NeuroEDDU Protocols

# Generation of Knockout Cell Lines Using CRISPR-Cas9 Technology

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# 1 Introduction

## 1.1 Objectives

This protocol describes a CRISPR-Cas9 genome editing method to generate knockout (KO) cell lines.

## 1.2 Protocol overview

Cells used in this protocol to generate a gene knockout harbor the Cas9 nuclease gene that is integrated in the AAV1 locus under the TetO inducible promotor ([1], [2]). In these cells, Cas9 is stably expressed in the presence of doxycycline (DOX). The cells chosen to knockout our gene of interest should have a high abundance for the protein expressed from this gene as determined from values on the PAXdb website.

After selecting the appropriate cells, a pair of synthetic guide RNAs (gRNAs) are designed to target the earliest constitutive exon of the target gene. Several online guide RNA design tools are used to ensure efficient guide design. Each pair of guides is designed to cut the gene within 100-200 bp of each other.

Next, parental cells are co-transfected with pair of gRNAs (**Figure 1**) to knockout the gene of interest. These cells are then cultured in media with doxycycline to turn on the expression of the Cas9 protein. In the presence of Cas9, gRNAs will target defined sites within the gene of interest, causing a DNA strand break.

Two days after transfection, the culture media of the polyclonal cell population of knockout (KO) and isogenic cells is switched to media without doxycycline, to turn off the expression of Cas9. The polyclonal population is subsequently screened for KO by PCR screening of genomic DNA extracted from the transfected cells using gene specific primers.

The polyclonal cell population is then selected by single cell FACS (Fluorescence-activated cell sorting) to seed individual cells that can be grown and expanded into monoclonal KO cells. Each sorted individual cell will produce a colony, also known as a single clone. Clones are genotyped by PCR and 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.

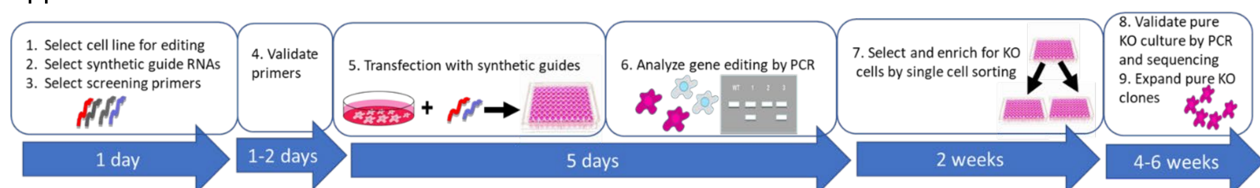



**Figure 1: Flowchart of the generation of knockout cell lines using CRISPR-Cas9 technology**

## 1.3 Technical and safety considerations

- The following information should be read before starting the procedure:
- This protocol is intended to be used only in cells that have a DOX-inducible CAS9 promoter integrated in the genome.
- Ensure that the cell culture is mycoplasma-free before starting the process.
- Use cells with low passage number as high passage number can have a negative effect on transfection efficiency and gene expression.
- For successful generation of 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 are recommended to 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% $CO_2$ 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 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.
- Make aliquots of primers and gRNAs to avoid contamination and repeated thawing and freezing.
- Good pairs of screening primers are needed to validate transfection efficiency and DNA editing by gRNAs. Thus, validate screening primers using wild type (WT) genomic DNA before doing transfection of synthetic guide. Test 2 primer pairs on WT DNA and look for a single band at the expected size (**Section 3.4**).
- gRNAs are transfected in pairs. When using pairs of gRNAs, make sure that the distance between the cleavage sites of the gRNAs is between 100-200 bp.
- Selected clones from Section 3.8 are sequence-validated by PCR and sequenced to reveal the exact gene editing on each allele. This is done in accordance with molecular cloning guidelines [3].

## 1.4 Abbreviation List

| Abbreviation | Full Name |
|---|---|
| bp | Base pair |
| cm | Centimeter |
| DMEM | Dulbecco's Modified Eagle's Medium |
| dNTP | Bis(5-nitro-2-pyridyl) disulfide |
| DOX | Doxycycline |
| EHS | Environmental Health and Safety |
| FACS | Fluorescence-activated cell sorting |
| Fetal bovine serum | FBS |
| g | Gram |
| gRNA | Guide RNA |
| h | Hour |
| KO | Knockout |
| L | Litre |
| mL | Millilitre |
| µL | Microliter |
| µM | Micromolar |
| min | Minute |
| M | Molar |
| Phosphate buffer saline | PBS |
| PCR | Polymerase chain reaction |
| PPE | Personal Protective Equipment |
| QE | Quick Extract |
| RT | Room Temperature |
| s | second |
| TBE buffer | Tris/Borate/EDTA buffer |
| Tm | Primer melting temperature |
| WT | Wild type |

# 2 Materials

Refer to the product data sheet from the supplier for further details on storage and preparation instructions.

## 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 |
| 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 |
| 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 |
| Pipet tips | VWR | 89079-444 |
| 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 |

| Item | Supplier | Catalogue # |
|---|---|---|
| Sterile syringe filter, 0.2 µm | VWR | 28-145-501 |
| Sterile syringe, 10 mL | BD | 302995 |
| Ice box | N/A | N/A |

## 2.2 Websites

| Name | Address |
|---|---|
| Addgene | https://www.addgene.org/ |
| Benchling | https://www.benchling.com/ |
| Bioinformatics | https://www.bioinformatics.org/sms/rev_comp.html |
| Ensembl | https://useast.ensembl.org/index.html |
| Expasy | https://www.expasy.org/ |
| Oligocalc | http://biotools.nubic.northwestern.edu/OligoCalc.html |
| PaxDb | https://pax-db.org/ |
| Primerblast | https://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| Promega | https://www.promega.ca/resources/pubhub/enotes/what-percentage-agarose-is-needed-to-sufficiently-resolve-my-dna-sample/ |
| Synthego | https://www.synthego.com/ |

## 2.3 Software

| Item | Supplier | Version |
|---|---|---|
| SnapGene | GSL Biotech LLC | N/A |

## 2.4 Reagents

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Agar | BioShop | AGA001.500 | N/A | N/A | Room Temperature (RT) |
| Agarose | BioShop | AGA001.1 | 100% | 0.5 – 2% | Stock: RT<br>Working: RT |
| Bis(5-nitro-2-pyridyl) disulfide (dNTP) | New England Biolabs | N0447L | 10 mM | 2.5 mM | Stock: -20°C<br>Working: RT |

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Bleach | Clorox | N/A | 100% | 10% | Stock: RT<br>Working: RT |
| Boric Acid | Bioshop | TRS001.5 | N/A | N/A | RT |
| CutSmart® Buffer | New England Biolabs (NEB) | B7204S | 10x | 1x | Stock: -20°C<br>Working: 4°C |
| Dimethyl sulfoxide (DMSO) | Sigma | D2650 | 100% | 10% | Stock: RT<br>Working: RT |
| DNA Ladder RTU, 100 bp | Froggabio | DM001-R500F | N/A | N/A | Stock: -20°C<br>Working: RT |
| Doxycycline | Bioshop | Dox444.1 | 1 mg/mL | 2 µg/mL | Stock: -20°C<br>Working: RT |
| Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/L Glucose L-glutamine + pyruvate | Multicell | 319-005-CL | 1x | 1x | Stock: 4°C<br>Working: 37°C |
| EcoRI restriction enzyme | New England Biolabs (NEB) | R3101 | 100x | 1x | Stock: -20°C<br>Working: RT |
| EDTA Dissodium | Multicell | 625-060CG | N/A | N/A | RT |
| FACSFlow | BD | 342003 | | | Stock: RT<br>Working: RT |
| Fluo-DNA loading Buffer | ZmTech | LB-001B | 6x | 1x | Stock: -20°C<br>Working: RT |
| Guide RNA | Synthego | N/A (Custom order) | 100x | 3 µM in RNAse-free water | Stock: RT<br>Working: -20°C |
| HiPerFect | Qiagen | 301704 | 100% | 1:175 | Stock: 4°C<br>Working: RT |
| Kanamycin | Bioshop | Kan201.5 | 100% | 50 mg/mL | Powder: 4°C<br><br>50mg/mL stock: -20°C |
| LB | Bioshop | LBL407.1 | N/A | N/A | RT |
| L-glutamine | Wisent | 609-065-EL | 100x | 1x | Stock: -20°C<br>Working: RT |
| Penicillin-Streptomicin | Wisent | 450-200-EL | 100x | 1x | Stock: -20°C<br>Working: RT |
| Primers | Invitrogen | N/A (Custom order) | Variable | 50 µM in DNA grade water | Stock: -20°C<br>Working: RT |

| Item | Supplier | Catalogue # | Stock conc. | Working conc. | Storage temp. |
|---|---|---|---|---|---|
| Q5 DNA Polymerase | New England Biolabs | M0491L | 100x | 1x | Stock: -20°C Working: 4°C |
| Q5 High GC Enhancer | New England Biolabs | M0491L | 5x | 1x | Stock: -20°C Working: RT |
| Q5 Reaction Buffer | New England Biolabs | M0491L | 5x | 1x | Stock: -20°C Working: RT |
| QIAquick PCR Purification Kit | Qiagen | 28104 | N/A | N/A | Varies (each reagent is stored at a different temperature) |
| QIAprep Spin Miniprep Kit | Qiagen | 27106 | N/A | N/A | Varies (each reagent is stored at a different temperature) |
| 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 |
| Tetracycline-Free Fetal Bovine Serum (FBS tet free) | Wisent | 081-150 | 100x | 10x | Stock: -20°C Working: RT |
| 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) |

## 2.5 Equipment

| Item | Supplier | Catalogue # |
|---|---|---|
| Advanced Dry Block Heaters | VWR | 75838-282 |
| Cell culture incubator | ThermoFisher Scientific | MPP50116050 |
| Cell culture water bath | ThermoFisher Scientific | IsoTemp GPD20 |
| Centrifuge | Eppendorf | 022626001 |

| **Item** | **Supplier** | **Catalogue #** |
|---|---|---|
| Digital Graphic Printer | Sony | UPP-110 |
| E-Box cell documentation imaging | Vilber | CXS.TS.26MX |
| Electrophoresis Apparatus | Biorad | 1658025FC |
| FACSAria Fusion cell sorter | BD | 6567OOG5 |
| Light microscope | Motic | AE2000 |
| LP Vortex Mixer | ThermoFisher Scientific | 88880017 |
| Luna-II Automated cell counter | Logos Biosystems | L40002 |
| Nanodrop | ThermoFisher Scientific | ND-ONE-W |
| Power supply | Biorad | 164-5070 |
| SterilGard Biosafety cabinet | Baker | SG303 |
| Shaking incubator New Brunswick™ Innova® 42 | Eppendorf | M1335-0000 |
| SimpliAmp™ Thermal Cycler | ThermoFisher Scientific | A24811 |
| Thermal Cycler | Biorad | 1861096 |

# 3 Protocol

## 3.1 Design of guide RNAs (gRNAs)

### 3.1.1 Websites and computer software used:

- Ensembl website: www.ensembl.org
- Synthego website: https://www.synthego.com
- Benchling website: https://www.benchling.com
- Addgene website: www.addgene.org
- Expasy website: https://web.expasy.org/translate
- Word processing program

### 3.1.2 Guidelines for gRNAs design:

- Use Synthego and/ or Benchling websites for gRNA design
- Choose gRNAs that have the lowest potential off target effect (on the Benchling web site the highest number for off target score indicates the lowest off target potential).
- The gRNAs forming a pair should be at least 100 bp apart from each other (ideal range is between 100 – 200 bp) in order to facilitate genotyping by PCR by yielding a product that can be distinguished from that of the WT when resolved on the agarose gel.
- When selecting gRNAs, it is important to ensure that the target sequences are within one of the initial exons within the gene.

### 3.1.3 Procedure:

1. Using the "Synthego" website, design guide RNAs (gRNAs) 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 (as in **Figure 2**). To visualize all the gRNAs available, click on "all guides" tab.
- Copy transcript ID (see example in **Figure 2**).

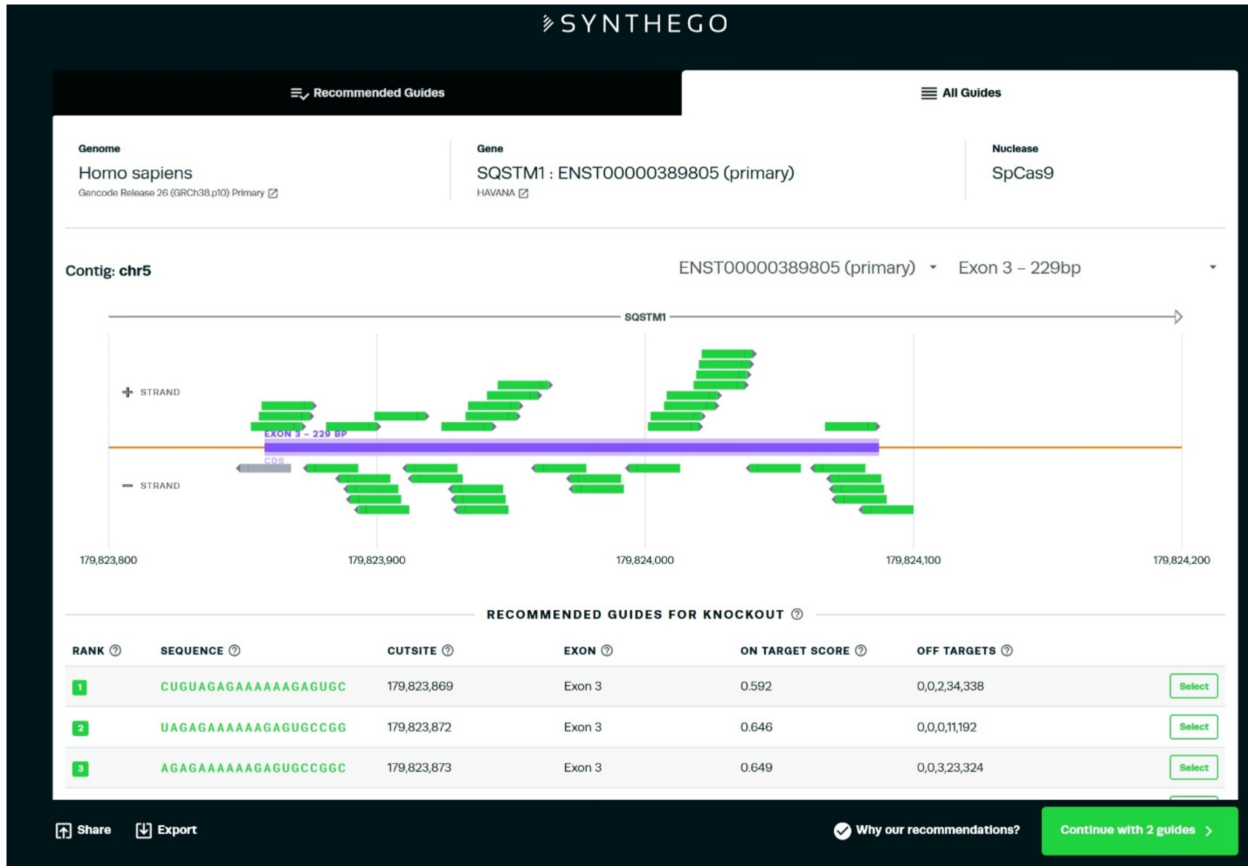



Figure 2: gRNAs designed at Synthego website are shown as green (recommended guides) and grey (other guides) arrows.

2. At “Ensembl” website verify that the gRNAs are designed to target the **early** exon:

- Paste the gene name in the search box and press “go” (see **Figure 3**).

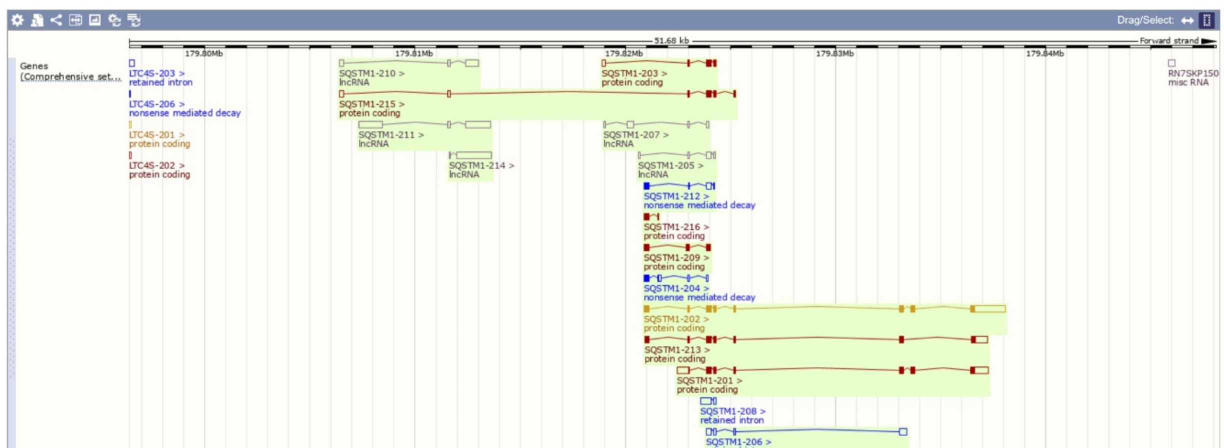



Figure 3: Transcript information of SQSTM1 gene on Ensembl website.

- The gene summary page includes a list of transcripts and only those highlighted in red or brown are protein coding. Choose from the list an early exon that is found in all transcript variants.
- Click on the longest protein-coding transcript.
- On side menu, click “Exons” (under “Sequence”). This will open the sequence map.
- To see the full intronic sequence, click on “Configure this page” on the side menu.
- Select “Show full intronic sequence”.
- Find in the sequence the first exon highlighted in blue (as shown in **Figure 4**). This will be the early exon. Verify which strand is being shown by looking at “Location”, for e.g., Chromosome 5: 179,806398 – 179,838,078 forward strand (which means the “+” strand).
- Verify if the gRNAs available at the Synthego website are designed for the early exon that is common for all the transcripts. If the gRNAs recommended by Synthego are designed against the early exon, go to (**3**), otherwise go to (**5**).

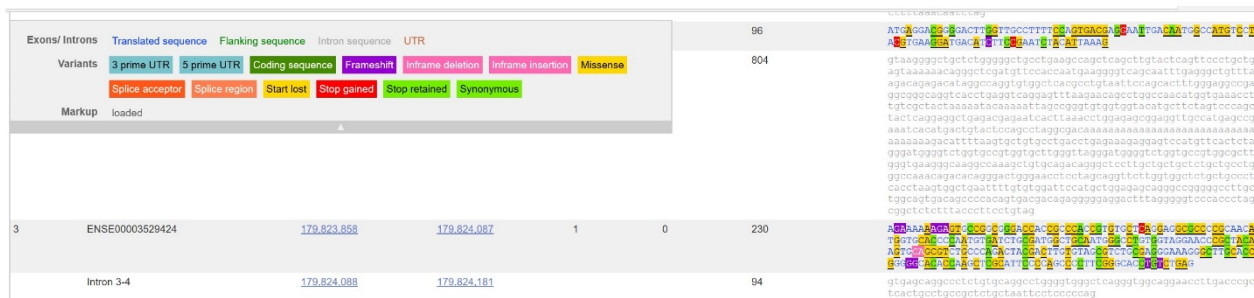
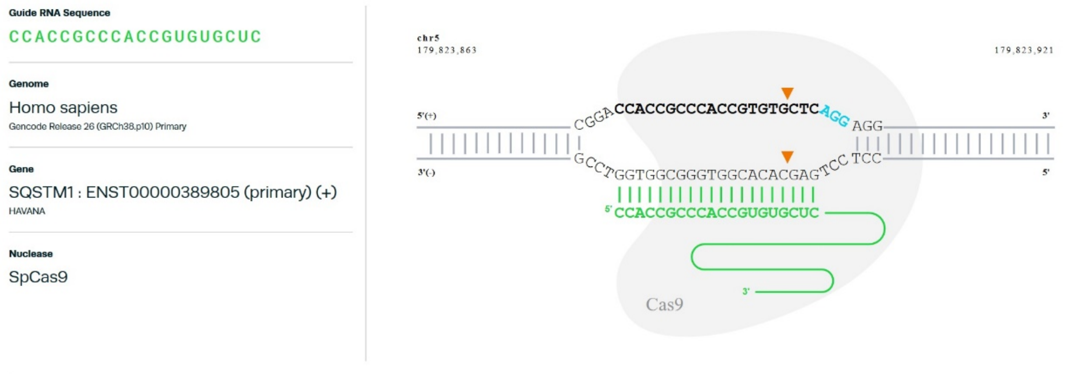



Figure 4: Genomic sequence of exon 3 of SQSTM1 gene. The coding region of the early exon is shown in blue.

3. At “Synthego” website select 3 guide RNAs (gRNAs) to be used in pairs to generate the knockout of the gene of interest:

- Select three gRNAs that can be used to identify the optimal pair of gRNAs that are 100-200 bp apart from each other.
- Find the gRNA target sequence by clicking on the gRNA (see **Figure 5**). To calculate the distance between two gRNAs, subtract the last three digits of the cleavage site of one gRNA from the three digits of the cleavage site of the other gRNA.




This guide will target a sequence in the gene SQSTM1 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 179,823,897.

EARLY CODING REGION | COMMON EXON | HIGH ACTIVITY | MINIMAL OFF TARGETS

**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 guides that have the lowest off-target scores.
- Copy the information about the gRNAs to a text document: it should contain the exon number, the size of the exon on base pairs (bp), the sequence of the gRNAs, the cleavage site and the strand, for example:

| gRNA target sequence | Exon number | Exon size | Cleavage site |
|---|---|---|---|
| CCACCGCCCACCGUGUGCUC | 3 | 229 bp | 179,823,897 |

- Make sure that all gRNAs are designed to target the same exon and will cut within a 100-200 bp distance from each other.
- If there are no gRNAs at Synthego that fit the criteria, design gRNAs (**4**) at Benchling website. Otherwise, go to (**5**).

4. Use “Benchling” website for gRNAs design:

- Sign in to the Benchling website at “Benchling”.
- 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.
- Select gRNAs that have the highest off target score since they have the lowest chance to cause the off target editing (see **Figure 6**).

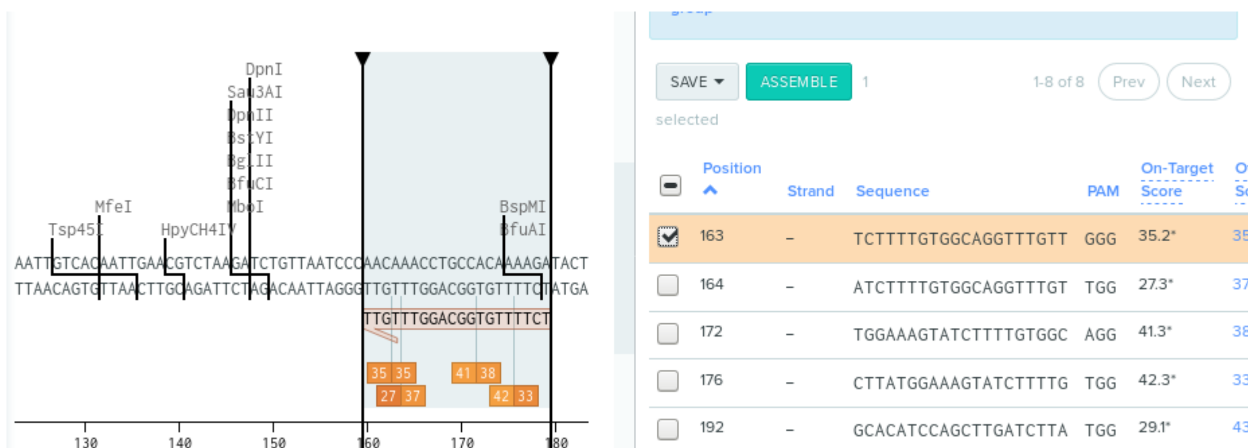


**Figure 6: Selection of gRNAs on Benchling website using off target score.**

- Benchling shows the gRNA target sequence below the template DNA sequence (see **Figure 7**). 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.

5. Paste your exon sequence into a Word processing program and highlight the gRNAs target sequence.

- Highlight the guide target sequence in the exon sequence and proceed to the primer design around this sequence.

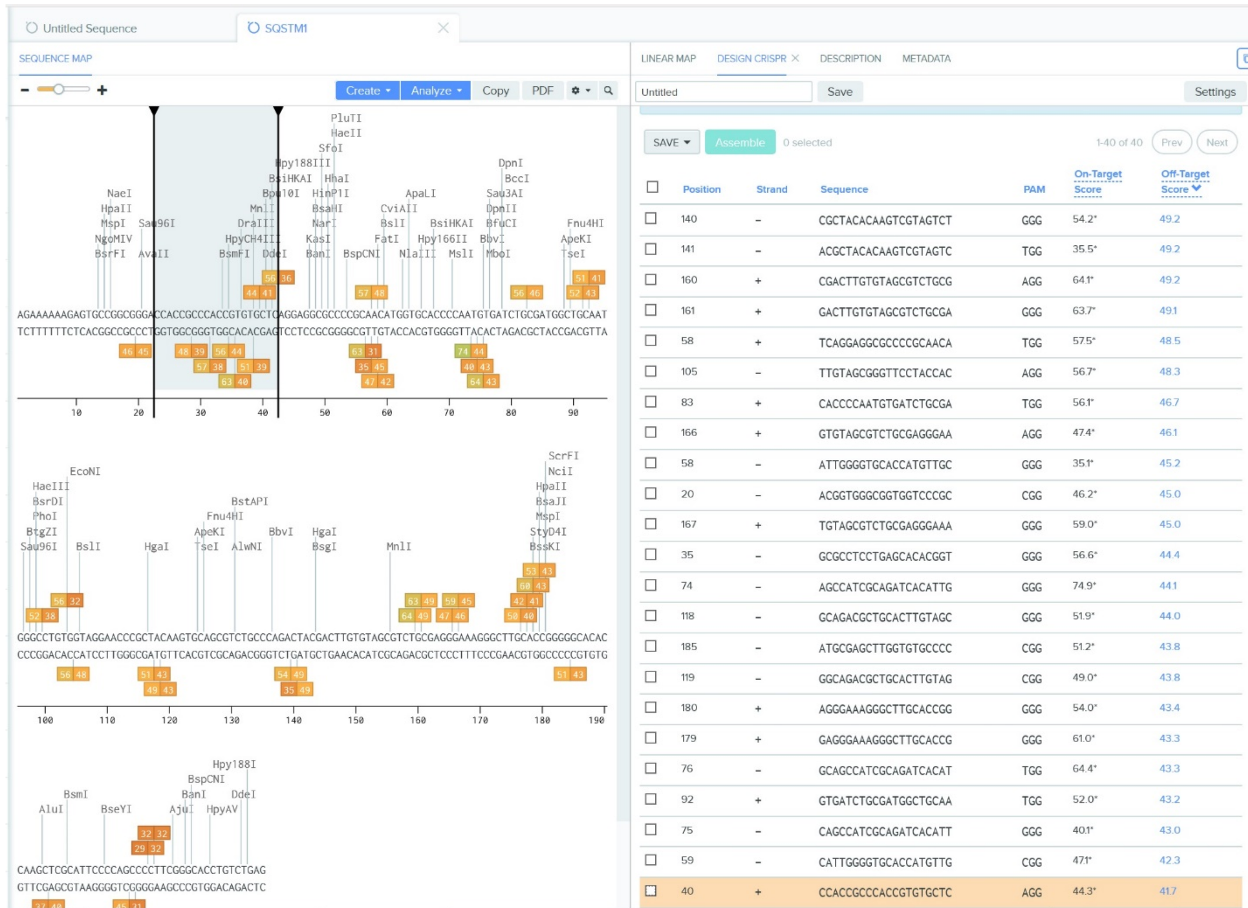


Figure 7: Selected gRNA target sequence shown at Benchling website.

## 3.2 Design of primers

**Website:**

- Primer-BLAST website

### 3.2.1 Guidelines to choosing primer pairs:

In order to assess Cas9 cutting at a specific locus, screening PCR primers are designed outside of the target region to produce a PCR product (amplicon) between 500 to 1000 bp. In order to ensure the detection of a clear single band corresponding to the expected product size, two primer pairs are designed and tested in parental cells in which the gene of interest is to be knocked out.

- Each primer should be a minimum of 150 bp upstream of its closest guide target sequence.
- 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 ideal length.
- GC% of a primer pair should be between 40 and 60, with an optimal of 50% GC.

- The distance between the forward and reverse primers should be 500 – 1000 bp.
- Primers can dimerize at defined melting temperatures, therefore, use the “Oligocalc” website to analyze the primer sequence for potential formation of hairpins and self-complementary.
- Primer melting temperature (Tm) is critical for efficiency of PCR. Optimal Tm of the primers is 60–64°C.The Tm of the two primers should not differ by more than 2°C for both primers to bind simultaneously and efficiently amplify the product during PCR cycles. A Tm calculator can be used to determine the annealing temperature for primers.
- Avoid primers that have unintended targets.

### 3.2.2 Procedure:

- At “Primer-BLAST” website design two pairs of primers (one forward primer and one reverse primer per pair) to generate a PCR product (amplicon) of 500-1000 bp that includes the region of the gRNAs cleavage site:
- Paste your sequence in the required field (including 200 bp upstream of the gRNAs area and 200 bp downstream of the gRNA’s area) under “PCR template” (see **Figure 8**).
- Change “PCR product size” to “500” Min and “1000” Max.
- Set lowest “primer melting temperature (Tm)” to 50°C and highest to 70°C. Make sure the melting temperature of the primer pairs is similar, within 3-5°C degrees, otherwise they will not anneal in the same temperature. Keep the remaining parameters unchanged.

**Figure 8: Example of input of target DNA sequence into Primer-BLAST website with modified parameters**

- After making the adjustments outlined above, press “get primers”.
- Select primer pairs that have:
  - GC content between 40 – 60%
  - Tm around 60–64°C. The Tm of the two primers should be similar (within 2°C)

- Minimal to no non-specific binding

- Go back to the exon sequence and highlight the primers sequence (see example in appendix). The forward primers must be upstream of the first gRNA (150 to 200 bp 5' of the first gRNA) and the reverse primers must be downstream of the last gRNA (150 to 200 bp 3' of the last gRNA).
- Make sure there is minimal to no overlap between primers from different pairs.
- To find the sequence for reverse primers, determine the reverse-complement using the "Bioinformatics".
- Check each primer sequence at "Oligocalc" website for self-annealing properties or potential for hairpin formation by pasting one primer sequence per time and pressing button "Check Self-Complementarity".

## 3.3 Culturing cells

### 3.3.1 Materials:

- Parental cell line
- DMEM
- Tetracycline-free FBS
- Penicillin-Streptomycin
- L-glutamine
- 10 cm cell culture dish
- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tetracycline-free FBS, 2mM L-glutamine, and 100 U/mL Penicillin-Streptomycin (complete DMEM)
- Trypsin
- Sterile PBS
- 15 mL conical sterile tube
- DMSO
- Sterile cryotubes
- Cryobox
- Sterile pipette
- Cell incubator
- Cell culture water bath
- Biosafety cabinet
- Centrifuge

### 3.3.2 Procedure

- Growing cells:
- Cells are cultured in a 10 cm culture dish containing 10 mL of culture media in a 37°C incubator with 5% $CO_2$. Culture media is complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine, and 100 U/mL Penicillin-Streptomycin) for U2Os, HEK293T cells (media may vary if using other cell-lines).
  - **IMPORTANT:** Cells used for this procedure must express Cas9 in the presence of doxycycline, and all examples in this SOP use HEK293T or U2OS cells which were generated to express CAS9 under a tetracycline inducible promoter. Since tetracycline also activates the expression of Cas9 in those cells, tetracycline-free serum is used to avoid Cas9 expression prior to and after transfection with gRNAs.
  - Pass cells once and grow until 70-80% confluence is reached.

### 3.3.3 Passaging cells

- Warm up sterile PBS, trypsin and complete DMEM in the water bath to 37°C.
- Transfer cells from incubator to biosafety cabinet.
- Inside the biosafety cabinet, remove the conditioned media by aspiration.
- Wash cells with 10 mL of sterile PBS and remove PBS by aspiration.

- Add 1.5 mL of trypsin and incubate in the 37°C/5% $CO_2$ incubator for 5-10 minutes.
- Verify under microscope that the cells have detached (see **Figure 9**). If not detached continue the incubation with trypsin for a few more minutes.

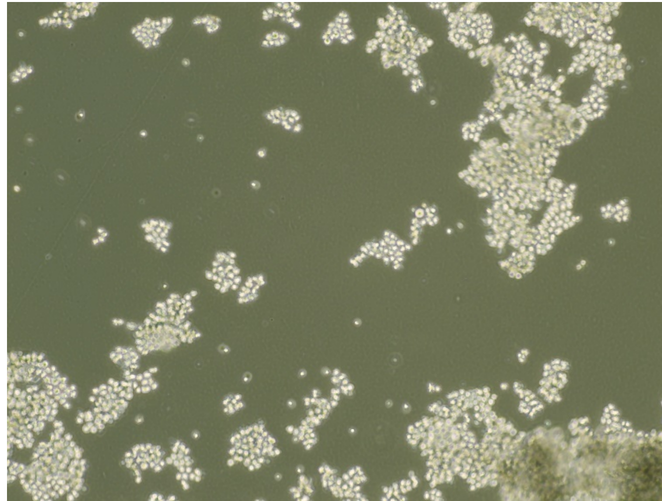


Figure 9: Image of detached HELA cells during trypsinization. 4x magnification.

- Once the cells are detached, 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.
- Place cells in the incubator.
    - Note: If at the time of passage cells are not 70% confluent, it is possible to transfer more than 0.5 mL of cell suspension to a new dish. The dilution of cell suspension in the new media can be up to 1:3.

### 3.3.4 Freezing cells

- Warm up trypsin and complete DMEM in the water bath to 37°C.
- Transfer cells from incubator to biosafety cabinet.
- Inside the biosafety cabinet, remove the conditioned media by aspiration.
- Wash cells with 10 mL of sterile PBS.
- Add 1.5 mL of trypsin and incubate at the 37°C/5% $CO_2$ incubator for 5-10 minutes.
- Verify under a microscope that the cells have detached.
- Once the cells detach, inactivate trypsin by adding 3.5 mL of complete DMEM.
- Pipet a few times up and down.
- Transfer cell suspension to a 15 mL conical tube.
- Centrifuge (spin down) at 1,200 rpm for 3 min.
- Aspirate supernatant.
- Re-suspend cell pellet in 10 mL of PBS to wash cells.
- Spin down at 1,200 rpm for 3 min.
- Aspirate supernatant.

- Re-suspend cell pellet in 10 mL freezing media (tetracycline-free FBS with 10% DMSO) as following: First re-suspend cells in 5 mL of tetracycline-free FBS and then add 5 mL of FBS tetracycline-free supplemented with 20% DMSO to yield a final DMSO percentage of 10%.
- Aliquot 1 mL of the cell suspension per cryotube.
- Store cryotubes at -80°C overnight inside a cryobox.
- Transfer cryotubes to liquid nitrogen tank.

### 3.3.5 Thawing cells

- Transfer cryotube with cells from liquid nitrogen to water bath at 37°C and thaw until the contents of cryotube are approximately 90% liquid.
- Inside the biosafety cabinet, transfer the cell suspension from the cryotube to a 15 mL sterile conical tube containing 4 mL of complete DMEM.
- Spin down at 1,200 rpm for 3 min.
- Remove supernatant by aspiration.
- Re-suspend pellet in 10 mL of complete DMEM.
- Transfer cell suspension to a 10 cm culture dish.
- Place cells inside incubator.
    - Allow cells to recover for at least one week before using them.

## 3.4 Extraction of genomic DNA

### 3.4.1 Materials:

- DMEM
- Tetracycline-free FBS
- Penicillin-Streptomycin
- L-glutamine
- QuickExtract™ DNA Extraction Solution (QE)
- Parental cell line
- P200 sterile tip
- Dry block heater
- 1.5 mL sterile conical tube

### 3.4.2 Procedure:

- To extract DNA from parental cell line using QE, grow parental cells (WT) until 70-100% confluent using complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine, 100 U/mL Penicillin-Streptomycin.
    - **IMPORTANT:** As cells used for this procedure express Cas9 in the presence of doxycycline, tetracycline-free serum is used to avoid Cas9 activity prior to and after transfection with gRNAs.

- Always work in a clean area. Use nuclease-free tubes, gloves and filter tips to avoid contamination of gRNAs and primers.

- Remove media and add Quick Extract (QE) for DNA extraction (50 µL per well of a 96-well plate or 100 µL to a well of a 24-well plate.
- Incubate with QE for 2 minutes and mix volume by pipetting up and down a few times.
- Scrape the cells using a P200 sterile tip.
- Transfer the cell suspension to a 1.5 mL sterile conical tube.
- Transfer the tube to a block heater previously warmed to 65°C and incubate for 5 minutes at 65°C.
- Incubate for 5 min at 95°C to inactivate QE reaction. If using the same block heater with tubes incubated at 65°C, it is possible to set the temperature to 95°C and leave the tubes in the block heater for 20 minutes allowing for a 15 min temperature ramp from 65°C - 95°C and the 5 min inactivation at 95°C.
- Remove tubes from block heater and let them cool down at RT or on ice for 5 minutes.
- Prepare one aliquot per condition of QE of a 1 in 10 dilution in DNase-free water.
- Centrifuge the tubes briefly.
- Tubes can be stored at -20°C for at least 2 years or at 4°C for several months.

## 3.5 Validation of primers

### 3.5.1 Materials:

- 2 pairs of primers
- Parental cell line DNA
- Q5 Reaction Buffer
- Q5 DNA Polymerase
- Q5 High GC Enhancer
- dNTPs 2.5 mM
- Fluo-DNA loading Buffer
- 100 bp DNA Ladder RTU
- Agarose
- TBE buffer 0.5x
- MicroAmp® Optical 8-Tube Strip
- PCR caps
- PCR Thermal Cycler
- Vortex
- Ebox cell documentation imaging
- Digital Graphic Printer
- Thermal Cycler (BioRad)
- Power supply
- Electrophoresis apparatus

### 3.5.2 Procedure:

- To determine the best annealing temperature for each primer pair and to validate primers in a PCR reaction containing template DNA extracted from parental cell line, thaw all reagents listed **Table 1**, except for Q5 DNA Polymerase.
  - **IMPORTANT:** Vortex reagents briefly and then centrifuge briefly to collect all the liquid at the bottom of each tube.

**Table 1: Suggested volume of reagents required for the preparation of one PCR reaction.**

| Reagent | Volume for one standard reaction (50 µL total) |
|---|---|
| | Volume per reaction (µL) |
| Template DNA (QE DNA) 1:10 dilution | 3 |
| Primer F (50 µM) | 1 |
| Primer R (50 µM) | 1 |
| DNase/RNase-free distilled water | 19.5 |
| Q5 Reaction Buffer 5x | 10 |
| Q5 High GC Enhancer 5x | 10 |
| dNTPs 2.5 mM | 5 |
| Q5 DNA Polymerase | 0.5 |
| Total volume | 50 |

- Considering the number of PCR reactions needed, prepare an appropriate amount of mastermix composed of all the reagents listed with the exception of template DNA. Refer to the values in **Table 1**. If preparing mastermix for eight reactions, for example, prepare mastermix for 9 reactions to avoid running out of mastermix.
  - **IMPORTANT:** Make sure to include a blank (composed of the mastermix only without template DNA) for each primer pair, as a control for contamination of primers with

ambient DNA. When preparing the blank, use DNAse-free water instead of template DNA.

- Mix the components on ice and add the Q5 DNA Polymerase last.
  - **IMPORTANT:** Q5 DNA polymerase must be kept at -20°C until the moment of use. When ready to use, transfer this reagent to ice and return it to the freezer right after using it.
  - In this protocol we use QE to purify genomic DNA and Q5 DNA polymerase for PCR reaction since this polymerase works well with DNA extracted by QE. Other DNA polymerases do not work with QE DNA. Therefore, a more thorough genomic DNA purification method is needed to yield a successful PCR with alternative DNA polymerases.
- At the Thermal Cycler, choose the parameters listed in **Table 2** except for the annealing temperature. In order to determine the best annealing temperature for each primer pair, run a temperature gradient. An example of a temperature gradient is illustrated in **Table 3**.

**Table 2: Program settings for PCR reaction.**

<table>
<tr><th>Step</th><th colspan="2">Description</th><th>Temperature (°C)</th><th>Time</th></tr>
<tr><td>1</td><td colspan="2">Denaturation</td><td>95</td><td>4 min</td></tr>
<tr><td rowspan="3">2</td><td rowspan="3">Amplification<br>(35 cycles)</td><td>Denaturation</td><td>95</td><td>30 sec</td></tr>
<tr><td>Annealing</td><td>63</td><td>30 sec</td></tr>
<tr><td>Elongation</td><td>72</td><td>10 min</td></tr>
<tr><td>3</td><td colspan="2">Hold</td><td>4</td><td>∞</td></tr>
</table>

**Note:** Annealing temperature in most cases is 63°C but can vary

**Table 3: Example of settings for temperature gradient parameters on a Biorad thermocycler.**

| Location of sample in a PCR instrument | Annealing Temperature (°C) |
|---|---|
| A | 65 |
| B | 64.3 |

| | |
|---|---|
| C | 63.1 |
| D | 61.3 |
| E | 59 |
| F | 57.3 |
| G | 56 |
| H | 55 |

- Prepare 10x TBE buffer: 1 M Tris base/ 1 M boric acid/ 0.02 M EDTA in distilled water (pH 8.3). Dilute TBE to 0.5x in distilled water.
- To resolve PCR products on agarose gel, prepare agarose gel in 0.5x TBE buffer, pour gel and let it solidify for 30 minutes.
    - **IMPORTANT:** The percentage of agarose to be used depends on the amplicon size. Consult the following link for information on the recommended percentage of agarose: Promega website.
- Prepare samples, mix 10 µL of PCR product with 2 µL of 6x Fluo-DNA loading buffer.
- Prepare the DNA ladder mix by adding 2 µL of 6x Fluo-DNA loading buffer to 10 µL of 100 bp DNA ladder. A good specific pair of primers at the optimal annealing temperature should yield a single PCR product at the expected size. Blank controls should not yield a PCR product unless there contaminating DNA is in the reaction.
- Transfer the gel to a horizontal electrophoresis cell (Biorad) containing 0.5x TBE buffer, remove comb carefully and load the samples on the gel.
- Run gel for about 1 to 2 hours at 90 V.
- To visualize the resolved DNA bands, transfer gel to the Ebox cell documentation imaging system and take a picture of the gel. Gel run is considered complete when the DNA bands of the analyzed sample are well separated by their length.
- Determine the optimal annealing temperature for each primer pair by looking for a single band at the expected size on the gel picture (as shown in **Figure 10**).
    - **IMPORTANT:** Good primer pairs at the optimal annealing temperature yield a single PCR product at the expected size. Blank controls should not yield a PCR product unless there is contamination with DNA. For example, for primer pairs tested to amplify fragment of UBQLN2 gene in Figure 9A, pair 1 appeared to be the optimal one at 63°C annealing temperature since single, thick PCR product at the expected size of 540 bp was seen. At lower temperatures, additional bands were seen indicating a non-specific binding of the primers to other DNA regions. In case of primer pair 2, the non-

specific bands appeared at all tested temperatures. The blank appeared clean, with no PCR product (**Figure 10B**) demonstrating a lack of DNA contamination.

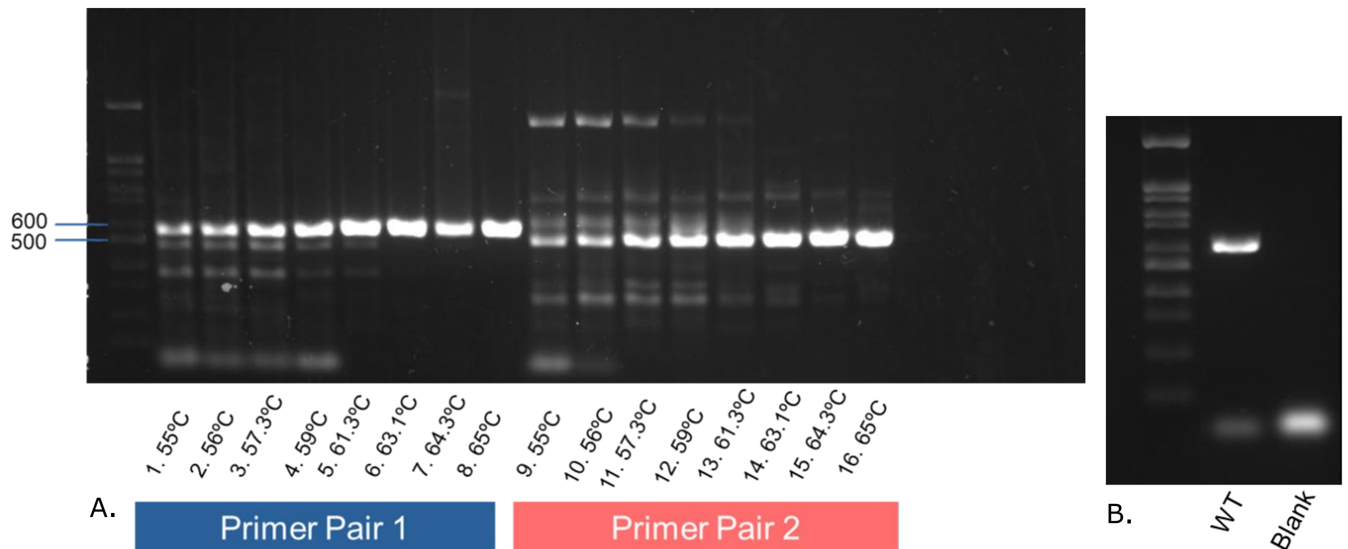



Figure 10: A. Agarose gel with PCR products of UBQLN2 gene using WT genomic DNA and 2 pairs of primers (pair 1: UBQLN2F GGGAACCGCAGTCTTCATCA and UBQLN2R GATTGTGATGGAGCCCGCAGC; pair2: UBQLN2F2 TCACAGAGGTACCGTGCTCC and UBQLN2R2 GGGAACTAACACTACCTCGGC at various annealing temperatures. B. Agarose gel with PCR products using pair 1 of primers to amplify UBQLN2 gene fragment at 63°C annealing temperature.

## 3.6 Transfection of cells with gRNAs

### 3.6.1 Materials:

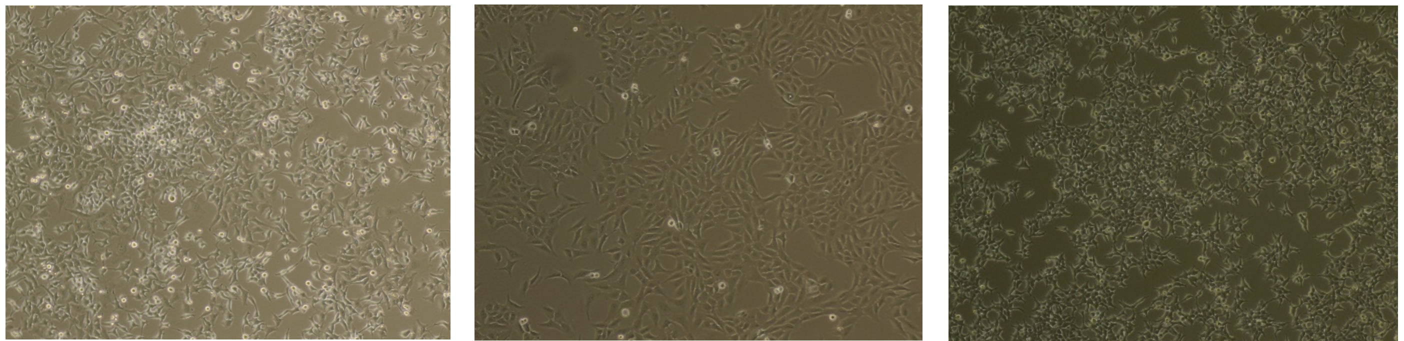
- Parental cells - use cells with low passage number as high passage number can negatively affect transfection efficiency and gene expression
- 10 cm cell culture dish
- gRNAs
- RNase free water
- HiPerFect transfection reagent
- Dulbecco's Modified Eagle's Medium (DMEM) without supplements
- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine and 100 U/mL Penicillin-Streptomycin (complete DMEM)
- Doxycycline (2 µg/mL final concentration)
- Trypsin
- Sterile PBS
- Trypan blue
- 15 mL conical sterile tube
- 1.5 mL conical sterile tube
- Luna cell counter slide
- Luna-II Automated cell counter.

### 3.6.2 Procedure:

- Grow parental cells (WT) in culture in complete DMEM in a 10 cm cell culture dish.

***Day 1***

- Once cells attain 50% confluence as shown in **Figure 11**, add 2 µg/mL doxycycline to cell media 48 hours before the transfection to activate the expression of Cas9.
  - **IMPORTANT**: Doxycycline is sensitive to light and temperature. Keep stock in freezer protected from light and prepare media with doxycycline fresh each time right before use.
  -



A. B. C.

Figure 11: Image of ~50%-60% confluent cells A. HELA, B. U2OS, C. HEK293T. 10x magnification. Images taken with EVOS XL Core microscope.

- To transfect cells with gRNAs, prepare gRNAs by re-suspending dry synthetic gRNAs to a concentration of 3 µM by adding 500 µL of RNase-free water per tube (RNase-free water is supplied along with the gRNAs).
- Set up reverse double transfection with two different combinations of gRNAs. Refer to **Table 4** for the experimental design.
- Each condition is done is duplicate (two wells/condition). One well will be used for DNA extraction and genotyping by PCR to check for the efficiency of transfection and the other well will be used for single cell FACs sorting to isolate single cell KO clones.
- Pipette the gRNAs into the corresponding wells, according to **Tables 4** and **5**.
- Add HiPerFect mix to each well (HiPerFect reagent with serum free media), except for the wells of control group, according to **Table 5**.
- Incubate the reagents for 10 minutes at room temperature to allow for gRNA-transfection reagent complex formation.
- While reagents are incubating, prepare DMEM with doxycycline and remove conditioned media from 10 cm dish by aspiration.
- Wash cells with 10 mL of sterile PBS.
- Remove PBS by aspiration.

- Add 1.5 mL of trypsin to the cells and incubate for 5-10 min at 37°C.
- Verify under microscope if cells have detached (See **Figure 11**).
- Once cells have detached, stop trypsinization by adding 3.5 mL of complete DMEM supplemented with doxycycline and mix by pipetting gently up and down 3 times with 5 mL pipette.
- Transfer cells to a 15 mL sterile conical tube.
- Count cells using Luna Cell Counter: In a sterile 1.5 mL conical tube, mix 20 µL of cell suspension with 20 µL of Trypan Blue. Pipette 10 µL of the mix into each side of the Luna cell counter slide (or other cell counter slide depending on one available). Count cells using Luna-II Automated cell counter.
- Follow the recommended values on **Table 5** to calculate the number of cells to plate per well. If needed, dilute the cells in complete DMEM with doxycycline.
- Plate the cells.
- Incubate transfected cells overnight at 37°C in the incubator with 5% $CO_2$.

***Day 4***

- Change media the next day to complete DMEM supplemented with DOX (2 µg/mL).
- Incubate the cells for an additional 24h.

***Day 5***

- Change media to complete DMEM without doxycycline.
- Grow cells until they are 70-80% confluent and proceed to the next step.

**Table 4: Recommended groups for a standard transfection.**

| **Group** | **Components** |
| --- | --- |
| Empty transfection | HiPerFect mix + cells |
| Control | Cells only |
| Transfection pair A | HiPerFect mix + cells + gRNA pair A |
| Transfection pair B | HiPerFect mix + cells + gRNA pair B |

**Table 5: Recommended amounts for a standard transfection.**

| Components | Amount per well of a 24-well plate | Amount per well of a 96-well plate |
|---|---|---|
| gRNA at 3 µM | 14 µL of each gRNA | 2.8 µL of each gRNA |
| HiPerFect reagent | 3.75 µL | 0.75 µL |
| Serum free media | 121.25 µL | 24.25 µL |
| Cells | 25,000-40,000 | 10,000 |

## 3.7 Guide RNA validation by screening transfected cells using QE and PCR

### 3.7.1 Materials:

- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tetracycline-free FBS, 2mM L-glutamine and 100 U/mL Penicillin-Streptomycin (complete DMEM)
- Two pairs of primers
- Quick Extract (QE) DNA extraction solution
- Parental cell line
- P200 sterile tip
- Dry block heater
- 1.5 mL conical sterile tube
- Q5 Reaction Buffer
- Q5 DNA Polymerase
- Q5 High GC Enhancer
- dNTPs 2.5 mM
- Fluo-DNA loading Buffer
- 100 bp DNA Ladder RTU
- Agarose
- TBE buffer
- MicroAmp® Optical 8-Tube Strip
- PCR caps
- PCR Thermal Cycler
- Vortex

### 3.7.2 Procedure:

- Perform DNA extraction for each group of transfected cells from **Section 3.5** (**Table 4**) using QE as outlined in **Section 3.4**.
- Perform PCR on the extracted DNA using validated primers as outlined in **Section 3.5**. Include a blank (master mix only, without template DNA).
- Resolve PCR products on agarose gel in TBE buffer as described in **Section 3.5**.
- When the transfection is efficient and both guides cause cleavage at their target DNA sites, a polyclonal cell population consisting of KO cells and the isogenic control (WT) is generated. The KO cells possess a shorter gene sequence (deleted version) while WT has a full-length sequence. In this case, PCR of DNA extracted from the mixed KO and WT cell population yield 2 PCR products, that is, 2 bands on a gel (**Figure 12A, Lane 1 and 3**). The efficiency of the transfection and gene editing can be estimated by looking at the intensity of the shorter and the full-length bands. When the shorter band is much thicker and more intense than the full-length band it indicates that gene cleavage and editing was efficient. In case of non-efficient or partial cleavage (when only one guide resulted in a cut), the PCR product size will correspond to the full-length WT gene sequence (**Figure 12A Lane 2**).
- An example of efficient gene-editing using a pair of gRNAs that target the SQSTM1 gene is illustrated in **Figure 12B**. The thick shorter band and the faint full-length band of post transfected cells indicate highly efficient transfection and double cleavage. An example of inefficient gene-editing is shown in **Figure 12C**.

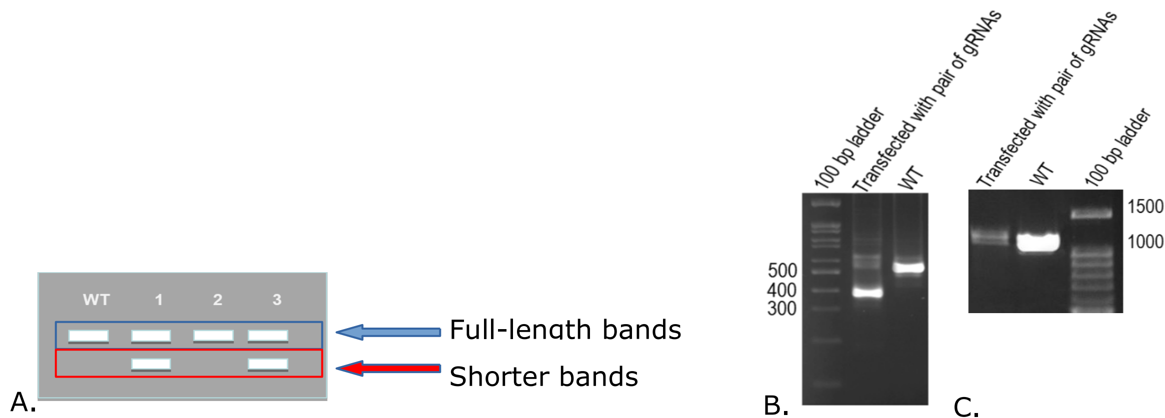



**Figure 12: A. Illustration of an agarose gel with PCR products from genomic DNA extracted from polyclonal population of isogenic and KO cells. B. Validation of cleavage efficiency of pair of guides that target SQSTM1 (p62) gene by resolving PCR products on agarose gel. C. Validation of cleavage efficiency for a pair of guides that target VCP gene.**

- If transfection and cleavage with both gRNAs was efficient, expand the cells by passing them gradually to a larger dish in order to prepare for isolation of individual cells from the KO plate by single cell sorting. From a well on 96-well plate, pass cells to a 24-well plate, from 24-well plate pass to a 6-well plate. Each well of a 6 well-plate will then be passed to one 10 cm dish. If needed, it is possible to freeze cells at this step when they reach 70-90% confluence.

## 3.8 Selection and enrichment for KO cells by single cell sorting

### 3.8.1 Materials:

- Dish with 70-90% confluent cells
- BD FACSDiva software
- BD FACSAria Fusion (Cell sorter)
- 30 µm sample filter
- 10% bleach
- 1.5 mL FACS tube
- Sterile FACS 5 mL tube with cap
- 96-well collection plates with border well around the plate (Eppendorf)
- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine and 100 U/mL Penicillin-Streptomycin (complete DMEM)
- Trypsin
- Pipet
- Sterile pipet tips
- Sterile PBS
- Trypan blue
- 15 mL conical sterile tube
- 1.5 mL conical sterile tube
- Luna cell counter slide
- Luna-II Automated cell counter

### 3.8.2 Procedure:

- Perform quality control of the equipment.
  - **IMPORTANT**: Cell sorting creates aerosols that may pose a risk to the operator and others in the facility. An aerosol manager system must be turned on to minimize risk.
  - **IMPORTANT:** A cell sorter (FACSAria Fusion is sorter used for this SOP) is used for this procedure. Before starting the procedure, make sure that the sorter is operational and that daily quality controls are done. Quality control of the equipment is performed daily as described in manufacturer user manual. Experiments should be performed by an experienced FACs operator. Our sorting proceeds using the following steps.
- To setup the cell sorter, open the browser window of the FACSDiva software and create a new folder. Rename the folder with the username.
- Inside the selected folder, create a new experimental layout and rename it with the cell line used for the transfection.
- Create a new specimen list within the experimental layout and rename it with the date of the experiment (e.g. 2019-06-13).
- Create a new sample tube within the specimen list and rename it as follows: *unstained_gene name_gRNA used* (i.e unstained_VCP_271-346) (See **Figure 13**).

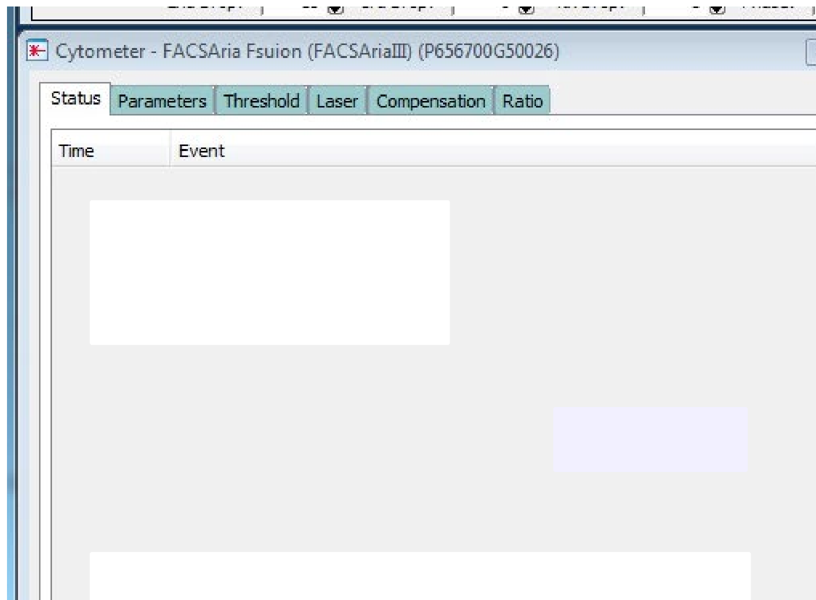



Figure 13: Experiment hierarchy in BD FACSDiva software.

- Select the desired cytometer settings for the experiment (if already available in the database). *All cell lines used are in the software database.*
- Apply the appropriate gating template for the cell line used.
- Go to the “Sort menu” and open a new sort layout. Select the “single cell sorting” protocol.
- In the same menu, select open “Custom devices”.
- Verify that the sorting stream (the far-left stream value is usually at 75) falls in the center of the sorting device. Make adjustments if needed.
- Open the device tray.
- Place a blank 96-well plate with lid on the device tray.
- Go to the “Custom devices” menu and select “Go to home”. Rapidly open and close the sorting stream to see a small droplet on the lid of the 96-well plate. Make sure the droplet falls in the centre of the well.
- Select “Furthest from home” in “custom devices” menu. Rapidly open and close the sorting stream to see a small droplet on the lid of the 96-well plate. Make sure the droplet falls in the center of the well. The plate is ready.
- Select eject button to remove the 96-well plate from the device.
- After setting up the equipment and under sterile conditions, prepare the appropriate number of 96-well plates to receive single cell clones by adding 200 µL complete DMEM per well.
  - **IMPORTANT:** When choosing how many plates to prepare, take in consideration that the recovery rate (the percentage of wells that have only one cell) is about 30-50%.
- Place the plates in the 37°C/5% $CO_2$ incubator until ready to sort the cells.
- Prepare a single cell suspension of a minimum of 0.5 million cells in at least 1 mL of complete DMEM following the recommended amounts on **Table 6**:

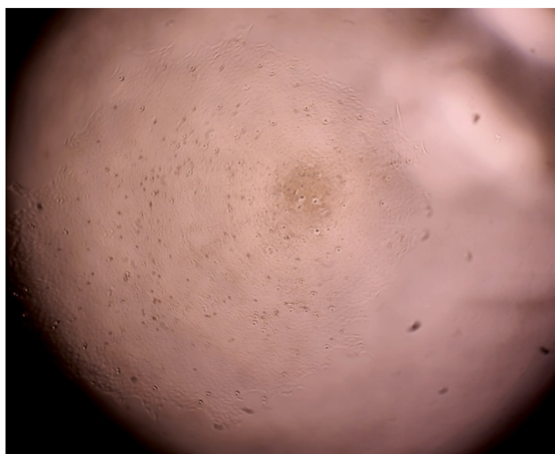
**Table 6: Recommended cell dilution for single cell sorting.**

| Dish/plate size | Trypsin volume | Complete DMEM volume | Volume for single cell sorting |
|---|---|---|---|
| 10 cm dish | 1.5 mL | 3.5 mL | 1mL |
| 6-well plate | 700 µL | 2 mL | 1mL |
| 24-well plate | 100 µL | 400 µL | 1mL |
| 96-well plate | 50 µL | 150 µL | 1mL |

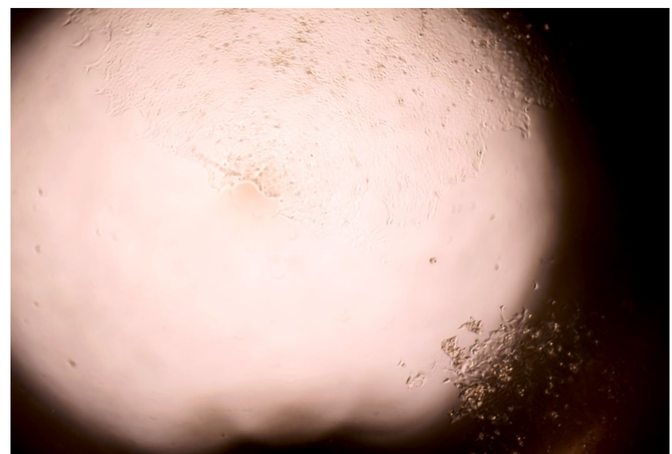
- Trypsinize a 70-90% confluent well of a 6-well dish, or a 10-cm dish, or several wells on a 24-well plate (for details, see **Section 3.5**)
- Re-suspend in complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% tetracycline free FBS, 2 mM L-glutamine, 100 U/mL Penicillin-Streptomycin) and pipet up and down to detach the cells.
- Count cells using Luna Cell Counter and Trypan Blue (see **Section 3.5** for details).
- Spin down the cells and re-suspend pelleted cells in appropriate amount of media in order to have a minimum of 0.5 million cells in at least 1 mL of suspension.
  - Depending on the cell type or cell line being sorted, cells may need to be filtered through a 30-µm filter. Filtering the cells greatly reduces the risk of clogging the cell sorter.
- Transfer cells to a sterile 5 mL FACS tube with cap and bring FACs tube to the sorter.
- To sort single cell/well using the cell sorter select the “100 µm/20psi” option for Nozzle Tip and Pressure (conditions may vary for different systems).
- Select “96-Well Plate” for collection device.
- Select “Room Temp” for Sort Temperature (inside the sample chamber).
- Acquire 50,000 cells to check for instrument settings.
- When settings are correct, retrieve one plate from the 37°C incubator to start the sorting process.
  - Sorting a 96-well plate takes about 5 minutes. Only bring the plates when ready to sort.
- Load the 96-well plate (with the lid on) onto the sorting device.
- Select “Go to home” in the sorting menu to place the sorting stream at the A1 position on the plate. Perform a test sort to make sure the stream falls in the center of the well.
- Select “Go to Last” in the sorting menu to place the sorting stream at the H12 position on the plate. Perform a test sort to make sure the stream falls in the center of the well.
- When all settings are correct, remove the plate lid.
- Re-position the plate at A1.

- Load the sample.
- Start the sorting by pressing the "sort" button.
- When the sorting is done, replace the lid on the plate.
- Remove plate from sorting device.
- Immediately replace the plate in a 37°C/5% $CO_2$ incubator.
- Repeat all previous steps until all plates and samples are done. It is recommended to perform an extra wash step with 10% bleach and $ddH_2O$ between the sorting of different samples.
- After the cell sorting is done, fill the border well of each 96-well plate with 4 mL of media to avoid evaporation of liquid from wells.
- Immediately return all the plates to the 37°C/5% $CO_2$ incubator.
    - **IMPORTANT:** Start tracking the sorted cells 6 days after sorting. At this time, colonies are usually big enough to be visualized under a regular cell culture brightfield microscope. Since colonies will grow bigger with time, as cells multiply, there is a chance that two or more colonies can merge. Therefore, it is important to start monitoring the colonies early, when they are relatively small, to accurately identify wells with a single colony/well. Only cells derived from a single colony will be used for clonal selection. To monitor colonies, check each well under the microscope and mark the colonies as follows:

- Circle in blue the wells that have a small single colony
- Circle in red the wells that have a big single colony
- Mark with an X the wells that have no colonies or more than one colony.
    - **IMPORTANT:** Two to three weeks after sorting, single large colonies (see **Figure 14**) start to form. It is important to note that some colonies will grow slower than others. Once there are about 20 single large colonies on a plate, the cells are ready for the next step, when they will be split into 2x96-well plates to generate duplicates of each clone (a colony derived from a single cell).



A.



B.

Figure 14: A. Image of a well of 96 well plate with one big colony derived from single U2OS cell. B. Image of two colonies in one well of 96 well plate. Images taken with EVOS XL Core microscope.

## 3.9 Selection of single KO clones

### 3.9.1 Materials:

- Multichannel pipette P200
- P200 pipette
- Freezing media (tetracycline-free FBS with 20% DMSO)
- Quick Extract (QE) solution
- Validated primers
- PCR machine
- Agarose
- TBE buffer
- P200 sterile tips
- Dry block heater
- 1.5 mL conical sterile tube
- Q5 Reaction Buffer
- Q5 DNA Polymerase
- Q5 High GC Enhancer
- dNTPs 2.5 mM
- Fluo-DNA loading Buffer
- 100 bp DNA Ladder RTU
- MicroAmp® Optical 8-Tube Strip
- PCR caps
- PCR Thermal Cycler
- Vortex
- 96-well plates
- Complete DMEM
- Sterile PBS
- Trypsin
- Bubble wrap
- Styrofoam box
- Parafilm

### 3.9.2 Procedure:

- Fill receiving plates (2 plates per one original 96-well plate) with 175 µL of complete DMEM/well.
- Remove media from wells to be split (wells with single colonies).
- Wash with 200 µL PBS/well.
- Trypsinize cells: add 50 µL of Trypsin/well and incubate for 5-10 minutes in 37°C/5% $CO_2$ incubator to detach cells. DO NOT aspirate trypsin.

- One well at a time, mix the trypsinized cells by pipetting up and down and transfer about 50% of cells (25 µL) to **an identically numbered well** (for example, from well A1 – column A, row 1 – in the original plate to well A1 in the receiving plate) on the first 96-well receiving plate.
  - The cells on this plate will be expanded and stored.
- Take the remaining 50% (25 µL) of cells and transfer to **an identically numbered well** on the second 96-well receiving plate.
  - The cells on this plate will be used for screening.
- Grow cells on the duplicate plates until they are 70%-90% confluent (this step usually takes between 2-8 days).
- When cells are 70%-90% confluent on the screening plate, proceed to screen the colonies by first extracting genomic DNA from each well using Quick Extract (QE). See **Figure 15** for the desired cell confluency.

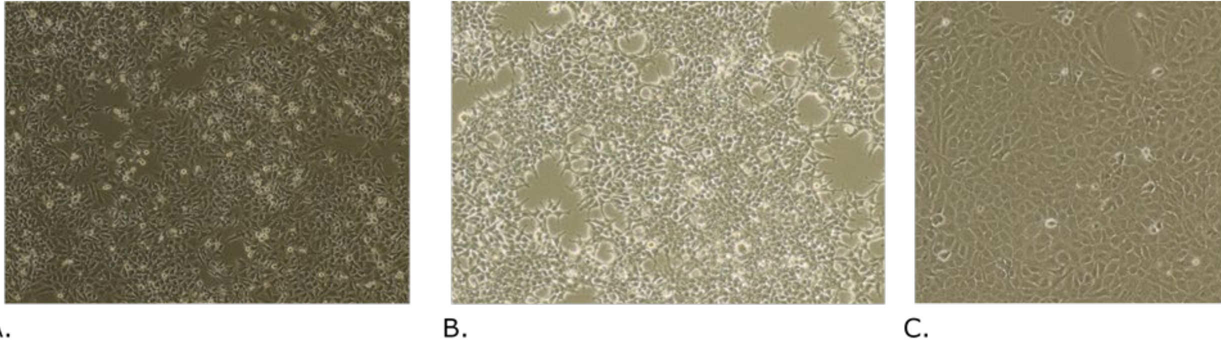



Figure 15: Image of 90% confluent cells. A. HELA cells on 96 well plate, 4x magnification. B. HEK293T cells, 10x magnification C. U2OS cells, 10x magnification. Images taken with EVOS XL Core microscope.

- Analyze clones by PCR using appropriate validated primers and Q5 DNA Polymerase.
  - For QE and PCR follow procedures in **Sections 3.3** and **3.4**.
- Resolve PCR products on agarose gel to identify KO clones.
- PCR products of KO clones will resolve on a gel as a single band with a size smaller than the WT (See **Figure 16A**. Lane 1 and B. Clones 1B2, 1C4 and 1C11).

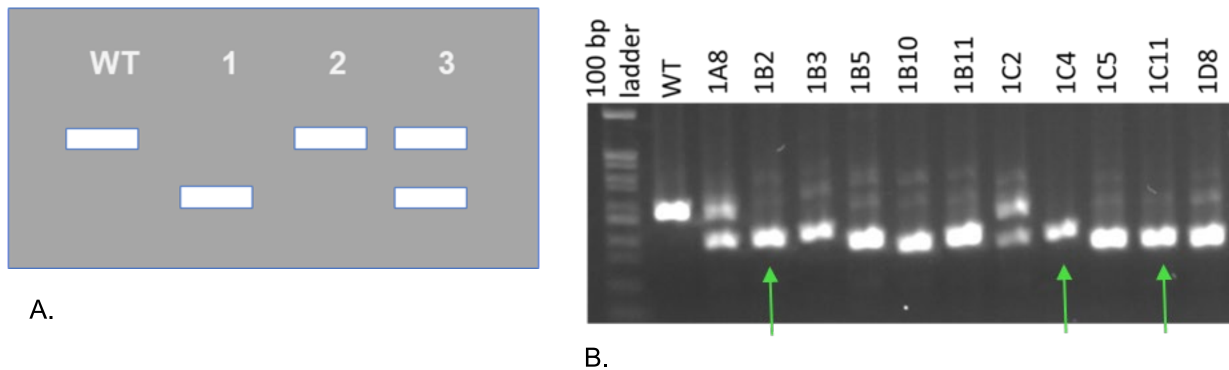



**Figure 16: A. Illustration of agarose gel with PCR products from genomic DNA extracted from single sorted clones. B. Gel with PCR products from single clones of CRISPR edited cells for SQSTM1 gene KO.**

- To freeze the cells, remove conditioned media by aspiration and wash cells with 100 µL PBS/well.
  - To facilitate genotyping by PCR in a timely manner, it is convenient to freeze down the whole 96-well plate with clones dedicated to cell growth and to do expansion later. Cells must be 70-90% confluent.
- Trypsinize the cells by adding 50 µL of trypsin/well and incubating cells for 5-10 min at 37°C/5% $CO_2$ incubator to detach cells.
  - **IMPORTANT: DO NOT** aspirate trypsin.
- Add freezing media to the wells on the plate: 50 µL/well.
- Cover the plate, seal the plate cover with parafilm and cover the whole plate in bubble wrap.
- Transfer wrapped plates to a Styrofoam box and store in -80°C freezer. Cells can be kept at -80°C for about 6 months.
- When PCR analysis of the clones in the screening plate(s) identifies specific clones in which the gene appeared to be knocked out (as shown in **Figure 16**), thaw the frozen 96-well plate and expand the appropriate wells with clones of interest. Prepare a 24-well receiving plate by adding 2 mL of complete DMEM/well and place plate at 37°C/5% $CO_2$ incubator.
- Thaw cells by placing the frozen 96-well plate into the 37°C/5% $CO_2$ incubator for 10-15 min.
- Transfer 100 µL of cell suspension/well from the thawed plate onto one well of 24 well plate filled with 2 mL warm media.
  - **IMPORTANT:** Transfer only KO clones.
  - **IMPORTANT:** Make sure to note the location of each clone in the new plate.
- Place cells back in the incubator and change the media the next day.
- Expand the KO clones with gradual passages: when cells in a well reach 60-90% confluence, transfer them to a larger dish (from 24-well to 6-well plate and from there to a 10-cm dish).
- Additionally, grow cells of these clones for genotyping by PCR and sequencing to ensure that the cells are indeed from a gene-deleted clone.

- Freeze expanded cells as described in **Section 3.3**.

## 3.10 Sequence validation of KO clones

### 3.10.1 Website:

- Expasy website

### 3.10.2 Materials:

- Qiagen PCR extraction kit
- Qiagen miniprep kit
- Zero Blunt TOPO PCR cloning kit (Invitrogen)
- LB agar plates with 50 µg/mL kanamycin
- LB media sterilized by autoclave
- Kanamycin
- Nanodrop instrument
- Sterile Eppendorf tubes
- Sterile 12 mL tubes with caps
- 37°C shaking and not shaking incubators
- EcoRI restriction enzyme (NEB)
- CutSmart® Buffer (NEB)

**Procedure:**

- Perform PCR following the protocol described in **Section 3.8**. Resolve 10 µL of PCR product on an agarose gel (as described in **Section 3.5**.) to verify presence of a single band at the expected size that is smaller than the WT band (see **Figure 16B** for an example).
- Purify PCR sample(s) with a single PCR product at a smaller size than the WT PCR product, as verified on the agarose gel, using a PCR purification kit. Follow the kit protocol and use at least 25 µL PCR sample volume.
- Determine DNA concentration of purified PCR product by Nanodrop.
  - The PCR products of the targeted gene have mixed alleles even though they possess similar sizes indistinguishable by agarose gel. Cells repair Cas9 cleaved DNA using the error-prone non-homologous end joining (NHEJ) repair pathway, which randomly inserts or deletes DNA bases to patch-up double-strand breaks in each allele. Therefore, it is critical to ensure that CRISPR-cas9 induced indels generated a frameshift on all the alleles. This is achieved by cloning the PCR products as single alleles, amplifying them in bacteria and then sequencing.
- Perform ligation of the purified PCR product to the vector and transform into bacteria using the Zero Blunt TOPO PCR cloning kit following manufacturer's instructions (see **Appendix**).

- Transformation and manipulation of bacteria should be done under sterile conditions near a flame. Autoclaved media and labware should always be used. Bench surfaces should be cleaned with 70% ethanol immediately before and after handling bacteria. Solutions that are heat-sensitive, such as stock antibiotics, should be sterilized by filtration prior to use.
- For bacterial transformation, use half the amount of reagents indicated in the manufacturer's instructions in the Zero Blunt TOPO PCR cloning kit (Invitrogen).
- For ligation and transformation reactions, appropriate controls should be included, as listed below:
  - Ligation control: Vector only
  - Transformation control: Cells only
- Restriction enzymes must be kept at -20°C until the moment of use. When ready to use, transfer the reagent to ice and return it to the freezer right after using it.
- To sequence validate each allele of a given gene CRISPR edited DNA, 10 bacterial colonies from each plate, transformed with PCR product from a single gene edited clone (KO), are sequence-analyzed. In some cases, some of the colonies yield one sequence editing pattern and some of them another. This means that the generated clone has several alleles of the gene. In cases where all 10 colonies show same gene editing pattern, all the alleles are identical (=homozygous).

# 4 Appendix

## 4.1 Example of the sequence of an exon of the gene

**GENE: OPTN ENSG00000123240**

**Description: optineurin**
**Gene synonyms:** FIP-2, FIP2, GLC1E, HIP7, HYPL, NRP, TFIIIA-INTP
**Location:** Chromosome 10:13098849:13138308:1
**Transcript:** OPTN-201 ENST00000263036

**Sequence of Exon 4 and the introns region around exon 4:**

ATTTAGATATTTGTGCTGTAGTGGCGGTACCCAAATCCACTTTATTTTCTTGGGATTTTT
AAGGACTAGAAATGATGTTCATCCCGCTAGTCTTTTCTGTAAGCAAAAACCACTTCGTCT
TTTTGCTGCTGACCCTTGGGCCAAGGCTAAGCATGGCATCTTTCAATTCAG**AGCCATGTG**
**GTCAAGTGGACTAGAGGGAGATTTGGTTCATCAGATCAAGTCCACTTTCCTGGTGTGTGA**
**CTCCATCACTCTGAACCTCCTGCAGAAGCCATGAAGCTAAATAATCAAGCCATGAAAGG**
**G**
**AGATTTGAGGAGCTTTCGGCCTGGACAGAGAAACAGAAGGAAGAACGCCAGTTTTTTGA**
**G**
**ATACAGAGCAAAGAAGCAAAAGAGCGTCTAATGGCCTTGAGTCATGAGAATGAGAAATT**
**G**
**AAGGAAGAGCTTGGAAAACTAAAAGGGAAATCAGAAAGGTCATCTGAG**GTGAGCAGAC
CG
ATCCATTGTGATGTTGTTTTTTTTTTTTCCCTTGACATTTGCAGTGGAATCTTACGTGTC
TAGACTCCTAGATCAAAACCTTTCATGGTTCAGTCTGGATTGGTGTTTTGCCTGGTCTTG
GAAGAAGTGCTTTTGCTGAAAAGATTGGTTGCCCTATTAAGGGTCATGGATAATCTCTTT
TAGAAGAAAGAAATTTGTAAAGCTTTGACCGTACTGATTGTAGGCAAAAGAACAGTAAGG
TTATAAATCATTGTATTGTATTCATTATAGATGGTGCAGATGGGCCTCTGCCTAGAACCA

Exon region is highlighted in red, guide RNA target sequences are underlined, pair 1 of gRNAs is highlighted in yellow and pair 2 in grey. Primer pairs 1 and 2 are highlighted in blue and green, respectively.

| **Guide RNA** | **Cut site** |
|---|---|
| 1. CUAAAUAAUCAAGCCAUGAA | 13,110,302 |
| 2. AGCCAUGAAAGGGAGAUUUG | 13,110,313 |
| 3. GAGAAAUUGAAGGAAGAGCU | 13,110,437 |
| 4. AACUAAAAGGGAAAUCAGAA | 13,110,462 |

Pairs of guides used for co-transfection:

1. CUAAAUAAUCAAGCCAUGAA + GAGAAAUUGAAGGAAGAGCU Should cut out ~135 bp
2. AGCCAUGAAAGGGAGAUUUG + AACUAAAAGGGAAAUCAGAA Should cut out ~ 149 bp

**Screening Primers:**

Pair 1: 10090F CAATTCAGAGCCATGTGGTC 10561R GGGCAACCAATCTTTTCAGC

Pair 2: 10069F CAAGGCTAAGCATGGCATC 10538RGCACTTCTTCCAAGACCAG

Primer descriptions from PrimerBlast website:

**Primer Pair 1:**

| | Sequence (5'->3') | Template strand | Length | Tm (°C) | GC% |
|---|---|---|---|---|---|
| **Forward primer** | CAATTCAGAGCCATGTGGTC | Plus | 20 | 56.78 | 50.00 |
| **Reverse primer** | GGGCAACCAATCTTTTCAGC | Minus | 20 | 57.92 | 50.00 |
| **Product length** | 471 bp | | | | |

**Primer Pair 2**:

| | Sequence (5'->3') | Template strand | Length | Tm (°C) | GC% |
|---|---|---|---|---|---|
| **Forward primer** | CAAGGCTAAGCATGGCATC | Plus | 19 | 56.74 | 52.63 |
| **Reverse primer** | GCACTTCTTCCAAGACCAG | Minus | 19 | 55.8 | 52.63 |
| **Product length** | 469 bp | | | | |

**Expected PCR products:**

| | Product size (bp) | | |
|---|---|---|---|
| | WT | Guide pair 1 | Guide pair 2 |
| **Primer pair 1** | 471 | 471-135=336 | 471-149=322 |
| **Primer pair 2** | 469 | 469-135=334 | 469-149=320 |

## 4.2 Preparation of LB media, LB Agar plates and Kanamycin stocks

### 4.2.1 Preparation of sterile LB media:

- Stir to suspend 25 g LB powder in 1 L of distilled water.
- Autoclave to sterilize using autoclave program liquid 20. Allow to cool before making additions, such as antibiotics.

### 4.2.2 Preparation of 50 mg/mL kanamycin stock:

- Stir or vortex to suspend 0.5 g of kanamycin in 10 mL of distilled water.
- Sterilize by passing through a 0.25 µm syringe filter under sterile conditions: in a biological hood or near flame and aliquot into sterile Eppendorf tubes, 500 µL per tube.
- Store at -20°C. Thaw before use. Can freeze-thaw multiple times as long as opened and used under sterile conditions.

### 4.2.3 Preparation of sterile LB Agar with kanamycin:

- Stir to suspend 12.5 g LB powder in 500 mL of distilled water. Transfer to Pyrex flask.
- Add 7.5 g agar and mix gently.
- Autoclave to sterilize using autoclave program liquid 20.
- Let agar cool to ~55°C (you should be able to pick up the flask without a glove) before making additions, such as antibiotics.
- Next steps should be done near flame: Add 500 µL of 50 mg/mL kanamycin stock to the 500 mL LB agar solution (dilution 1:1000) and mix gently by agitating the flask.

### 4.2.4 Pouring LB agar plates:

- Remove sterile Petri dishes from plastic bag (save the bag for storage).
- Pipette 20 mL of LB agar into each plate with 25 mL sterile pipette. Avoid bubbles.

- Let each plate cool until it is solid (~20 minutes). Plates can be left overnight on a bench at RT.
- Flip plates to avoid condensation on the agar.
- Store plates in plastic bags or wrap in fridge with name, date and contents.

## 4.3 Zero Blunt TOPO PCR cloning kit protocol steps at a glance.

- Set up the TOPO® Cloning ligation reaction using the reagents in the order and volume shown in **Table 7**. As a negative control, set up an additional single ligation reaction with vector only (without PCR product). For the control reaction, use water instead of pCR™II-Blunt-TOPO® Vector. *Vector should not undergo self-ligation without PCR product.*
- Mix the reaction gently and incubate for 5 minutes at room temperature.
- Place the reaction on ice and proceed to transformation. You may store the TOPO® Cloning ligation reaction at -20°C for 1 month if needed.
- Prepare for transformation: For each transformation use half a vial of competent cells (TOP10 cells from the TOPO® Cloning kit) and 1 µL of the TOPO® Cloning ligation reaction. Bring the temperature of a block heater to 42°C. Warm LB plates containing 50 µg/mL kanamycin at RT. Thaw on ice 1 vial of One Shot® chemically competent *E. coli* cells for every 2 transformation reactions. Have ready one control reaction of cells only. Turn on Bunsen flame as transformation and bacteria handling should be done under sterile conditions near flame.
- Add 1 µL of the TOPO® Cloning ligation reaction to sterile Eppendorf tube. Add 1 µL of sterile water to a control tube.
- Add half a vial of One Shot® chemically competent *E. coli* to each tube with ligation or control and mix gently (do not pipette up and down).
- Incubate on ice for at least 10 minutes.
- Heat-shock the cells for 30 seconds at 42°C without shaking.
- Transfer the tubes to ice immediately. Incubate on ice for 10 minutes.
- Add 125 µL of room temperature S.O.C. medium (supplied with the kit).
- Cap the tube tightly and shake the tube in shaking incubator at 200 rpm, at 37°C for 1 hour.
- Spread 10–50 µL from each transformation on a pre-warmed selective LB agar plate with kanamycin and incubate overnight at 37°C. An efficient TOPO® Cloning reaction will produce several hundred colonies. No colonies should be seen on the control plates.
- Pick 10 colonies from each plate and inoculate each single colony in 3 mL of LB medium containing 50 µg/mL kanamycin. Grow cultures overnight in 37°C/200 rpm shaking incubator. As a sterility control, incubate 3 mL of LB medium only containing 50 µg/mL kanamycin (without bacterial colony) under the same conditions. After overnight incubation, the tubes with bacterial growth will be turbid while the control should remain transparent.
- Extract plasmid from each 3 mL bacteria culture using miniprep kit (Qiagen) and determine DNA concentration using Nanodrop.

- Analyze the plasmids by restriction analysis with EcoRI enzyme to confirm the presence of an insert. The insert will contain the sequence of ligated PCR product. The EcoRI enzyme cuts the pCR™II-Blunt-TOPO® Vector twice, once upstream and once downstream of the insertion site.
- Thaw all reagents listed in **Table 8**, with the exception of EcoRI. Vortex the thawed reagents and spin them down briefly.
- Set up the restriction reaction, as follows:
    - Add 10 µL of extracted plasmid DNA to a 1.5 mL conical tube.
    - Prepare mastermix (reagents listed in **Table 8**) while taking in consideration the number of restriction reactions needed. For example, for 10 reactions prepare mastermix for 11 reactions to avoid running out of mastermix. Mix briefly and spin down briefly.
    - Add 40 µL of mastermix to each tube, mix it briefly and spin down briefly.
    - Incubate at 37°C for 1 hour.
- Run 5-10 µL of restriction reaction on a 1% agarose gel. In the case of a successful ligation, two bands will be seen on the gel: one a smaller size corresponding to the insert and another larger one corresponding to the vector.
- Analyze the DNA from each extracted plasmid with an insert, as validated by restriction analysis, by sequencing the DNA insert.
- To sequence the DNA, send to the sequencing facility. For sequencing use M13 Forward or M13 Reverse primers.
- Analyze sequencing results using SnapGene. Make sure that the sequence deletion of a gene, the indel of a gene, produces a frameshift mutation. The indel will yield a frameshift unless the length of an indel is a multiple of 3. Frameshift mutations are an indication of a successful gene KO. It is possible that different sequence deletions occurred in each allele of a gene.
- Use Expasy translate tool to make sure that translated product will not yield a truncated protein. Verify that a stop codon is introduced early in the predicted amino acid sequence in all the possible reading frames.

**Table 7: Recommended amounts of reagents for ligation.**

| Reagent | Volume (µL) |
|---|---|
| Purified PCR product | 1 |
| Salt Solution | 1 |
| Water (nuclease free from the kit) | 3 |
| pCR™II-Blunt-TOPO® Vector | 1 |
| Final Volume | 6 |

**Table 8: Suggested volume of reagents required for the EcoRI enzymatic reaction.**

| Reagent | Volume for one standard reaction (50 µL total) |
|---|---|
| | Volume per reaction (µL) |
| Plasmid DNA | 10 |
| CutSmart® Buffer (NEB) | 5 |
| Water | 34.5 |
| EcoRI Restriction Enzyme | 0.5 |

## 5 References


1. Mandegar, M.A., et al., *CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs.* Cell Stem Cell, 2016. **18**(4): p. 541-53.
2. Laflamme, C., et al., *Implementation of an Antibody Characterization Procedure and Application to the Major ALS/FTD Disease Gene C9ORF72.* Elife, 2019. **8**.
3. Sambrook, J. and D.W. Russell, *MOLECULAR CLONING : A LABORATORY MANUAL*. 2001, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.