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NeuroEDDU Protocols

CRISPR-mediated introduction of single nucleotide changes in iPSCs

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Table of Contents

1 Introduction

1.1 Objectives

This protocol describes how to edit an individual nucleotide in the genome of human induced pluripotent stem cells (iPSCs), combining CRISPR-Cas9 editing, droplet digital PCR (ddPCR) technology and antibiotic-free selection.

1.2 Protocol overview

Precise editing of human iPSCs by homology-directed repair (HDR) occurs at low frequency. This protocol presents an efficient method to capture rare editing events, and to facilitate isolation of edited cells with single-base substitutions without antibiotic selection. Specific criteria to increase editing efficiency are suggested regarding the design of guide RNA (gRNA) and repair template. Nucleofection of ribonucleoprotein (RNP) complex is proposed to increase delivery of the CRISPR components into iPSCs. Accurate detection of edited alleles is achieved using ddPCR through a specific TaqMan hydrolysis assay. The enrichment of edited cells relies on limited-dilution cell passaging.

1.3 Technical and safety considerations

The following information should be read before starting:

- iPSCs must be handled within a Class II biosafety laminar flow hood to protect the worker from possible adventitious agents. Appropriate Environmental Health and Safety (EHS) office regulations must be followed.
- Use autoclaved tubes, plates, pipets and tips to avoid contamination.
- Keep thawed reagents on ice during use. Do not leave reagents at room temperature for extended periods.
- Protect fluorescent DNA probes from light to avoid photobleaching. Cover tubes and plates containing probes with aluminum foil.

2 Materials

Refer to the product datasheet from the supplier for further details on storage and preparation instructions. Pay attention to the lot number for some reagents, which can vary sometimes and affect the efficiency of the protocol.

2.1 Labware

Item	Supplier	Catalogue #
96-well Clear Flat Bottom TC-treated Culture Microplate	ThermoFisher	353072
Culture plate, 6-well	ThermoFisher	087721B
ddPCR™96-Well Plates	BioRad	12001925
DG8 Cartridge Holder	BioRad	1863051
DG8 Cartridges and Gaskets	BioRad	1864007
Pierceable Foil Heat Seal	BioRad	1814040
Pipet-Lite Multi Pipette L8-50XLS+	RAININ Mettler Toledo	17013804
Tips GP-LTS-A-250µL	RAININ Mettler Toledo	30389278

2.2 Reagents

Item	Supplier	Catalogue #	Storage temp.
Accutase™	StemCell Technologies	07922	–20°C
Alt-R® HDR Enhancer	IDT	1081073	–20°C
Alt-R® S.p. HiFi Cas9 Nuclease V3	IDT	1081061	–20°C
Amaxa™ P3 Primary Cell 4D Nucleofector™ X Kit S -P3 Primary Cell Nucleofector™ Solution -Supplement 1 -16-well Nucleocuvette™ Strips	Lonza	V4XP-3032	4°C
Antibiotic-Antimycotic	Gibco	15240062	Stock: –20°C Working: 4°C
ddPCR Supermix for Probes (no dUTP)	BioRad	1863024	–20°C
DMEM/F12	Gibco	10565018	4°C
Droplet Generation Oil for Probes	BioRad	1864110	RT
Droplet Reader Oil	BioRad	1863004	RT
Guide RNA	Synthego	NA (custom order)	–20°C
LNA™ Probes	IDT	NA (custom order)	–20°C†
Matrigel Matrix hESC-qualified	Corning Millipore	354277	Stock: –80°C Working: 4°C*
mTeSR1™	StemCell Technologies	85850	–20°C
PCR Primers	Invitrogen	NA (custom order)	–20°C
Phosphate-Buffered Saline (PBS)	ThermoFisher	10010023	RT
QuickExtract™ DNA Extraction Solution	Lucigen	QE09050	–20°C
ReLeSR™	StemCell Technologies	05872	RT

Item	Supplier	Catalogue #	Storage temp.
ssODN	IDT	NA (custom order)	–20°C
UltraPure™ DNase/RNase-Free Distilled Water (H_2O)	ThermoFisher Scientific	10977015	RT
Y-27632 (Rock Inhibitor)	Selleckchem	S1049	Stock: –80°C Working: 4°C

*Matrigel working solution must be used immediately or stored at –20°C for later use.
†Protect from light

2.3 Equipment

Item	Supplier	Catalogue #
Biosafety Cabinet	Baker	SG404P
C1000 Touch™ thermal cycler with 96–deep well reaction module	BioRad	1851197
Cell Counter	Logos Biosystems	L40002
Cell culture incubator	ThermoScientific	Steri-Cycle Model 370
Centrifuge	Eppendorf	022626001
Heat Block	VWR	12621-088
Microscope	Motic	110010380054
Nucleofector 4D™ Core Unit	Lonza	AAF-1002B
PCR Plate Spinner	VWR	89184-608
PX1™ PCR Plate Sealer	BioRad	1814000
QX200™ Droplet Generator	BioRad	1864002
QX200™ Droplet Reader	BioRad	1864003
Vortex Mixer	Fisher Scientific	88880017

3 Protocol

3.1 Strategy Setup

Figure 1 shows an overview of the method used to design the CRISPR components and to isolate properly edited iPSC clones.

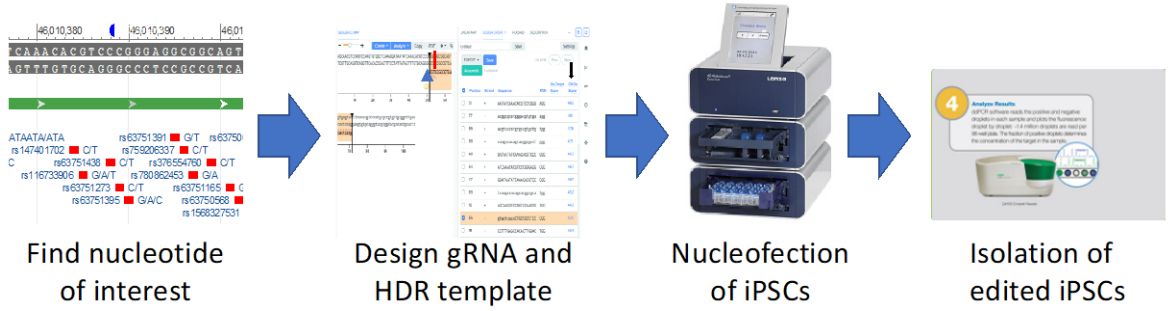

Figure 1. Graphical workflow of the strategy for gene editing in iPSCs.

3.1.1 Characterization of the target nucleotide

Locate the genomic DNA sequence of interest using a genome browser such as ensembl.org or genome.ucsc.edu. The identification of the nucleotide to edit within the reference genome is not always straightforward. The nomenclature of a given mutation often reflects the first transcripts discovered for a given gene but might not be coherent with newly-discovered transcripts. For instance, if your objective is to introduce the missense mutation P301L on the human protein Tau, you will need to find a proline as the 301st amino acid on a protein-coding transcript corresponding to the gene *MAPT*. However, there are at least 12 different known protein-coding transcripts for *MAPT* (**Figure 2**).

Figure 2. Several known protein-coding transcripts for the human *MAPT* gene. Credit: Ensembl Comparative Genomics Data

Only one of these transcripts has a proline at position 301, i.e., transcript MAPT-205. In any case, ensure you find the corresponding genomic DNA sequence on the human reference genome. In this example, tools like snpedia.com and dbSNP, i.e., ncbi.nlm.nih.gov/snp/rs63751273, are very helpful to find the target nucleotide (**Figure 3**).

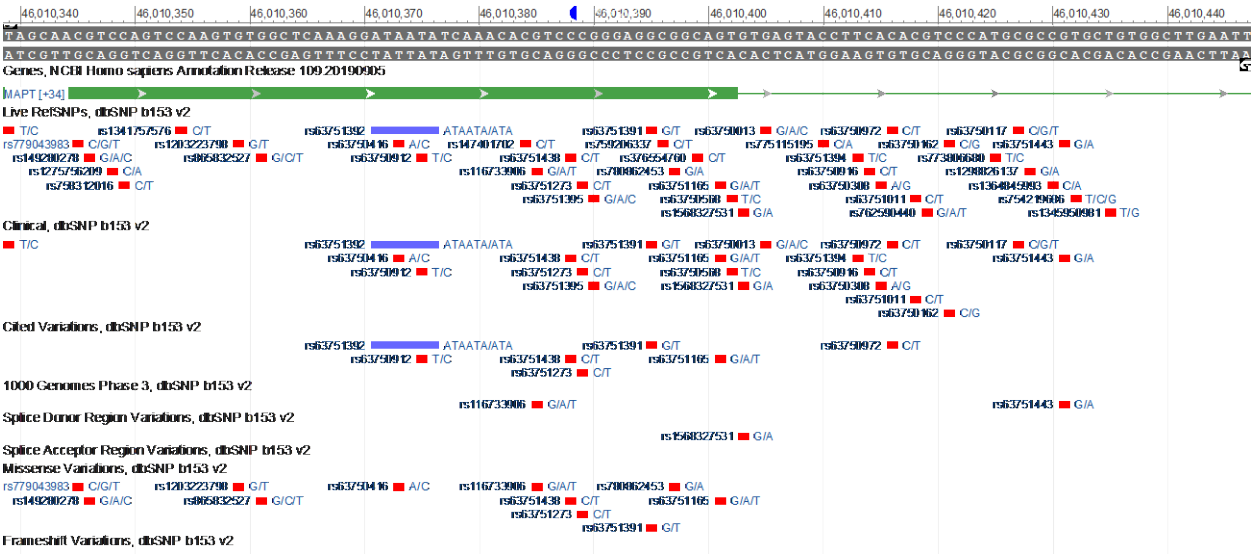

Figure 3. dbSNP representation of the target nucleotide in *MAPT*. Credit: Ensembl Comparative Genomics Data

3.1.2 Design of gRNA

Copy ~50-75 base pairs (bp) of genomic DNA sequence on each side of the mutation to edit, and search for the best gRNA sequences using tools such as benchling.com, crispor.tefor.net, deskgen.com, chopchop.cbu.uib.no, etc. A good gRNA normally complies with these two criteria:

1. It cuts less than 10 bp away from the mutation to edit [keep in mind that Cas9 from *S. pyogenes* cuts three bp upstream of the proto-adjacent motif (PAM) sequence].
2. It has a low off-target event potential (= highest off-target score in Benchling).

However, you might want to design 2-3 of the best-scored gRNAs, i.e., highest off-target score in Benchling and a cut site less than 10bp from the target nucleotide (**Figure 4**). Choose the most effective gRNA using a mismatch assay such as the T7 endonuclease 1 cleavage assay from New England Biolabs (NEB) in HEK293 cells. T7 endonuclease I cleaves DNA heteroduplexes formed in the presence of an indel. Briefly, a PCR reaction is performed surrounding the CRISPR gRNA target site. If a NHEJ repair event following CRISPR-Cas9 cleavage has introduced a mutation, denaturing and annealing will form a fraction of heteroduplexes containing mutant and wildtype PCR amplicons. T7 endonuclease I will cleave DNA mismatches in those heteroduplexes generating fragments that can be resolved on an agarose gel. Be mindful that some single-nucleotide polymorphisms (SNPs) present in the genome of the cell-line to be edited, but not in the reference genome in which the design is made, might create a mismatch in the gRNA sequence, and vice versa, so try to avoid any SNP within the chosen gRNA sequence using tools such as ensembl.org.

Figure 4. An example of highest-scored (black arrow) gRNA is depicted on the minus strand for *MAPT*-P301L, with the cut site (red line) <10bp away from the target nucleotide (blue arrow). Credit: Benchling

3.1.3 Design of single-stranded oligodeoxynucleotide (ssODN)

For HDR-based gene editing, you need to design a DNA template with homology arms flanking the appropriate codon to change. For instance, the codon to change is CCG>CTG for an introduction of *MAPT*-P301L. Plasmids, BACs or viruses can be used as a template to insert large sequences, with typically ~500 bp homology arms. However, synthesizable ssODN fragments have proven highly efficient and simple to use for relatively small edits. Typically, a 40-60 bp homology arm on each side of the mutation to edit works best with ssODN (**Figure 5**).

MAPT-P301L
AGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTGCTGGGAGGCGGC
AGTgtgagtaccttcacacgtcccatgcgccgtgctgtggctt

Figure 5. Design of ssODN for *MAPT*-P301L with 40-60bp homology arms on each side of the target codon to change, which is highlighted in green.

The company Integrated DNA Technologies (IDT) can synthesize Ultramer® ssODN fragments up to 200 bp in length, and Megamer® up to 2 kb. You might also need to disrupt the PAM of your gRNA sequence that is embedded within the ssODN sequence by introducing silent mutation(s) to prevent Cas9 from cutting the template and the newly engineered allele. A silent mutation is introduced by changing one of the two Gs within the PAM sequence of the gRNA with another nucleotide resulting in no amino acid change after translation. Thus, changing the codon CCG for CCC will still encode a proline. When introducing silent mutations, pay close attention to the human codon usage frequency as some codons are rarely used in humans, and

also try not to create new donor or acceptor splicing sites, i.e., new GT or AG, that might disrupt the expected splicing pattern.

3.1.4 Design of primers and probes

The detection of the modified nucleotide, or single-nucleotide polymorphism (SNP), is based on a TaqMan® assay including two PCR primers and two DNA probes fused with different fluorophores. One probe is specific to the original allele and the other probe to the modified allele. Locked Nucleic Acid (LNA®) probes synthesized by IDT are particularly efficient for this.

1. Ensure that both probes target the same strand to avoid probe complementarity.
2. The SNP of interest should be positioned in the center of the probe.

- Avoid placing SNPs in the first couple or last couple bases, if possible.

3. ΔTm = The difference between melting temperature (Tm) of match and Tm of mismatch hybridizations. ΔTm is of particular importance in applications where probes are used for specific detection of mutations, or homologous sequences. A large ΔTm is required to ensure specific detection of the sequence of interest. As an example, if the wildtype allele is an A, and the mutant allele is a G, mismatches would be A:C and G:T. Use a thermodynamics calculator (www.idtdna.com/calc/analyzer/lna) to calculate match and mismatch Tm's of possible probe configurations. Enter the Mg+2 and dNTP concentrations for qPCR conditions (3mM MgCl2 and 0.8mM dNTPs).
4. Enter the probe target sequence with LNA bases denoted with a "+" in front of the base of interest (+A, +C, +G, +T). Start with an LNA base on the SNP, and one on each adjacent base. Match Tm: 64-68°C. Mismatch Tm: minimum ΔTm of 8°C, greater is better. No higher than 56°C. It may be necessary to design towards the antisense strand if Tm mismatch is troublesome for the sense strand. If this is the case, make sure to design both probes to the same strand so they are non-complementary.
5. Additional LNA bases are added to achieve optimal Tm mismatch. Some mismatches can make achieving optimal thermodynamics challenging: examples include designs involving G-T and C-A mismatches.

Ideal configuration:

- LNA on SNP and on adjacent bases (3 LNAs in a row), e.g., ACGT+A+C+TATCG.
- Do not place more than 4 LNAs in a row. No more than 6 LNAs per probe.
- Spread out the LNAs to get a greater effect on Tm.
- Avoid stretches of 3+ C's or G's.
- Placing LNAs on G and C will give the greatest Tm increase.
- No LNA on first base. Avoid LNA on second base.
- Avoid probes with a 5' G residue (can quench fluorophore).
- Probes can be designed to target either the sense or antisense strand.

- Shorter LNA probes (10-14nt) are more effective at single base discrimination (ensure Tm ≥ 64°C).

6. Use PrimerQuest or PrimerBlast to design a set of primers surrounding SNP of interest: Primer Tm: Aim for 62^0C (60-63^0C). Amplicon size ~80-150 bp.
7. Check primers for dimers, hairpins, heterodimers using OligoAnalyzer. BLAST primers/probe to check for specificity/off target transcripts.

3.2 iPSC maintenance

For more details on iPSC culture, see protocol #EDDU002-01 at https://www.mcgill.ca/neuro/open-science/open-science-platforms/eddu/resources-researchers)

Materials:

- 96-well Clear Flat Bottom TC-treated Culture Microplate
- Culture plate, 6-well
- Phosphate-Buffered Saline (PBS)
- Matrigel Matrix
- mTeSR1™
- ReLeSR™
- Antibiotic-Antimycotic
- DMEM/F12

3.2.1 Coating culture vessels

Prepare Matrigel working solution on ice by adding 1 aliquot of Matrigel (150 µL) and 150 µL of Antibiotic-Antimycotic to 15 mL of cold DMEM/F12. Mix solution well.

- **IMPORTANT**: Thaw Matrigel on ice. Keep DMEM/F12 with Antibiotic-Antimycotic on ice.

1. Use immediately after preparation. Matrigel working solution can be stored at –20 °C for up to 1 week. Thaw the Matrigel working solution on ice and use immediately. Do not re-freeze.
2. Immediately apply recommended volume of Matrigel solution to culture vessel and swirl to spread across surfaces.
3. Incubate culture vessel at 37°C for at least 1 hour.
4. If plating cells on culture vessel immediately, gently tilt culture vessel to one side to allow excess Matrigel solution to collect in a corner. Remove the Matrigel solution with a serological pipet or by aspiration. Ensure coated surface is not scratched. Immediately add an appropriate volume of culture media to the culture vessel and then plate cells. If not plating cells on culture vessel immediately, do not aspirate Matrigel solution, seal with Parafilm, and store at 4°C for up to 7 days.

3.2.2 Culturing iPSCs

1. Change mTeSR daily (2 ml per well for 6-well plates).
2. When 60-70% confluency is attained for your cells, aspirate media, rinse with 2 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic, and aspirate media.
3. Add 1 mL ReLeSR, distribute evenly and aspirate the majority of the ReLeSR, leaving a small film to prevent drying.
4. Transfer the plate into the incubator at 37°C for 7 minutes
5. Add 1 ml per well mTeSR. Cell aggregates should all lift off. If not, tap gently on the side of the plate until all attached clumps of cells lift off.
6. Mix gently, avoid breaking aggregates, and transfer ~100 µL in a new Matrigel-coated well of a 6-well plate with 2 ml mTeSR.
7. Place the cells in a 37°C incubator. Move the culture vessel in several quick back-and-forth and side-to-side motions to distribute the cell aggregates.
8. Change media daily until next passage.

3.3 Nucleofection

Materials:

- 96-well Clear Flat Bottom TC-treated Culture Microplate
- PBS
- Accutase
- Alt-R® HDR Enhancer
- Alt-R® S.p. HiFi Cas9 Nuclease V3
- Amaxa™ P3 Primary Cell 4D Nucleofector™ X Kit S (P3 Primary Cell Nucleofector™ Solution, Supplement 1, 16-well Nucleocuvette™ Strips)
- Guide RNA
- Matrigel
- mTeSR
- Rock Inhibitor
- ssODN
- Microscope
- Centrifuge
- Cell Counter
- Nucleofector 4D™ Core Unit
- Biosafety Cabinet

3.3.1 Preparation of RNP complex

The CRISPR components [Cas9 + gRNA + ssODN] are best introduced into human iPSCs through nucleofection. Incubation of 1 µl of Cas9 protein (stock 61 µM) with 3 µl of gRNA (stock 100 µM) at RT for 10-20 minutes will form a Cas9: gRNA RNP complex. After formation of the RNP complex, add 1 µl of ssODN (stock 100 µM) and 20 µl of buffer P3 to the RNP mix.

3.3.2 iPSC nucleofection

1. When cells attain 50-60% confluency in a 6-well plate, aspirate media, rinse two times with 2 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic, and aspirate media.
2. Add 1 mL Accutase and transfer the plate into the incubator at 37°C for 10 minutes.
3. Cells should all lift off. If not, leave a few more minutes in the incubator.
4. Mix gently by pipetting up and down 2-3 times and transfer the mix into a 15-ml conical tube containing 5 ml mTeSR.
5. Mix well by inverting the tube gently a few times, and count cells.
6. Centrifuge 500,000 cells in a separate tube at ~250g for 3 minutes.
7. Aspirate supernatant, add 5 ml of PBS, mix by inverting the tube, and centrifuge again.
8. Aspirate supernatant completely.
9. Resuspend the pellet very gently with the 25 µl RNP-ssODN-buffer mix.
10. Transfer this resuspension in a cuvette and nucleofect quickly using the program CA137 on a Nucleofector 4D device (protocols might vary if using different systems).
11. Add 75 µl of [mTeSR + rock inhibitor 10 µM final] on top of the cells in the cuvette. Next, transfer the entire contents of the cuvette into a new 15-ml conical tube containing 10 ml of nucleofection media [mTeSR + rock inhibitor 10 µM final + HDR enhancer 30 µM final]. Rock inhibitor is used to prevent apoptosis during single-cell dissociation.
12. Mix gently by inverting a few times and distribute 100 µl per well in a Matrigel-coated 96-well plate, after aspirating the Matrigel.
13. Change media with 100 µl per well of iPSC maintenance media [mTeSR + rock inhibitor 10 µM final] after 24 hours, and then daily with mTeSR only after 48 hours.

3.4 ddPCR Mutation Detection

The procedures presented in this section have been optimized for the Bio-Rad ddPCR QX200™ system.

Materials:

- 96-well Clear Flat Bottom TC-treated Culture Microplate
- 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
- PBS
- Accutase
- ddPCR Supermix for Probes (no dUTP)
- Droplet Generation Oil for Probes
- Droplet Reader Oil

- Matrigel
- mTeSR
- Primers
- Probes
- QuickExtract™ DNA Extraction Solution
- Rock Inhibitor
- H_2O
- 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

3.4.1 Genomic DNA extraction

1. When near-confluent, i.e., 10-14 days post-nucleofection, wash twice with PBS, and treat with 30 µl/well of accutase for 10 minutes at 37°C.
2. Transfer half (15 µl) of each well in a new matrigel-coated 96-well plate pre-filled with 150 µl/well of [mTeSR + rock inhibitor 10 µM final], and store this "backup" plate in the incubator at 37°C.
3. Add 35 µl of QuickExtract solution to the remaining cells/accutase mix in the original plate, heat at 65°C for 10 minutes, followed by 95°C for 5 minutes, and dilute 1/20 with H_2O: this is the diluted DNA extract that will be used to evaluate the proportion of edited alleles using ddPCR.

3.4.2 ddPCR reaction mixture

To prepare a typical ddPCR master mix for a whole 96-well plate:

1. Mix 1,170 µl of "ddPCR Supermix for Probes (no dUTP)", 975 µl H_2O, 20.8 µl of each primer (stock 100 µM) and 6.5 µl of each probe (stock 100 µM).
2. Distribute 19 µl per well of this master mix in a new 96-well ddPCR plate.
3. Add 2µl per well of the diluted DNA extract in each corresponding well.
4. Spin briefly.

3.4.3 Droplet generation

- **IMPORTANT:** The generation of the ddPCR droplets requires the preparation of everything upfront (section 3.4.3) 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. Transfer 20 µl from each well of column 1 of the ddPCR reaction mixture plate into the appropriate wells of a new DG8 cartridge, previously inserted in a cartridge holder (**Figure 6**).

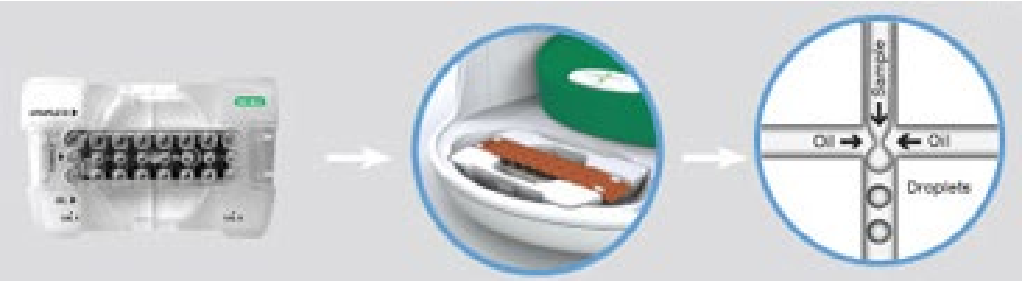

Figure 6. Droplet generation using the DG8 cartridge and the QX200 Droplet Generator. Credit: BioRad

2. Add 65 µl of "Droplet Generation Oil for Probes" in the appropriate wells of the DG8 cartridge.
3. Install a ddPCR gasket on top of the cartridge.
4. Place the cartridge inside the "QX200 Droplet Generator" and close the door.
5. When the green light appears after 1-2 minutes, open the door and remove the cartridge from the droplet generator.
6. Remove the gasket to re-use it one more time at a later point on the other side.
7. Transfer 42 µl of the droplet mix **VERY** slowly (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-250µL". The droplets are very fragile before the PCR reaction is completed.
8. Repeat the same process for columns 2 to 12.
9. Seal the plate with the "Pierceable Foil Heat Seal" and place it in a PCR machine.
10. Start the appropriate ddPCR program while ensuring that you set the proper Tm corresponding to your probe/primer set, previously established using the gradient mode. A typical ddPCR program would be 1x[95°C, 10'], 45x[95°C, 30''; 62°C, 1'; 72°C, 30''], 1x[98°C, 10'], 1x[12°C, ∞], at 2.5°C/sec ramp rate.

3.4.4 Droplet reading

- When the PCR reaction is completed, the droplets are stable for several days if kept at 4-10°C in the dark.
- When ready, read the plate using the "QX200 Droplet Reader" in the absolute quantification mode of the QuantaSoft software.
- Select the appropriate reporter fluorophores according to your probes, e.g., FAM and HEX.
- Ensure there is enough "Droplet Reader Oil" in the instrument and the waste is empty before a run.

3.5 Data Analysis

Materials:

- QuantaSoft software

3.5.1 Quantification of positive droplets

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

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

You might have to adjust the threshold manually for positive droplets on a well-by-well basis or across the entire plate (**Figure 7**). Please refer to the ddPCR application guide from BioRad for more information.

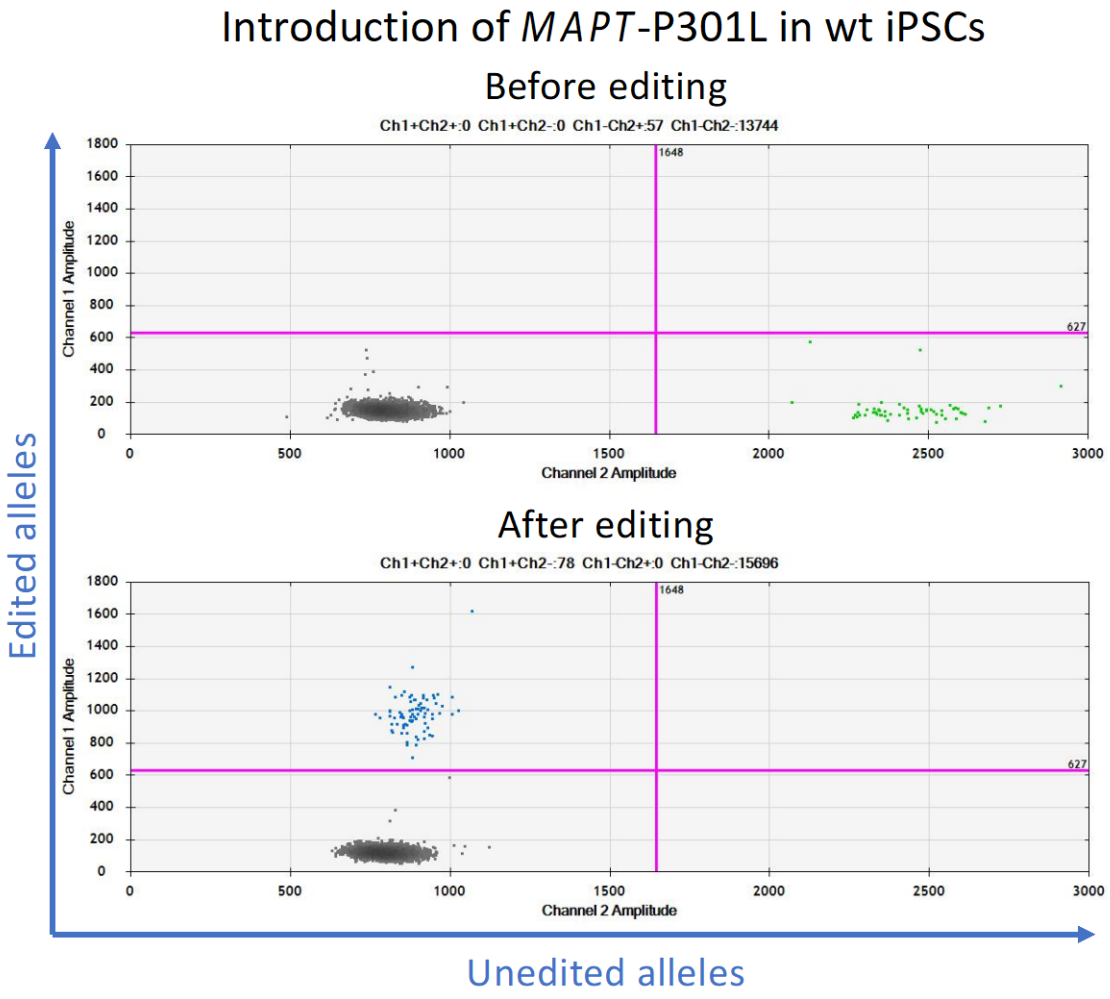

Figure 7. Only wt alleles in green are detected before the introduction of the *MAPT*-P301L mutation (x-axis on upper panel) while only mutant alleles in blue are detected after introducing this mutation homozygously (y-axis on bottom panel).

3.5.2 Data export

Export a .csv file including the analysed data. The column “Concentration” represents the number of wt or mutant alleles per µl of reaction mixture, for each well. Calculate the proportion of mutant vs wt alleles in each well. The well containing the highest proportion of mutant alleles will be used for subsequent enrichment steps.

3.6 Enrichment of mutant iPSCs

Materials:

- 96-well Clear Flat Bottom TC-treated Culture Microplate
- PBS
- Accutase
- Matrigel
- mTeSR
- Rock Inhibitor
- Microscope
- Centrifuge
- Cell Counter
- Biosafety Cabinet

3.6.1 Passage at limiting dilution

If you have not been able to find at least one well containing 100% mutant alleles following QuantaSoft analysis, identify the well or wells with the highest proportion of edited alleles. Go back to your “backup” plate previously placed in the incubator in the **section 3.4.1** .

1. When the identified well has reached 60-70% confluency, aspirate media, wash twice with 100 µl PBS per well, and aspirate PBS.
2. Add 30 µl per well of accutase for 10 minutes at 37°C. Cells should all lift off, if not, leave a few more minutes in the incubator.
3. Add 250 µl of [mTeSR + rock inhibitor 10 µM final], mix gently by pipetting up and down 2-3 times.
4. Count cells.
5. Transfer 100-200 cells into a new 15-ml conical tube containing 10 ml of [mTeSR + rock inhibitor 10 µM final].
6. Mix gently by inverting the tube a few times.
7. Distribute 100 µl/well in a new matrigel-coated 96-well plate.

Keep in mind that the number of cells to transfer can differ considerably between different iPSC lines depending on robustness, survival, growth rate, etc. This can be assessed beforehand with the goal of ending up with ~1 colony per well. Freeze the remaining cells as well as the

second highest well. Discard the rest of the plate. Repeat **sections 3.4; 3.5; 3.6** until you have identified a well containing 100% edited cells. Each round takes about 2 weeks to complete.

3.7 Final characterization

Materials:

- 96-well Clear Flat Bottom TC-treated Culture Microplate
- Matrigel
- mTeSR
- Biosafety Cabinet
- PBS

3.7.1 Expansion and banking

Once you have a minimum of one, and ideally three independent, 100% edited clones, pass the whole well into a 6-well plate for expansion and freezing stocks.

3.7.2 Target sequence integrity

You will have to spend some time characterizing your isolated clones for sequence integrity and homozygosity/heterozygosity of the mutation using PCR cloning and Sanger sequencing. You can use the same genomic DNA that was prepared above, or use commercial kits to get purer DNA. Design and order PCR primers that will give rise to a ~300-500 bp single/clean band on agarose gel, with the mutation of interest located in the middle of this amplified fragment. Use this fragment for PCR cloning or directly for Sanger sequencing using the same PCR primers. For example, **Figure 8** shows Sanger sequencing chromatograms confirming the expected editing.

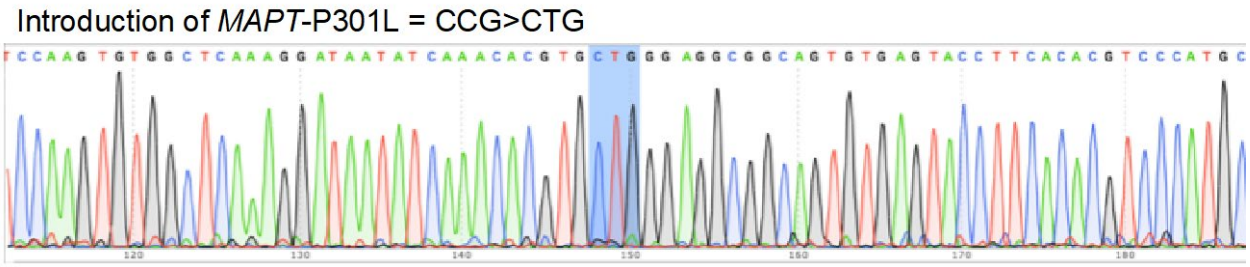

Figure 8. Chromatograms from Sanger sequencing confirming the introduction of homozygous CTG codon corresponding to P301L in *MAPT*.

3.7.3 Off-target events

To ensure that any of the phenotypes you end up studying are not the result of off-target effects, look at potential off-target sites by PCR based on mismatches between your gRNA sequence and the whole genome.

3.7.4 Genome integrity

Check the integrity of the genome by karyotyping and/or targeted PCR. Ultimately, the level of characterization you opt for will depend on your specific research project.