	N/A (Custom order)	N/A	100 µM in TE buffer	Stock: RT Working: -20°C
L-glutamine	Wisent	609-065-EL	100x	1x	Stock: -20°C Working: RT
LNA™ Probes	IDT	N/A (custom order)	N/A	100 µM	-20°C
Penicillin-Streptomycin	Wisent	450-200-EL	100x	1x	Stock: -20°C Working: RT
Primers	Invitrogen	N/A (Custom order)	Variable	100 µM in DNA grade water	Stock: -20°C Working: RT
QuickExtract™ DNA Extraction Solution (QE)	Mandel	LGN-QE09050	100x	100x	Stock: -20°C Working: RT
Sterile Phosphate Buffer Saline (PBS)	Multicell	311-010-CL	1x	1x	Stock: RT Working: RT
ssODN	IDT	N/A (Custom order)	N/A	100 µM	-20°C
Tris	Bioshop	BOR001.1	N/A	N/A	RT
Trypsin, 0.05% with 0.53 mM EDTA	Multicell	325-542-EL	100x	100x	Stock: 4°C Working: 37°C
UltraPure™ DNase/RNase-Free Distilled Water	ThermoFisher Scientific	10977015	1x	1x	Stock: RT Working: RT
Zero Blunt TOPO PCR cloning kit	Invitrogen	K287520	N/A	N/A	Varies (each reagent is stored at a different temperature)

2.3 Equipment

Item	Supplier	Catalogue #
Advanced Dry Block Heater	VWR	75838-282
C1000 Touch™ thermal cycler with 96–deep well reaction module	BioRad	1851197
Cell Culture Incubator	ThermoFisher Scientific	MPP50116050
Cell Culture Water Bath	ThermoFisher Scientific	IsoTemp GPD20
Centrifuge	Eppendorf	022626001
Electrophoresis Apparatus	Biorad	1658025FC
FACSAria Fusion Cell Sorter	BD	656700G5
EVOS FLoid Cell Imaging Station	ThermoFisher Scientific	4471136
Light Microscope	Motic	AE2000
LP Vortex Mixer	ThermoFisher Scientific	88880017
Luna-II Automated Cell Counter	Logos Biosystems	L40002
Nanodrop	ThermoFisher Scientific	ND-ONE-W
Nucleofector 4D™ Core Unit	Lonza	AAF-1002B
PCR Plate Spinner	VWR	89184-608
Power Supply	Biorad	164-5070
PX1™ PCR Plate Sealer	BioRad	1814000
QX200™ Droplet Generator	BioRad	1864002
QX200™ Droplet Reader	BioRad	1864003
SterilGard Biosafety Cabinet	Baker	SG303

2.4 Websites

Name	Address
Benchling	https://www.benchling.com/
Bioinformatics	https://www.bioinformatics.org/sms/rev_comp.html
Ensembl	https://useast.ensembl.org/index.html
IDT (LNA probes)	https://www.idtdna.com/calc/analyzer/lna
IDT OligoAnalyzer tool	https://www.idtdna.com/pages/tools/oligoanalyzer
PAXdb	https://pax-db.org/
Primer-BLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
Synthego	https://www.synthego.com/

2.5 Software

Item	Supplier	Version
SnapGene	GSL Biotech LLC	N/A
QuantaSoft	BioRad	N/A

3 Protocol

This protocol was developed to identify CRISPR-generated gene knockout by ddPCR analysis. Thus, strategy design includes features such as probes and oligonucleotides that work best when using ddPCR method. The ddPCR method has been successfully used for the analysis of gene editing in iPSC previously. For details, see protocol [EDDU-009-01 CRISPR-mediated introduction of single nucleotide changes in iPSCs](#).

3.1 Design strategy

3.1.1 Design of gRNA

Guidelines for gRNAs design:

- Use [Synthego](#) and/ or [Benchling](#) websites for gRNA design.
- The gRNA cutsite is 3 nucleotides upstream from the protospacer adjacent motif (PAM) sequence.
- Choose the gRNA that have the lowest off-target effect potential (on the [Benchling](#) website the highest number for the off-target score indicates the lowest off-target potential).
- Targeted exon should be the constitutive exon (exon that is present in all mRNA transcripts of a gene). If not possible, choose the most common exon to all coding transcripts.
- The gRNA target sequence should be at least 50 bp away from intron-exon junction and 200 bp after the start codon (ATG) or before the stop codon. Otherwise, if the gRNA is designed to cut to close to intron-exon junction, the resulting transcripts can escape nonsense mediated decay (pathway that degrades mRNA transcripts with premature stop codons) and end up being expressed, therefore, halting the knockout strategy.
- Check gRNA sequence with the IDT [OligoAnalyzer tool](#) for possible self-dimerization and hairpin loop formation.

Procedure:

1. Using the [Synthego](#) website, design a gRNA to target the gene of interest:
 - Click on “Bioinformatics”.
 - Select “CRISPR design tool”.
 - Press “Launch”.
 - Type the genome name or ID in the field “Genome”.
 - Type the name of the gene of interest in the field “Gene”.
 - Click on “Search”.
 - “Recommended guides” will be shown in green (**Figure 2**). To visualize all the gRNAs available, click on “all guides” tab.
 - Copy transcript ID (**Figure 3**).

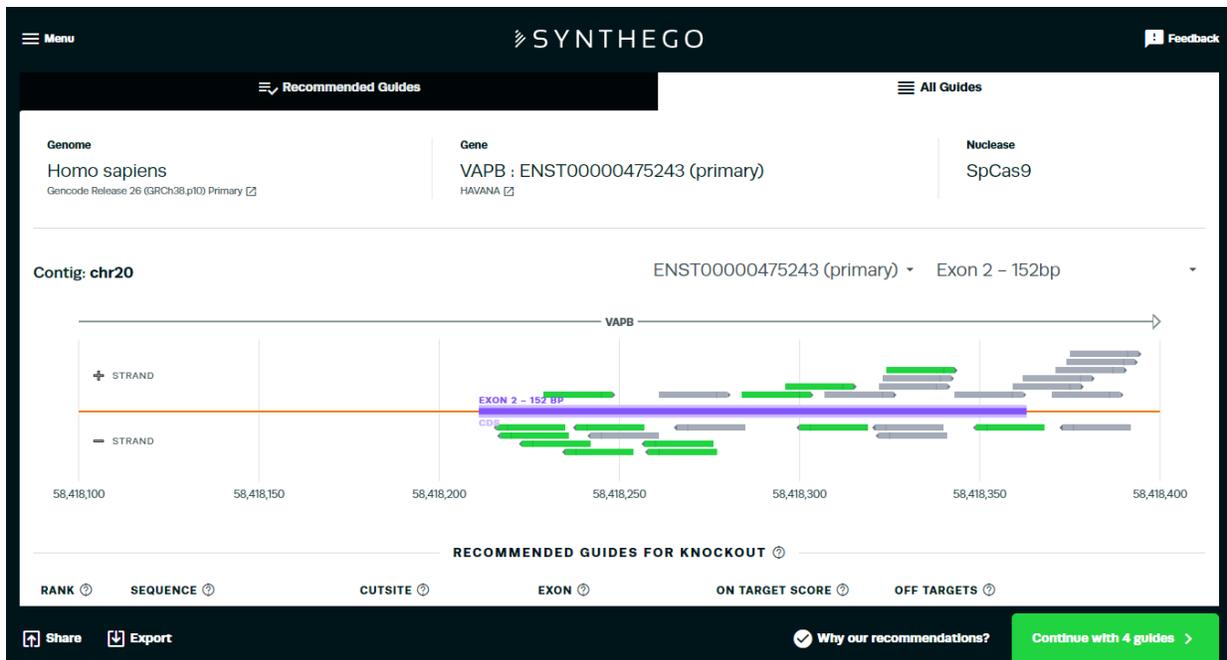


Figure 2. gRNAs designed at Synthego website are shown as green (recommended guides) and grey (other guides) arrows.

2. At [Ensembl](https://www.ensembl.org) website verify that the gRNAs are designed to target the early common exon:
 - Paste the gene name in the search box and press “go”.

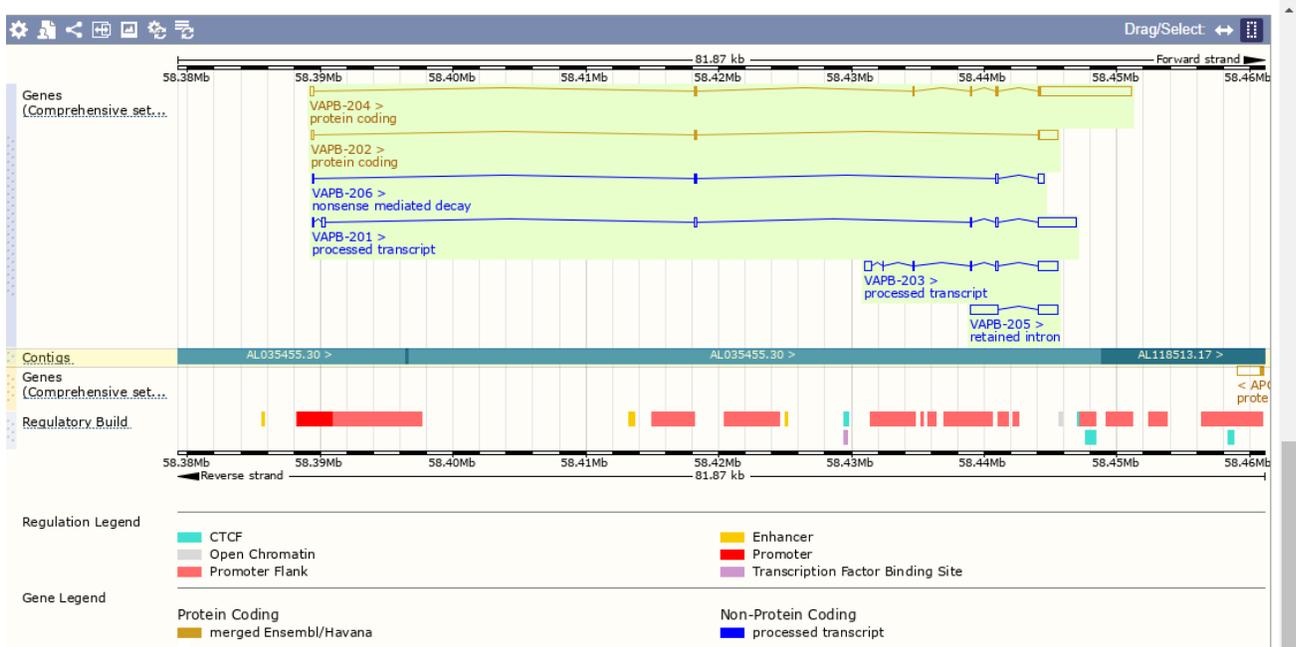
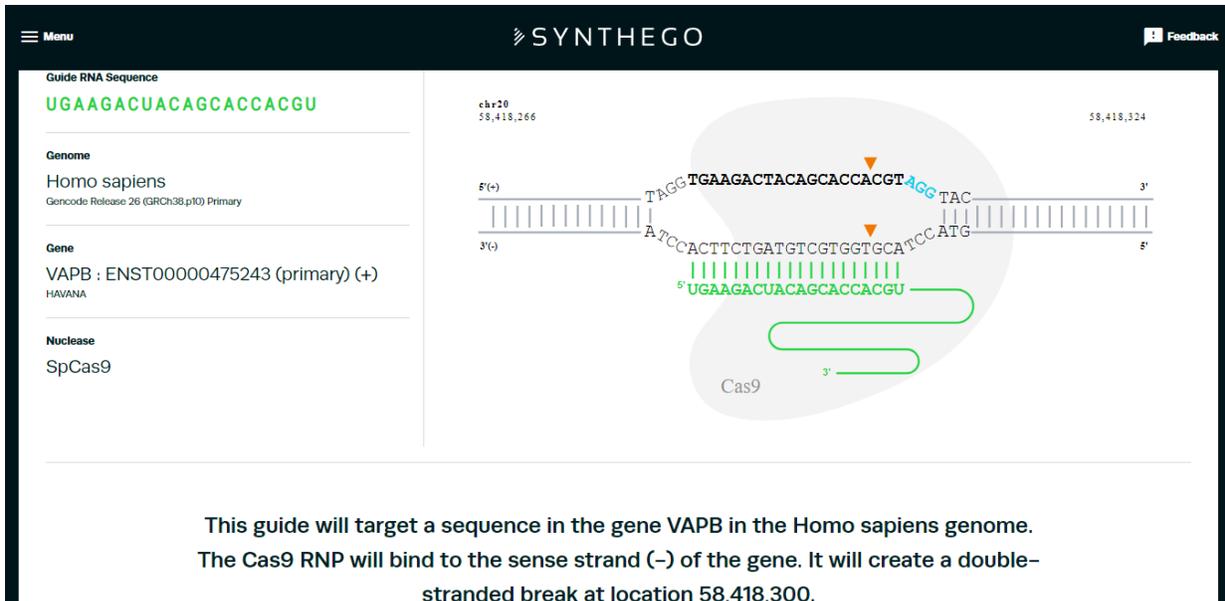


Figure 3. Transcript information of VAPB gene on Ensembl website.



This guide will target a sequence in the gene VAPB in the Homo sapiens genome. The Cas9 RNP will bind to the sense strand (-) of the gene. It will create a double-stranded break at location 58,418,300.

Figure 5. Example of gRNA design on Synthego website. PAM sequence is highlighted in blue. The orange arrows indicate the cleavage site by Cas9 using this gRNA.

- Choose the gRNA that have the lowest off-target score.
- Copy the information about the gRNA to a text document: it should contain the exon number, the size of the exon in base pairs (bp), the sequence of the gRNAs, the cleavage site and the strand (**Table 1**).

Table 1. gRNA information

gRNA target sequence	Exon number	Exon size	Cleavage site
TGAAGACTACAGCACCACGT	2	152 bp	58,418,300

- If there are no gRNAs at Synthego [CRISPR Design Tool](#) that fit the criteria, design gRNA at [Benchling](#) website (**step 4**). Otherwise, go to **step 5**.
4. Use the [Benchling](#) website for gRNAs design:
- Sign in to the Benchling website.
 - Click “+” in the blue bar.
 - Select “CRISPR”.
 - Go to “CRISPR guides”.
 - Select “Raw bases”.
 - Copy the coding (blue) sequence of the early exon from Ensembl website and paste into the “Bases” field in the Benchling website.
 - Click “Next”.

- Choose “Single guide” in the Design Type field.
- Choose Guide length: 20/ Genome: Human/ PAM: NGG.
- Click “Finish”.
- Go to the sequence map and select the full exon.
- Click on the green “+” button on the right.

From the list of gRNAs, click on “off target” to sort by off target score (**Figure 6**).

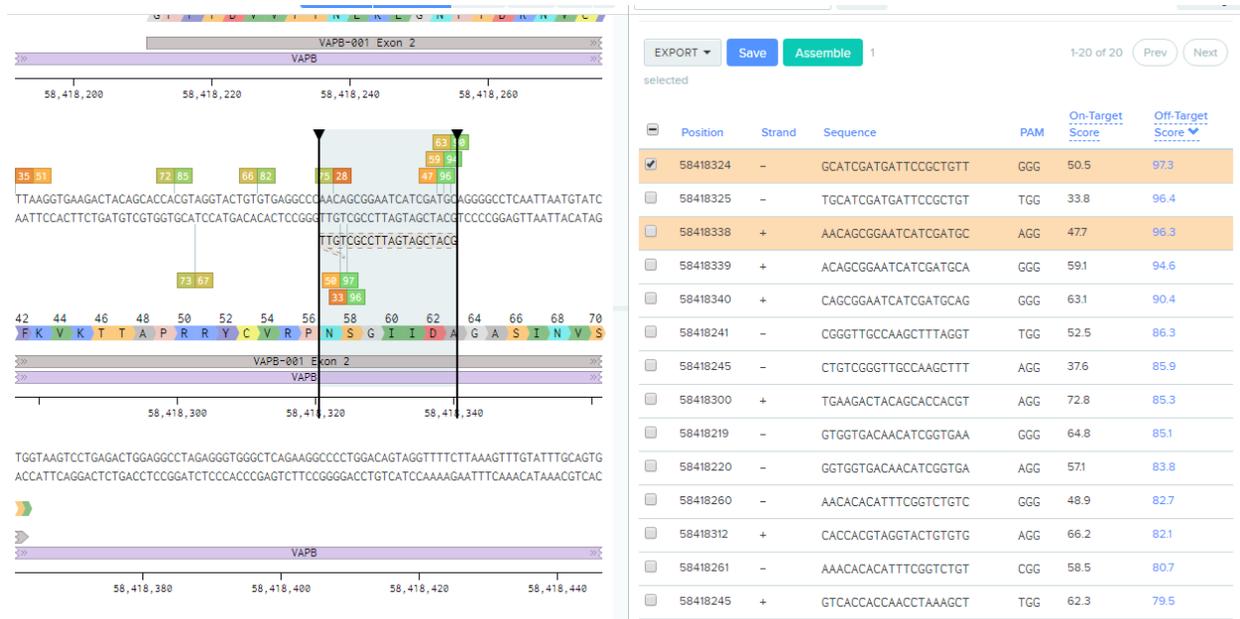


Figure 6. Selection of gRNA on Benchling website using off-target score.

- Benchling shows the gRNA target sequence below the template DNA. Considering the strand of the exon sequence, locate the gRNA target sequence. For instance, if the gRNA targets the minus strand and the sequence extracted from Ensembl is in the plus strand, then determine the reverse-complement.
- Select gRNAs that have the highest off-target score since they have the lowest chance to cause the off-target editing (**Figure 6**).
 - By clicking on the off-target score it is possible to visualize which gene(s) is(are) also going to be affected. If it is an important gene, another gRNA should be used instead. The extent of the off-target activity is highly dependent on the gRNA, and the number of off-targets varies from 0 to >150 (**Figure 7**).

Off-Target Sites ✕

Copy TSV

Sequence	PAM	Score	Gene	Locus
GTGGTGACAACATCGGTGAA	GGG	100.0	VAPB (ENSG00000124164)	chr20:-58418219
GTGGAGACAAAGATCGGTGAA	CAG	3.7		chr17:+76615485
GTGGTGCAACATCTGTGAA	AAG	1.4		chr3:-18225316
CTGGTACAACATCGGGGAA	TGG	0.9		chr22:-38611094
GTGCTGATGGCATCGGTGAA	CAG	0.8		chr20:-330362
GTAGTGACTACATCTGTGAA	GGG	0.5	ABHD2 (ENSG00000140526)	chr15:-89201716
GTAGTGACTACATCTGTGAA	GGG	0.5	VAPA (ENSG00000101558)	chr18:-9931818
AAGGAGACAACATCTGTGAA	GAG	0.5		chr6:+1656387
CCGGTGACACCATCTGTGAA	AAG	0.5		chr13:+81053320
GCGGTGACAGCATGGGTGAA	GGG	0.5	GOLGA7 (ENSG00000147533)	chr8:+41490704
GTGTTGAGAAATAGCGGTGAA	AAG	0.3		chr9:-21506025
GTGGTGACATCACCGGTGCA	GGG	0.3		chr6:+99847347
TTGGTGCCAGCATCAGTGAA	GAG	0.3		chr15:-95839694
GTGTTGTAACATCAGTGAA	TAG	0.3		chr6:-148271445
ATGGTGCAACATCGGTAAA	AAG	0.3		chr11:-93635750
GTGGGGAGGACATCAGTGAA	CAG	0.3		chr2:-10587819
TGGTTGACAACATAGGTGAA	GAG	0.3		chr17:+29382101
CTGCAGACAACATAGGTGAA	AGG	0.3		chr10:+71295120
GGGATGAGAACATGGGTGAA	GGG	0.3	SEPW1 (ENSG00000178980)	chr19:-47781019
GTGGTGACCAGATCTGTGAA	CAG	0.2		chr7:-142600142

Figure 7. Example of a list of off-target genes for a VAPB gRNA.

- Paste your exon sequence into a text document and highlight the gRNA target sequence.

3.1.2 Design of ssODN

Guidelines for ssODN design:

- In this protocol, synthesizable ssODN is used to insert a StopTag into the target exon, thus adding a premature stop codon to knockout expression of the target gene.
- The recommended size of the homology arms is between 40 and 60 bp on each side of the StopTag sequence.
- The sequence of the ssODN is identical to the exon sequence (to serve as DNA template for HDR repair) minus the PAM and the gRNA target sequences that must be replaced by the StopTag sequence. The PAM sequence is removed to prevent Cas9 from cutting the template and the newly engineered allele.

Procedure:

- Locate the gRNA target sequence within the exon sequence (**Figure 8, A**).
- Select 40 to 60 bp upstream and downstream from the StopTag sequence (50 bp is the optimal) - these nucleotides will form the homology arms.

3. Replace the gRNA target sequence and the PAM sequence with the StopTag sequence (Figure 8, B).

A. VAPB gene sequence of exon 2 for transcript 204:

```
GTCCCTTCACCGATGTTGTCACCAACCTAAAGCTTGGCAACCCGACAGACCG  
AAATGTGTGTTTTAAGG TGAAGACTACAGCACCACGTAGGTACTGTGTGAGGCC  
CAACAGCGGAATCATCGATGCAGGGGCCTCAATTAATGTATCTG
```

B. ssODN sequence:

```
ACCAACCTAAAGCTTGGCAACCCGACAGACCGAAATGTGTGTTTTAAGG CGTAACT  
AGCTGATACTGTGTGAGGCCCAACAGCGGAATCATCGATGCAGGGGCCTCAATTA
```

Figure 8. Design of ssODN sequence to generate VAPB KO. Target gRNA sequence and PAM sequence are highlighted in red and blue, respectively. StopTag sequence is highlighted in green.

3.1.3 Design of probes

Fluorescence probes are used in ddPCR analysis to detect droplets containing target DNA sequence. Droplet positivity is determined by fluorescence intensity of the respective probe. As the droplet volume is known, ddPCR allows for the direct absolute quantification of a target gene in each sample through the calculation of the fraction of positive droplets [1].

It is important to note that the maximum fluorescence amplitude of the probes differs for each fluorophore (FAM and HEX, for example), being also dependent on the thermocycling conditions and the avidity of each probe for its target sequence [2]. In this protocol, the StopTag is coupled with the FAM fluorophore while the wild type probe has HEX as a fluorophore.

Guidelines for probes design:

- To detect the StopTag sequence once it has been inserted into the gene, two PCR primers and two specific DNA probes fused with different fluorophores are used. One probe is specific to the original allele and the other one to the mutated allele.
- In this protocol, we use Locked Nucleic Acid (LNA) probes synthesized by IDT. They are shorter probes with high melting temperature (T_m) and exhibit increased specificity relative to traditional 5' nuclease probes as they have high efficiency.
- To avoid probe complementarity, both probes must target the same strand.
- Probes should be 10-14 nucleotides and sequence should not start with a G (can quench fluorophore).
- Probe melting temperature (T_m match) should be between 64-68°C. The difference in T_m match between the two probes should be minimal (less than 2°C).
- Avoid having more than 4 LNAs in a row and more than 6 LNAs per probe. Placing LNAs on G and C will give the greatest T_m increase.

- Avoid stretches of 3+ C's or G's.
- Do not insert LNA on first base and avoid inserting LNA in the second base.

Procedure:

1. To design the LNA probes, open the [LNA analyzer tool](#) on IDT website and enter the sequence to be targeted (**Figure 9**).
2. Enter a plus (+) symbol in front of the base of interest to denote the LNA bases (for example, +A, +C, +G or +T).
3. Enter the Mg²⁺ and dNTP concentrations for the reaction conditions (3 mM MgCl₂ and 0.8 mM dNTPs).

TM Mismatch

The screenshot shows the LNA analyzer tool interface. On the left, there is a 'Sequence' input field containing 'ACA+G+CA+C+CA CG'. Below the input field, it indicates '# Bases: 11' and lists 'Base Sequence Symbols' as 'Native DNA: A, C, G, T', 'Inosine: I', and 'Locked nucleic acids: +A, +C, +G, +T'. To the right of the input field are several input fields for reaction conditions: 'Oligo Conc' (0.25 μM), 'Target Conc' (0 μM), 'Na⁺, K⁺ Conc' (50 mM), 'Mg²⁺ Conc' (3 mM), and 'dNTPs Conc' (0.8 mM). On the far right, there is a vertical stack of four buttons: 'TM MISMATCH' (orange), 'TM MATCH' (blue), 'RESET' (grey), and 'ADD TO ORDER' (blue).

Figure 9. Example of LNA probe design. Credit: IDT.

4. Press the button "Tm match" and then select "calculate" to see the match Tm. Repeat this step for the Tm mismatch. The Tm match calculator is used to get the optimal Tm for each probe while Tm mismatch is used to compare the Tm between the 2 probes used.
5. To achieve the recommended Tm, add or remove LNA bases. Spread out the LNAs to get a greater effect on Tm.
6. Use IDT [OligoAnalyzer tool](#) to check the probe sequence for possible dimer and hairpin.

3.1.4 Design of primers

Guidelines for primers design:

- Primers should be outside of the ssODN region and not too close to it, to ensure a PCR product containing the targeted sequence will be amplified during the ddPCR procedure. If not possible, design reverse primer outside of ssODN region and forward inside ssODN region but not too close to the StopTag sequence.
- Amplicon size should be between 80 and 200 bp.
- Each primer pair is composed of a forward primer (from 5' to 3') and a reverse primer (3' to 5').
- The length of each primer should be at least 18 bp, while 20 bp would be the optimal.
- GC% of a primer pair should be between 40 and 60, with an optimal of 50% GC.
- T_m is critical for the efficiency of PCR. Optimal T_m of the primers is 60–63°C. Ideal T_m is 62°C.
- Avoid primers that have unintended targets.
- Melting temperature of primers should be about 2°C lower than the probe T_m.
- The IDT [OligoAnalyzer tool](#) can be used to calculate the T_m. If needed, remove nucleotides to decrease temperature.

Procedure:

On the [Primer-BLAST](#) website, design two pairs of primers (one forward primer and one reverse primer per pair) to generate a PCR product (amplicon) of 80-200 bp that includes the region of the gRNA cleavage site:

1. Paste your sequence in the required field under “PCR template” (**Figure 10**).
2. Change “PCR product size” to “80” Min and “200” Max.
3. Set lowest “primer melting temperature (T_m)” to 60°C and highest to 63°C. Keep the remaining parameters unchanged.

Finding primers specific to your PCR template (using Primer3 and BLAST).

PCR Template [Reset page](#) [Save search parameters](#) [Retrieve recent results](#) [Publication](#) [Tips for finding specific primers](#)

Enter accession, gi, or FASTA sequence (A refseq record is preferred) [Clear](#) Range
From To [Clear](#)

```

agtggagacatgataaattaaagcttactctactagtagcattaaacctcagctcatctctt
tcctccattgacatatttaataaataatcgtgaattctcaaatcttcccttaactatattt
acagctctctttccacaaaccttctaaactttccctcagaagaccctcccaatcagctctc
tctcattctcaag
gtcccttcaccgatgttctcaccaccaaccttaagcttgscacccgacagaccgaaatg

```

Or, upload FASTA file No file chosen

Primer Parameters

Use my own forward primer (5'→3' on plus strand) [Clear](#)

Use my own reverse primer (5'→3' on minus strand) [Clear](#)

PCR product size
Min Max

of primers to return

Primer melting temperatures (T_m)
Min Opt Max Max T_m difference

Exon/intron selection A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span

Exon junction match
Exon at 5' side Exon at 3' side

Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

Intron inclusion Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range
Min Max

Note: Parameter values that differ from the default are highlighted in yellow

Primer Pair Specificity Checking Parameters

Specificity check Enable search for primer pairs specific to the intended PCR template

Figure 10. Example of input of target DNA sequence into Primer-BLAST website with modified parameters.

4. After making the adjustments outlined above, press “get primers”.
5. Select primer pairs that have:
 - GC content between 40–60%.
 - T_m around 60–64°C. The T_m of the two primers should be similar (within 2°C).
 - Minimal to no non-specific binding.
6. Go back to the exon sequence and highlight the primers sequence.
7. To find the sequence for reverse primers, determine the reverse-complement using the [Bioinformatics](#) reverse complement website.
8. Check primers for dimers, hairpins, heterodimers using IDT [OligoAnalyzer](#) tool.

3.2 Culturing cells

This protocol has been optimized for HEK293T, HELA and U2OS cells. Conditions such as media, reagents and cell manipulations may vary for other cell lines. For more details on culturing and freezing cells, see protocol [EDDU-010-02 Generation of knockout cell lines using CRISPR-Cas9 technology](#).

Materials

- 10 cm cell culture dish
- 15 mL conical sterile tube
- Sterile cryotubes
- Sterile pipets 10 mL or 5 mL
- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tetracycline-free FBS, 2mM L-glutamine, and 100 U/mL Penicillin-Streptomycin (complete DMEM)
- Phosphate-Buffered Saline (PBS)
- Trypsin
- Dimethyl sulfoxide (DMSO)
- Fetal Bovine Serum (FBS)
- Cryobox
- Biosafety cabinet
- Cell incubator at 37°C with 5% CO₂.
- Centrifuge
- Microscope

Procedure:

- **IMPORTANT:** All cell manipulations must be done in a Class II biosafety cabinet with sterile media and solutions prewarmed to 37°C.
1. Cells are cultured in a 10 cm dish containing 10 mL of media in a 37°C incubator with 5% CO₂. Culture media is complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL Penicillin-Streptomycin).
 2. Passage cells when they are 70%-90% confluent (usually every 5-7 days). To passage cells, aspirate media, wash cells with 10 mL of PBS and aspirate.
 3. Add 1.5 mL trypsin and transfer the plate to the 37°C/5% CO₂ incubator for 5-10 minutes.
 4. Verify under microscope that the cells have detached and then inactivate trypsin by adding 3.5 mL of complete DMEM and pipetting up and down two or three times to create a cell suspension. Transfer 0.5 mL of cell suspension to a 10 cm culture dish containing 9.5 mL of complete DMEM and place the cells in the incubator. It is possible to transfer more than 0.5 mL of cell suspension to a new dish if needed and the dilution of cell suspension in the new media can be up to 1:3.

Freeze cells when needed to store them in liquid nitrogen for later usage. To freeze cells use freezing media: FBS with 10% DMSO and follow the steps outlined in [EDDU-010-02 Generation of knockout cell lines using CRISPR-Cas9 technology](#).

3.3 Nucleofection of cells

This protocol is optimized for HEK293T, HELA and U2OS cells. Conditions and nucleofector programs may vary for other cell lines.

Materials:

- Culture plate, 6-well
- Complete DMEM
- PBS
- Trypsin
- Alt-R® HDR Enhancer
- Alt-R® S.p. HiFi Cas9 Nuclease V3
- Amaxa™ SE Cell Line 4D-Nucleofector™ X Kit (SE Cell Line Nucleofector™ Solution, Supplement, 16-well Nucleocuvette™ Strips, pmaxGFP™ Vector)
- Guide RNA
- ssODN
- Microscope
- Centrifuge
- Cell counter
- Nucleofector 4D™ Core Unit
- Biosafety cabinet
- Cell incubator at 37°C with 5% CO₂
- 1.5 mL conical sterile tube
- 15 mL conical sterile tube
- Gilson Pipetman P10G, P20G and P200G
- P10, P20 and P200 sterile tips
- Sterile pipets 10 mL

3.3.1 Preparation of RNP complex

Procedure:

1. Resuspend gRNA and ssODN to 100 µM in TE buffer.
 2. Thaw Cas9 protein at room temperature in the biosafety cabinet.
 3. Assemble Cas9-gRNA RNP complex: in a 1.5 mL conical tube mix 1 µl of the Cas9 protein with 3 µL of gRNA and 5 µl of Amaxa™ SE cell line solution with supplement and incubate at RT for 10-20 minutes.
 4. Add 1 µl of ssODN to the RNP mix.
- For nucleofection positive control, use pmaxGFP™ Vector to transfect the cells: mix 0.5 µl of GFP Vector with 5 µL of SE cell line solution with supplement and incubate for 10 minutes at RT.

5. Meanwhile, prepare cells for nucleofection: detach cells as indicated in **Section 3.2**, transfer cell suspension to a 15 mL conical tube and count cells.
6. Centrifuge 150,000 cells in a separate 15 mL conical tube at 1,200 rpm for 3 minutes and aspirate supernatant (150,000 cells are used per single nucleofection reaction).
7. Add 5 mL of PBS, mix by inverting the tube, centrifuge again and aspirate supernatant completely.
8. Resuspend the cell pellet in 15 μ L of Amaxa™ SE cell line solution with supplement and add to the RNP-ssODN mix. Mix gently. For the nucleofection control add the 15 cell-SE μ L suspension to the pmaxGFP™ Vector-SE mix in a separate tube.
9. Transfer the RNP-ssODN-cell suspension to one well on a Nucleocuvette strip. Transfer the GFP Vector-cell suspension to another well and then click to close the lid and tap gently to ensure no bubbles in the cuvette.

3.3.2 Nucleofection and cell recovery

Procedure:

1. Program the Nucleofector 4D device with the appropriate program code depending on the cell type (CM130 for HELA and HEK293 while CM104 is recommended for U2OS). Choose option SE in the nucleofector settings.
2. Place the Nucleocuvette strip with closed lid into the retainer of the 4D-X Core unit. Check for proper orientation of the strip. The larger cut out is the top (wells A1 and A2) of the cuvette and the smaller cut out is the bottom (wells H1 and H2).
3. Press start and wait until screen displays a green “+” over the wells with successfully transfected cells.
4. Remove strip with cells from the Nucleofector instrument and add 70 μ L of recovery media (complete DMEM + HDR enhancer at 100 μ M final concentration) to each well with cells. Mix gently by pipetting up and down and transfer the entire content of each well from the strip into a sterile 15 mL conical tube containing 6 mL of recovery media.
5. Wash each well with the additional 70 μ L of recovery media and combine with 6 mL media and cells from the same well. This is done to recover most of the nucleofected cells.
6. Mix gently by inverting a few times and distribute 3 mL of each transfected well per 2 wells of 6-well plate. One well with cells will be used to screen the edited DNA and another well will be used for the clonal selection.
7. Place plates with seeded cells in the 37°C/5% CO₂ incubator.
8. Change media with 3 mL per well of complete DMEM after 24 hours.
9. Monitor post-nucleofected cells daily under the light microscope. When cells reach 50-90% confluence (takes 3-10 days depending on the gene) they can be screened for gene editing and separated by single cell sorting.
10. The GFP transfection efficiency in the control group can be assessed using fluorescence microscopy 24-48 hours post-transfection (**Figure 11**).

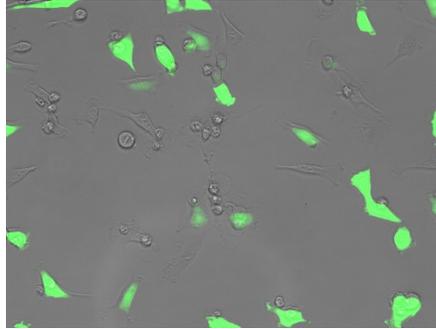


Figure 11. GFP transfected HeLa cells 24 hours post-transfection.

3.4 Isolation and Selection of Knockout Cells

The cell sorting procedures presented in this section have been optimized for the BD FACS Aria Fusion system and can vary with different systems.

Cells are separated from the polyclonal cell population after the nucleofection (3-10 days in post-nucleofection culture) by single cell sorting to seed individual cells per well. The sorted single cell produces a single clone colony of either edited or wild type (WT) genotype.

Materials:

- BD FACSDiva software
- BD FACS Aria Fusion (Cell sorter)
- 30 μ m sample filter
- Sterile FACS 5 mL tube with cap
- 96-well collection plates with border well around the plate (Eppendorf)
- Complete DMEM
- Trypsin
- Sterile 5 mL pipets
- Gilson Pipetman P200G Pipette
- Sterile pipet tips P200
- Sterile PBS
- 15 mL conical sterile tube
- 1.5 mL conical sterile tube
- Gilson Pipetman G, 8 x 200 Multichannel Pipette
- Freezing media (FBS with 20% DMSO)
- Quick Extract (QE) solution
- Vortex
- 96-well plates
- Biosafety cabinet
- Cell incubator at 37°C with 5% CO₂

- Centrifuge
- Microscope

3.4.1 Single cell sorting

Procedure:

For more details on single cell sorting, see protocol [EDDU-010-02 Generation of knockout cell lines using CRISPR-Cas9 technology](#)

1. Prepare the appropriate number of 96-well plates with border wells to receive single cell clones after sorting. Add 200 μ L of complete DMEM per well. Place the plates in the 37°C/5% CO₂ incubator until ready to sort the cells.
2. Trypsinize a well of a 6-well plate with post-nucleofected cells (see **Section 3.3**) when they reach at least 50% confluence. Aspirate media by vacuum, wash cells with 3 mL of PBS and aspirate PBS.
3. Add 0.6 mL of trypsin and transfer the plate with cells to the 37°C/5% CO₂ incubator for 5-10 minutes.
4. Verify under microscope that the cells have detached, add 3.4 mL of complete DMEM pipette up and down two times and transfer cell suspension to a 15 mL conical tube.
5. Centrifuge at 1,200 rpm for 3 minutes, aspirate supernatant, resuspend cell pellet in 1 mL of complete DMEM and transfer cell suspension to a sterile 5 mL FACS tube with cap.
6. Bring the FACS tube with cells along with the 96-well collection plate(s) from the incubator to the FACS sorting facility.
7. Sort single cell per well onto 96-well plate.
8. After cell sorting, bring the 96-well plate to biosafety cabinet and fill the border well of the plate with 4 mL of media to avoid evaporation of liquid from wells.
9. Carefully transfer the 96-well plate to the 37°C/5% CO₂ incubator to allow growth of single colonies. This usually takes 1-3 weeks.
10. Monitor the sorted cells under brightfield microscope starting on day 6 after sorting and identify and mark wells with single colonies. Some wells will not produce colony and some wells could produce more than one colony. Only cells derived from a single colony will be used for clonal selection.

3.4.2 Selection of single clones

Procedure:

Two to three weeks after the sorting, large colonies are formed (**Figure 12**). When substantial number of wells on 96-well plate have single large colonies (at least 20 wells per plate) cells are passaged onto two duplicate plates: one to be used to screen for edited alleles and another one for backup storage.

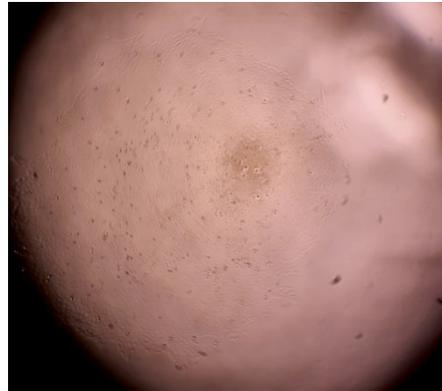


Figure 12. Large single colony derived from single U2OS cell on a well of 96-well plate. Image taken with EVOS XL Core microscope.

1. Remove media from wells with single colonies on a 96-well plate.
2. Wash cells: Add 200 μ L PBS per well and aspirate.
3. Trypsinize cells: add 50 μ L of trypsin per well and incubate for 5-10 minutes in 37°C/5% CO₂ incubator to detach cells.
4. Mix the trypsinized cells by pipetting up and down and transfer half of the volume (25 μ L) of each well to the corresponding well on the receiving plate.
5. Take the remaining 25 μ L of cells and transfer it to a corresponding well on the second 96-well receiving plate. Make sure that cells from each single colony well are passaged to the well with identical location on both receiving plates.
6. Grow cells on the duplicate plates in the 37°C/5% CO₂ incubator until they are 70%-90% confluent (usually takes 2-8 days).
7. Use one of the duplicate plates to screen the clones: extract DNA using QuickExtract and screen the DNA of the clones by ddPCR (see **Section 3.5**).
8. The second “backup” plate with single clones could be frozen down and conveniently stored until the screening results. To freeze cells on a 96-well plate, aspirate media, wash cells with 100 μ L PBS per well and detach cells by adding 50 μ L of trypsin per well and incubating for 5-10 min at 37°C/5% CO₂.
9. Add 50 μ L per well of freezing media (FBS with 20% DMSO) to the detached cells, cover the plate, seal it with parafilm, wrap plate with bubble wrap and store in a -80°C freezer in Styrofoam box for up to 6 months.

3.5 Validation of gene editing by ddPCR

Droplet Digital PCR (ddPCR) analysis is a digital PCR method based on water-oil emulsion droplet technology. A sample containing primers along with fluorescent probes is fractionated into about 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet. This is a multiplex experiment in which two targets are amplified by PCR. Following PCR, each droplet is read to determine the fraction of PCR-positive droplets (containing at least one copy of the target) in the sample. This data is then analyzed to determine the proportion of edited allele in the sample. ddPCR technology can be used for extremely low target quantification from variably contaminated samples. ddPCR technology will produce more precise, reproducible, and statistically significant results.

The procedures presented in this section have been optimized for the Bio-Rad ddPCR QX200™ system. Similar procedures have been used for the gene editing analysis in iPSC and are outlined in the protocol [EDDU-009-01 CRISPR-mediated introduction of single nucleotide changes in iPSCs](#).

Materials:

- 96-well cell culture plates
- ddPCR™ 96-well plates
- DG8 Cartridge Holder
- DG8 Cartridges and Gaskets
- Pierceable Foil Heat Seal
- Pipet-Lite Multi Pipette L8-50XLS+
- Tips GP-LTS-A-250µL
- P200 sterile tips
- Gilson Pipetman Pipette P200G
- Gilson Pipetman G, 8 x 200 Multichannel Pipette
- PBS
- ddPCR Supermix for Probes (no dUTP)
- Droplet Generation Oil for Probes
- Droplet Reader Oil
- Primers
- Probes
- QuickExtract™ DNA Extraction Solution
- DNase free H₂O
- Microscope
- C1000 Touch™ thermal cycler with 96–deep well reaction module
- Heat Block
- PCR Plate Spinner
- PX1™ PCR Plate Sealer
- QX200™ Droplet Generator
- QX200™ Droplet Reader

- Biosafety cabinet
- Vortex Mixer

3.5.1 Genomic DNA extraction

Procedure:

1. When the cells are 70%-90% confluent, remove media from the wells and add QuickExtract (QE) solution (50 μ L per well of a 96-well plate or 250 μ L for a well of a 6-well plate).
2. Incubate with QE for 3 minutes and mix by scrapping the cells with P200 pipet tip and pipetting up and down a few times. Leave QE in the wells of the plate or transfer volume of wells to individual 1.5 mL sterile conical tube.
3. Incubate plate or tube at 65°C for 5-10 minutes followed by 95°C for 5 minutes to inactivate QE solution.
4. Cool down and store at 4°C. Can also be stored at -20°C for long-term storage.
5. Prepare a 1/10 dilution with DNase free H₂O; this is the diluted DNA extract that will be used to evaluate the proportion of edited alleles using ddPCR. DNA extract can be stored at 4°C.

3.5.2 ddPCR reaction mixture

Procedure:

1. Prepare ddPCR master mix by adding reagents listed in **Table 2**. Use the ddPCR mastermix for screening of edited gene and use the additional ddPCR mix (**Table 2**, right column) at the later stage for gene knockout evaluation.
2. Distribute 19 μ L per well of this master mix in a 96-well ddPCR plate.
3. Add 2 μ L per well of the diluted DNA extract in each corresponding well.
4. Spin briefly using PCR plate spinner.

Table 2. Suggested volume of reagents required for the preparation of ddPCR master mix

Reagent	ddPCR master mix		Additional ddPCR mix to evaluate ratio of KO gene versus another non edited gene
	Volume for a whole 96-well plate (µL)	Volume for one 8-well column on 96-well plate (µL)	Volume for one 8-well column on 96-well plate (µL)
ddPCR Supermix for Probes (no dUTP)	1170	90	90
Primer F (100 µM)	20.8	1.6	1.6
Primer R (100 µM)	20.8	1.6	1.6
DNase-free H ₂ O	881.4	67.8	64.1
StopTag Probe FAM	6.5	0.5	0.5
WT Probe HEX	6.5	0.5	0.5
Primer F for another gene (100 µM)	–	–	1.6
Primer R for another gene (100 µM)	–	–	1.6
Probe for another gene	–	–	0.5
Total volume per well	19	19	19

3.5.3 Droplet generation

Procedure:

- **IMPORTANT:** The generation of the ddPCR droplets requires the preparation of everything upfront as once you start the droplet generation process, the PCR reaction needs to be started within one hour, otherwise the droplet quality will degrade significantly.

1. Insert a new DG8 cartridge in its holder and transfer 20 μL of the ddPCR reaction mixture from each well of column 1 of the 96-well plate into the “sample” wells (8 wells in total) of the cartridge (**Figure 13**).



Figure 13. Generation of droplets using the DG8 cartridge and the QX200 Droplet Generator. Credit: BioRad.

2. Add 65 μL of “Droplet Generation Oil for Probes” in the “oil” wells (8 wells in total) of the DG8 cartridge.
 3. Leave the “droplet” wells empty.
 4. Install a ddPCR gasket on the top of the cartridge. The gasket can be re-used, but it should be properly dried prior to each use.
 5. Place the cartridge inside the “QX200 Droplet Generator” and close the lid. The droplet generation is accompanied with a buzzing sound.
 6. When the green light appears after 1-2 minutes, open the door of the equipment, and remove the cartridge from the droplet generator.
 7. Remove the gasket and let it dry. It can be re-used later when using the other (dry) side.
 8. Transfer 42 μL of the droplet mix by pipetting it **VERY** slowly, gently and continuously (to not disrupt the newly-generated droplets) into the corresponding wells, i.e., column 1, of a new 96-well ddPCR plate using the “Pipet-Lite Multi Pipette L8-50XLS+” and the “Tips GP-LTS-A-250 μL ”. Prior to the completion of the PCR reaction, the droplets are very fragile.
 9. Repeat the same process for columns 2 to 12 of the 96-well plate (as needed depending on the number of samples).
 10. Seal the plate with the “Pierceable Foil Heat Seal” using PX1™ “PCR Plate Sealer” preheated to 180°C and transfer the plate to PCR machine. To avoid droplet degradation, let the Plate Sealer block ejected during the pre-heating step. The lid needs to be hot only during sealing process.
 11. Start the appropriate ddPCR program while ensuring that you set the proper T_m corresponding to your probe/primer set, previously established using the gradient mode. A typical ddPCR program would be: 1x[95°C, 10 minutes], 45x[95°C, 30 seconds; 62°C, 1 minute; 72°C, 30 seconds], 1x[98°C, 10 minutes], 1x[12°C, ∞], at 2.5°C/second ramp rate.
- When the PCR reaction is completed, the droplets are stable for several days if kept at 4-10°C in the dark.

3.5.4 Droplet reading

Following PCR amplification of the nucleic acid target in the droplets, the QX200 droplet reader analyze each droplet individually using a two-color detection system (set to detect FAM and either VIC or HEX).

Procedure:

- Read the ddPCR plate with droplets using the “QX200 Droplet Reader” in the absolute quantification mode of the QuantaSoft software.
 - Ensure there is enough “Droplet Reader Oil” in the instrument and the waste is empty before starting a run.
1. Insert the ddPCR plate with droplets into the “QX200 Droplet Reader”, close the metal gasket and close the lid of the instrument.
 2. In QuantaSoft software pull up a new template and on plate map select the wells to be analyzed.
 3. Select the appropriate reporter fluorophores according to the used probes, e.g., -FAM for mutant and -HEX for WT.
 4. Select run to start the droplet reading.

3.6 Data Analysis

Materials:

- QuantaSoft Software
- SnapGene Software
- Microsoft Office Excel

After the “QX200 Droplet Reader” has finished interrogating all wells, use QuantaSoft software to analyze the data in each well.

The data analysis interface is separated into three windows (**Figure 14**):

- Result table
- Well selector
- Processed data/graphical display

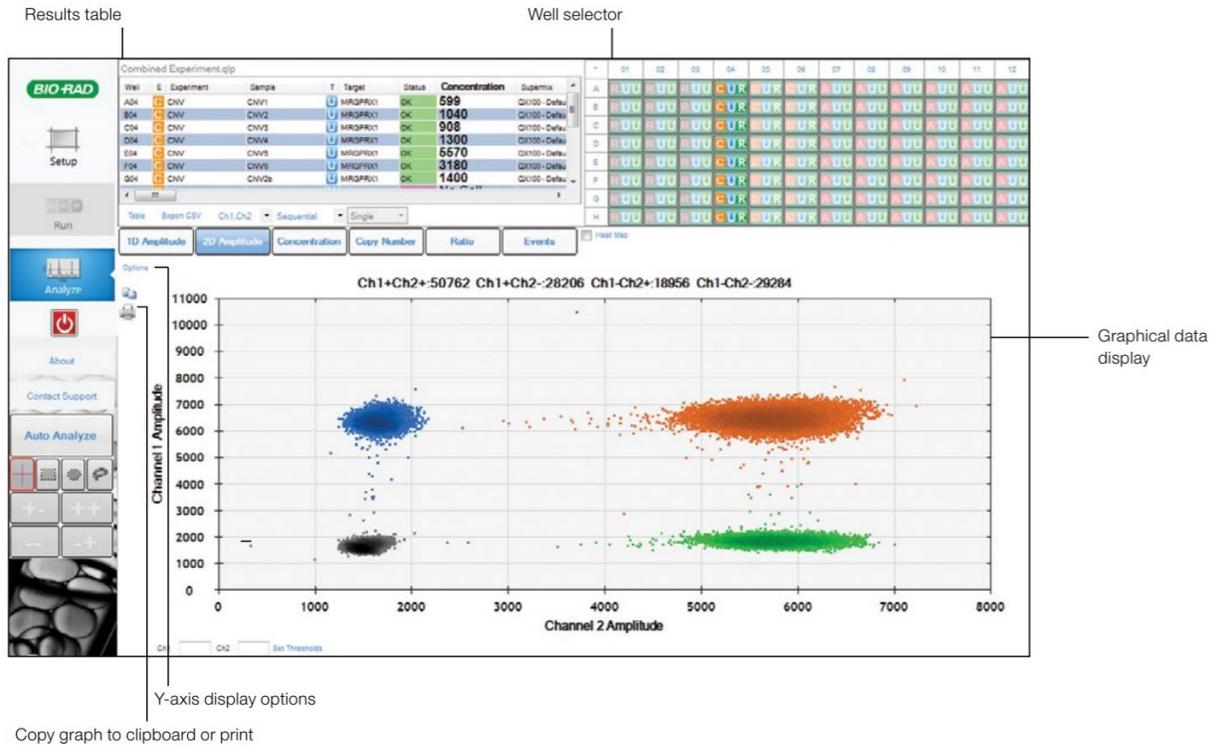


Figure 14. Analysis interface. Credit: BioRad.

3.6.1 Viewing events

Procedure:

Select Events to view the number of droplet events counted for selected wells/samples. Use the radio buttons to select the channels displayed. View positive, negative, or total droplet counts, or any combination of these (**Figure 15**).

- **IMPORTANT:** The total number of droplets per well should be between 10,000 and 20,000 for an accurate analysis

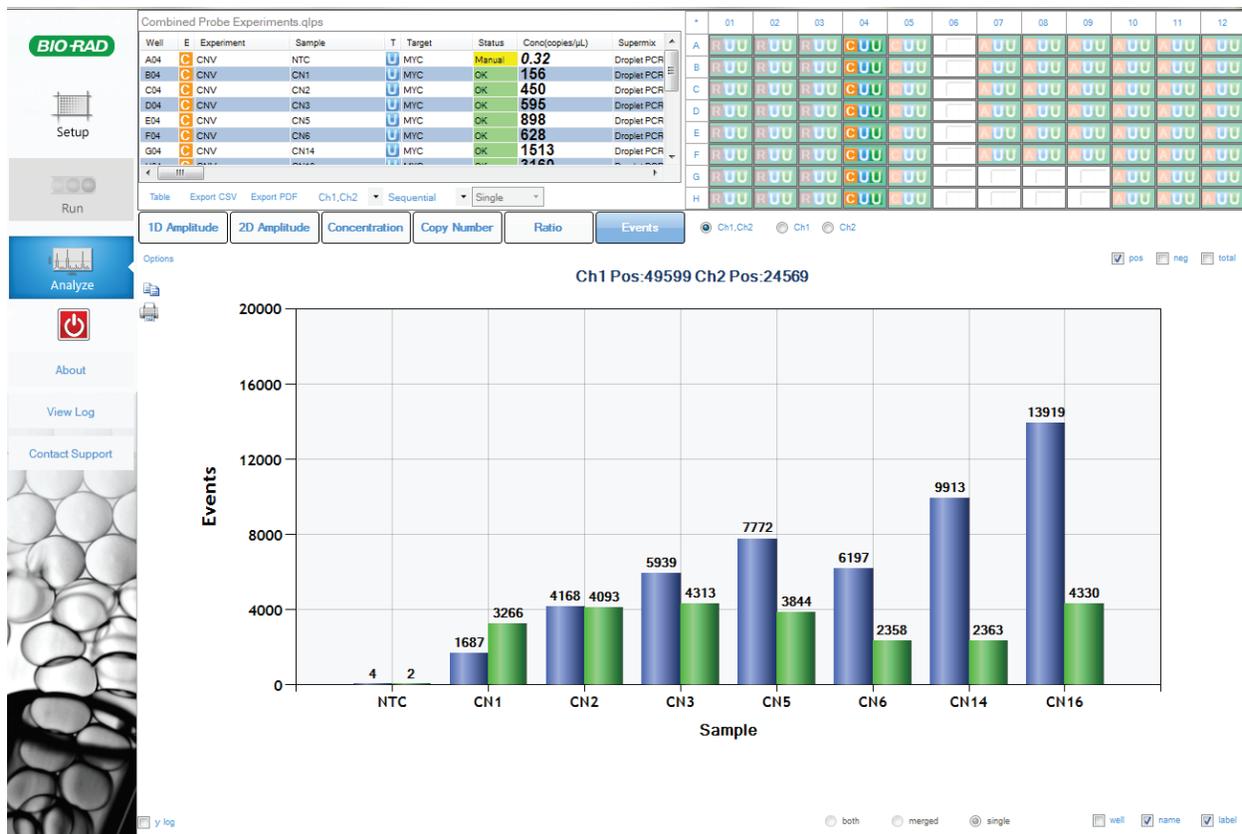


Figure 15. Viewing event data. Credit: BioRad.

3.6.2 Quantification of positive droplets

Droplet Digital PCR data from a multiplex experiment in which two targets are PCR amplified can be viewed as a 2-D plot in which FAM fluorescence is plotted versus HEX fluorescence for each droplet. The droplets are clustered into four groups.

Procedure:

Select 2D Amplitude to view the channel 1 (StopTag probe-FAM) vs. channel 2 (WT probe-HEX) clustering plot and enable options for manually or automatically adjusting the thresholds used in assigning positives and negatives for each detection channel (**Figure 16**).

- To reset automatic thresholds for positives and negatives, click on Auto Analyze.
- To manually assign thresholds:
 - Use the thresholding crosshair to assign classification regions for the whole plot.
 - Use the ellipse, rectangle, or lasso threshold adjustment tool to classify a region of the plot.

- Mouse over any well in the well selector to preview data from that well in the clustering plot.
- Axis scale can be adjusted by selecting Y-axis display options (**Figure 16**).

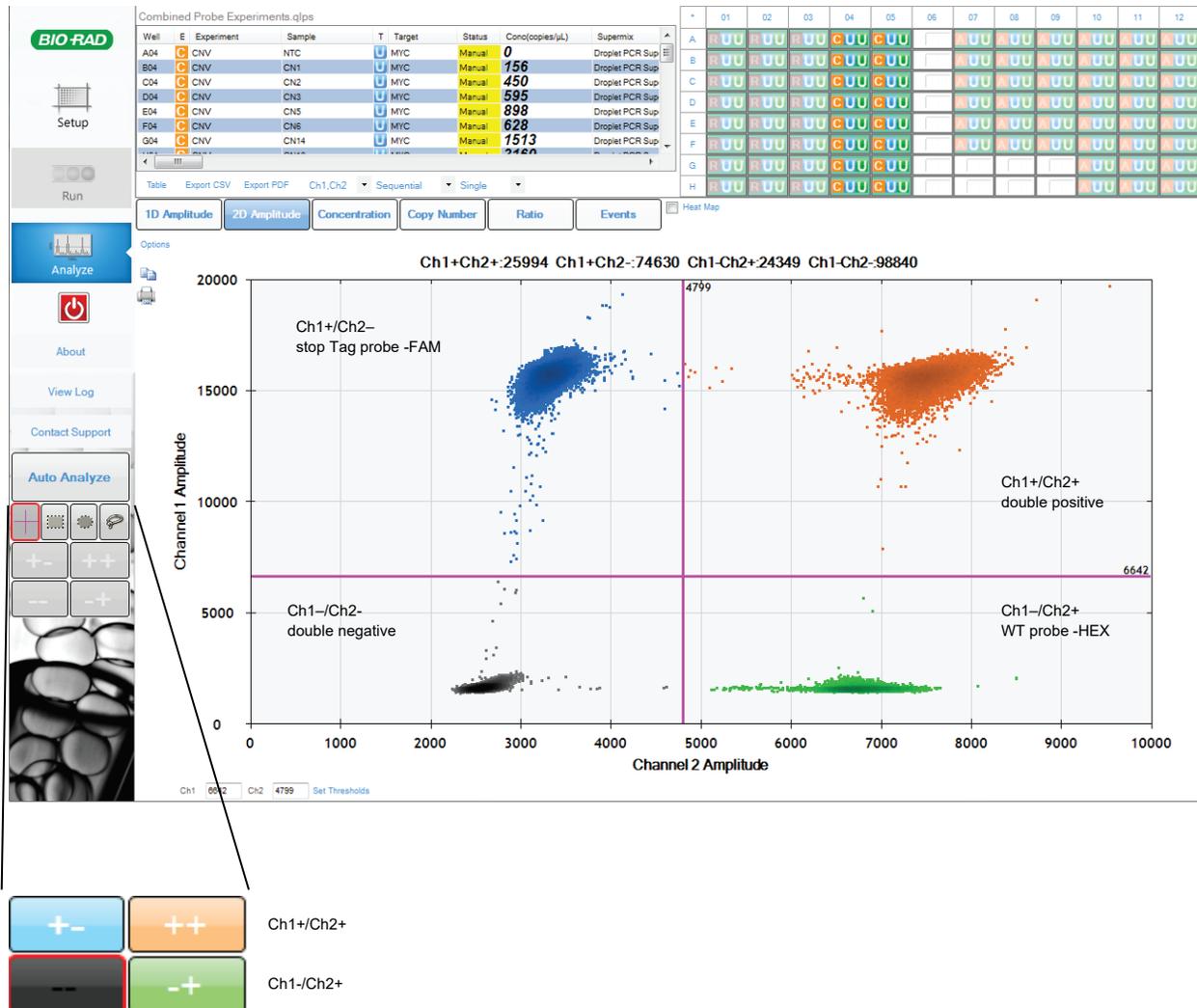


Figure 16. Threshold adjustment options available in the clustering plot. Credit: BioRad.

3.6.3 Determining KO edited proportion

3.6.3.1 Using a specific designed WT gene probe

The designed WT probe (-HEX) will bind a part of DNA supposed to be replaced by the StopTag fragment inserted. While the StopTag (-FAM) probe will bind the edited alleles (**Figure 17**).

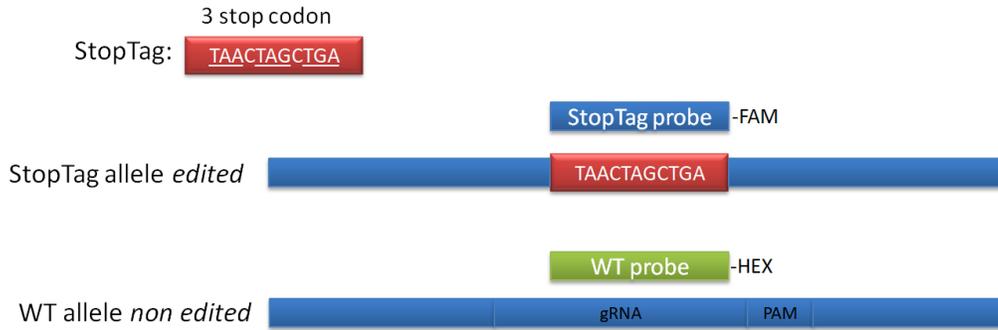


Figure 17. StopTag and WT probes binding.

- A ratio between StopTag probe and non edited WT probe concentration will indicate the percentage of edited allele. A 1:0 ratio indicates that 100% cells have edited allele.
- An example of ddPCR detection of 100% edited VAPB gene KO with StopTag probe (-FAM) and VAPB WT probe (-HEX) is shown in **Figure 18**.

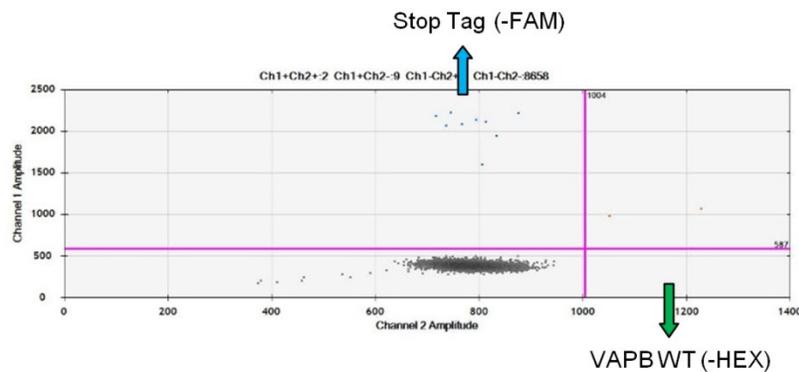


Figure 18. VAPB gene KO ddPCR analysis with StopTag / VAPB WT probes.

3.6.3.2 Using a non-edited different WT gene probe

- When the WT probe is designed too close to the cut site, it could happen that the WT probe is not able to bind the site. This is due to small indels generated on the cut site during the editing. The specific designed WT probe is then not able to bind the site even if the StopTag is not inserted (**Figure 19**). This resulting in a skewed ratio and an inaccurate percentage of edited cells.

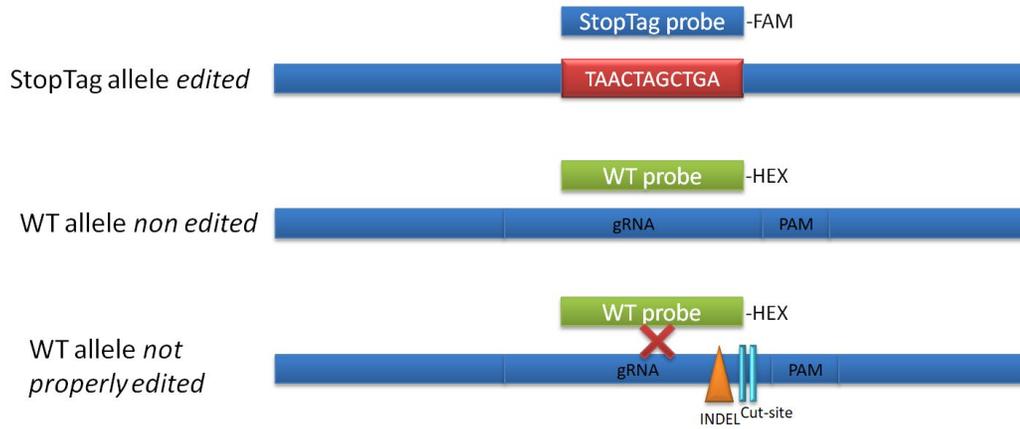


Figure 19. INDEL around cut site prevents binding of the WT probe.

- Another – HEX probe targeting a different not edited WT gene can then be used to accurately estimate the percentage of edited cells. This “control gene” probe will indicate the total number of alleles present in the reaction (**Figure 20**).



Figure 20. StopTag and control WT gene probe binding.

- A ratio between StopTag probe and control gene WT probe concentration will indicate the percentage of edited allele. A 1:1 ratio indicates that 100% cells have edited allele.
- An example of ddPCR detection of 100% edited VAPB gene KO with StopTag probe (-FAM) and a non edited control WT gene probe (-HEX), which is targeting all alleles, is shown in **Figure 21**.

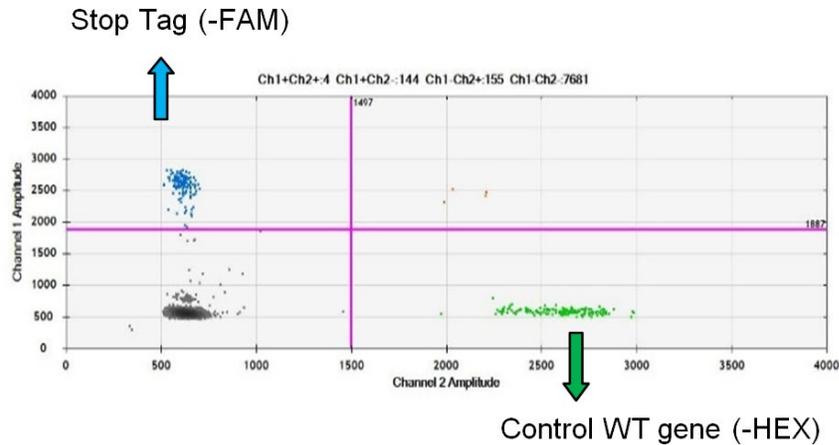


Figure 21. VAPB gene KO ddPCR analysis with StopTag / Control WT gene probes.

3.6.4 Data export

Procedure:

Once the threshold is adjusted for each well, analyzed data are exported into .csv file.

- .csv file can be opened with Microsoft Office Excel software.
- The column “Concentration” represents the number of WT or edited alleles per μl of reaction mixture, for each well.
- Calculate the proportion of edited vs WT alleles in each well (**Table 3**).
 - $\% \text{ edited allele} = \frac{\text{concentration mut}}{\text{concentration mut} + \text{concentration WT}} \times 100$
- Analysis can be done with:
 - genomic DNA of post-nucleofected cells to estimate the percentage of edited cells (StopTag inserted).
 - genomic DNA of single clone after single cell sorting (**Section 3.4.1**); to select clones 100% edited and expand them.

Table 3. Example of .csv file calculation.

Well	Gene	Target	Concentration	Target	Concentration	% mut/total
A01	VAPB	mut	2.7	wt	0	100
A02	VAPB	mut	1	wt	0	100
A03	VAPB	mut	1.9	wt	0	100
A04	VAPB	mut	1.5	wt	0	100
A05	TUBA4A	mut	0.8	wt	4.2	16
A06	TUBA4A	mut	0.25	wt	3.6	7
A07	TUBA4A	mut	0	wt	4.1	0
A08	TUBA4A	mut	1	wt	4.3	19

3.7 Final characterization

Materials:

- 6-well plate
- Complete DMEM
- PBS
- Agarose gel
- PCR polymerase kit
- Zero Blunt TOPO cloning kit
- Sequencing Analyzer

3.7.1 Expansion and banking

Once 1-3 independent 100% edited clones are identified, these clones are expanded further, and cells are banked and processed for further DNA sequence analysis to verify the presence of the StopTag.

Procedure:

1. Thaw the cells in the 96-well backup plate by placing the frozen 96-well plate into the 37°C/5% CO₂ incubator for 10-15 min.
2. Prepare a 24-well receiving plate by adding 2 mL of complete DMEM per well and place plate at 37°C/5% CO₂ incubator.
3. Transfer 100 µL of the clone of interest from the thawed plate onto one well of 24-well plate filled with 2 mL warm media. Place plate with cells in the incubator and change the media the next day.
4. Expand the KO clones for freezing stocks with gradual passages: when cells in a well reach 60-90% confluency, transfer them to a larger dish (from 24-well to 6-well plate and from there to a 10-cm dish).
5. In parallel, validate the KO alleles by Sanger sequencing analysis.

6. Prepare freezing stocks of KO cells (**Section 3.2**).

- **IMPORTANT:** Prior to freezing, cells must be tested for mycoplasma to ensure they are free of contamination.

3.7.2 Target sequence integrity

Once isolated clones are expanded, they are tested for sequence integrity and homozygosity / heterozygosity of the mutation using PCR cloning and Sanger sequencing.

- PCR primers are designed to give rise to a ~300-500 bp single/clean band on agarose gel, with the StopTag insertion located in the middle of the fragment.

Use this fragment for PCR cloning or directly for Sanger sequencing using the same PCR primers.

- DNA sequencing is the process of determining the sequence of nucleotides in a fragment of DNA. In Sanger sequencing, the target DNA is copied many times, making fragments of different lengths. Fluorescent “chain terminator” nucleotides mark the ends of the fragments and allow the sequence to be determined by laser during capillary gel electrophoresis.
- Sequencing data are obtained following the protocol [EDDU-005-02 SeqStudio DNA Sequencing](#).
 - PCR cloning: used to isolate and sequence each allele in different bacterial clone to ensure the homozygosity. The PCR product is ligated in a plasmid using TOPO cloning kit, amplified in bacteria then the plasmid is extracted and sequenced.
 - Direct Sanger sequencing: check if the chromatogram is not mixed to ensure the homozygosity.
- Sequencing data are analyzed using SnapGene program (**Figure 22**).

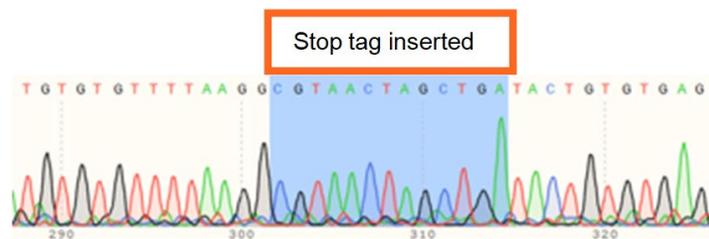


Figure 22. Sequence of 100% edited cell line with StopTag inserted.

The clone with StopTag inserted in all alleles shows a clean chromatogram and therefore can be used as KO for further experiment.

3.7.3 Off-target events

CRISPR-Cas9 of a specific single gRNA can edit at the right region of its target gene, however it may also bind to other genes and edit their sequences. The latter is called off-target editing.

To ensure that the phenotypes observed are not the result of off-target effects, it is advised to investigate potential off-target sites by PCR based on mismatches between the gRNA sequence and the whole genome (**Section 3.1.1, Figure 7**).

Off-targets that are predicted to be into coding region of the genome, or at least into important genes, should be verified by Sanger sequencing. This method is fast and simple, but a lot of information is ignored as only a few off-target sites are checked.

However, various advanced methods for the off-target detection including deep sequencing, and CHIP-seq have been developed [3] (**Table 4**).

Table 4. Non exhaustive list of off-target detection methods.

Methods	Advantages	Disadvantages
Whole Genome Sequencing	Unbiased, accurate, credible	More expensive than biased methods
Whole Exome Sequencing	Unbiased, cheaper than WGS	Mutations in non-coding sites are ignored
Chip-seq	Unbiased, genome-wide detection of gRNA/Cas9 binding loci	High rate of false positives

4 References

1. Hindson, B.J., et al., *High-throughput droplet digital PCR system for absolute quantitation of DNA copy number*. *Anal Chem*, 2011. **83**(22): p. 8604-10.
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3. Zischewski, J., R. Fischer, and L. Bortesi, *Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases*. *Biotechnol Adv*, 2017. **35**(1): p. 95-104.