Chapter 2
Methods & Materials

This research was conducted from January 2016 to September 2016 at the Wellcome Trust Sanger Institute. Tissue culture experiments with human induced pluripotent stem (iPS) cells were performed in containment level 2 laboratories of the institute. The protocols and methods used in this study are described in this chapter. I conducted all the experiments, unless otherwise stated.

Induced pluripotent stem cells

Human induced pluripotent stem (iPS) cell line, KOLF2 (ID: HPSI0114i-kolf_2), was obtained from HipSci (Human Induced Pluripotent Stem Cell Initiative; www.hipsci.org). This cell line was subcloned and karyotyped by FISH (Fluorescence in situ hybridization) prior to genome editing experiments. The subline, KOLF2-C1, obtained was used for the tissue culture experiments described herein. Both the parental KOLF2 and KOLF2-C1 subline have a normal 46; XY karyotype (Koutsourakis, Bushell & Skarnes; unpublished data).

Bioinformatics

CRISPRs were designed using the HTGT WGE CRISPR Search (Hodgkins et al. 2015) (http://www.sanger.ac.uk/htgt/wge/find_crisprs), and InFusion primers were designed using WGE Gibson Designer (http://www.sanger.ac.uk/htgt/wge/gibson_designer). All other PCR primers were designed using Primer3 (http://bioinfo.ut.ee/primer3/) or manually selected.

Synthetic DNA nucleotides, recombinant proteins, and services

All PCR, InFusion, sequencing primers, and CRISPR gRNA oligos were manufactured by Sigma. For the AAAS point mutation experiment, crRNAs, tracrRNA, and *AAAS* point mutation ssODN were all purchased commercially from Integrated DNA Technologies. Recombinant Cas9 nuclease was purchased from two commercial sources, Thermoscientific and Feldan. HTT synthetic plasmid was obtained from Invitrogen. All Sanger sequencing samples were sent to Eurofins in Germany for capillary sequencing.

Molecular biology techniques

Extraction of genomic DNA

The cells from one well of a confluent 6-well plate (BD Biosciences) were washed with 3ml DPBS (Gibco Life Technologies), and then lysed in 0.5ml TENS buffer (10mM Tris pH 8/ 1mM EDTA/ 150mM NaCl/ 0.5% SDS) containing freshly added Proteinase K (1-1.5 mg/ml; Sigma) at 60^{o}C for at least 4 hours. The cell lysate was transferred to a 1.5 ml microfuge and

an equal volume of phenol: chloroform (1:1; Amresco) was added to the cell lysate and mixed by inversion for 5 minutes. Following centrifugation for 5 minutes at 13000rpm, the aqueous phase was transferred to a new 1.5ml tube. The above step was repeated once more and the genomic DNA was precipitated with 0.8 volume of isopropanol after which the spooled DNA was washed twice in 1ml of 70% ethanol. The DNA was resuspended in TE buffer (10mM Tris/ 1mM EDTA) and placed in a 60ºC incubator for 20 minutes to evaporate any ethanol. The sample was left to resuspend overnight at room temperature (RT) and the DNA concentration was determined from its absorbance at OD_{260nm} on the following day.

Transformation of chemically competent DH5α and plasmid isolation

Plasmid DNA was added to thawed 50µl of *E. coli* DH5α cells (NEB) and incubated on ice for 20 minutes. The cells were then heat-shocked at 42ºC for 45 seconds followed by another 2-minute incubation on ice, after which the cells were allowed to recover in 500µl of SOC medium (NEB) for an hour in a 37ºC shaking incubator. The cells were then plated at two different densities on LB agar plates (Sanger media kitchen) containing the appropriate concentration of antibiotic and incubated overnight at 37ºC.

Following overnight culture of transformed cells, the desired number of colonies from the plates were used to set up overnight liquid cultures at 37ºC in 2xLB broth (Sanger media kitchen) containing any antibiotic at the appropriate concentration. Plasmid DNA was extracted from these cultures using the QIAprep spin miniprep kit (QIAGEN) as per the manufacturer's instructions. To prepare glycerol stocks, 50% glycerol (Sanger media kitchen) was added to cell cultures at a ratio of 3:2 and stored at -80ºC. Additionally, diagnostic restriction digests, where necessary, were performed using buffers and units of enzymes recommended by the manufacturer and run on 0.7% agarose (Invitrogen) gel to confirm the presence of the correct-sized DNA fragments.

Sequencing and ethanol precipitation of samples

Sanger sequencing reaction

For sequencing reactions, equal volumes of DNA and sequencing reaction master mix (Table 2.1) were added together (usually 2µl of DNA with 2µl of sequencing master mix). The reaction mixture was then subjected to sequencing reaction in a PCR machine (program in Appendix, Table A4).

Table 2.1: Sequencing reaction master mix

Component	**Volume (µl) for 1x reaction**
H_2O	0.9225
SRD (sequencing reaction diluent)	0.9225
BigDye V3.1 terminator mix (Applied Biosystems)	0.125
dGTP BigDye V3.0 terminator mix (Applied Biosystems)	0.02
Sequencing primer (100µM)	0.01
Total	**2µl**

***** SRD (0.3M Tris Base pH 9.0/ 6mM $MgCl_2$/ 0.18%v/v Tween-20/ 5.9% v/v Glycerol / 1.1% v/v Formamide (Sigma)/ 9.9% v/v Tetramethylene Sulfone (Sigma)/ 7.8mM Pottassium Glutamate (Sigma)/ 79µg/ml BSA (Sigma))

Ethanol precipitation of sequencing reaction

To the sequencing reaction from the preceding step, 30µl of precipitation mix (80% ethanol/ 50mM sodium acetate) was added and centrifuged for 25 minutes at 4000rpm and 4ºC. The plate was then inverted on a blotting pad and spun at 400rpm for a minute. To the precipitated DNA, 30µl of 80% ethanol at -20ºC was added and spun again for 10 minutes at 4000rpm and 4ºC. The plate was inverted again on a blotting pad and spun for 1 minute at 400rpm, and finally dried overnight at RT or for 20 minutes at 60ºC before sending the plates for analysis on a capillary sequencing machine (service provided by Eurofins, Germany).

Construction of CRISPR gRNA expression plasmids

Annealing CRISPR gRNA oligos

CRISPRs were chosen using the WGE CRISPR search website, in that, the target site in the genome composed of the sequence complementary to the first 20 bases of the guide RNA plus the adjacent PAM sequence. The gRNA oligos were appended at their 5'ends with a 4-base sequence compatible with the BsaI cloning site of the U6_gRNA (AU flip) expression plasmid, containing a modified guide RNA backbone (based on Chen et al. 2013 by Koutsourakis & Skarnes, unpublished; refer Appendix Figure A9). The oligos were resuspended in TE at a concentration of 1µg/µl. 2.5µg of the forward strand gRNA oligo was annealed to the same amount of reverse strand (or complementary) oligo in 50µl of annealing buffer (10mM Tris, pH 7.5-8; 50mM NaCl; 1mM EDTA). The mixture was heated at 98ºC in a heating block for 5 minutes followed by cooling down slowly to RT inside the block. The annealed oligos were then diluted 1:10 in H_2O.

Cloning annealed gRNA oligos into U6_gRNA (AU flip) expression plasmids

Ten micrograms of U6_gRNA (AU flip) plasmid was digested with BsaI (NEB), overnight at 37ºC. The linearized plasmid was then purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. 20ng of each of the annealed diluted gRNA oligos and the linearized plasmid were ligated with Takara ligase/ buffer mix (Takara ClonTech) and incubated at RT for 1-2 hours. The ligated plasmids were then transformed into chemically competent DH5α cells and cultured overnight at 37ºC on LB agar plates containing 50µg/ml Kanamycin (kan) (Sigma).

Following transformation, overnight liquid cultures were set up in 2xLB containing 50µg/ml kan, and plasmids were extracted from the culture. Subsequently, diagnostic restriction digests were performed on the extracted plasmids and agarose gels run to confirm the presence of correct gRNA expression plasmids. The plasmids with correctly sized bands on an agarose gel were then subjected to sequencing reaction with U6_gRNA (AU flip) forward and reverse sequencing primers (listed in Appendix, Table A8) and ethanol precipitated to send for capillary sequencing. Bacterial cultures for CRISPR gRNA plasmids with perfectly accurate sequences were then grown in maxiprep cultures (100ml of 2xLB containing 50µg/ml kan) overnight in a 37ºC shaking incubator. The plasmids were extracted from the culture on the following day using QIAGEN Plasmid Maxi Kit as per the manufacturer's instructions, and the concentration determined from OD_{260nm} in a UV spectrophotometer. The plasmids were then used for subsequent nucleofection experiments.

InFusion cloning

pUC19_RV plasmid used for InFusion cloning is a modification of pUC19 (NEB) that contains a unique EcoRV linearization site (Koutsourakis & Skarnes, unpublished; refer Appendix Figure A10). Gene-specific PCR primers for InFusion cloning were designed with the WGE Gibson Designer (for AAAS KO experiment) or Primer 3 (for HTT experiment). Each primer was appended, at its 5' end, with a 15-base extension homologous to the ends of the linear pUC19_RV plasmid. Thus, the PCR products generated by these InFusion primers would contain ends that are homologous to those of the vector. The primers were resuspended in TE and 5µM of each was used to amplify the target fragment from 50ng of wild-type KOLF2_C1 genomic DNA with CloneAmp HiFi PCR premix (Takara Clontech) according to the manufacturer's instructions (see Appendix, Table A1 for PCR program). 10µg of pUC19_RV plasmid was digested with EcoRV (NEB) restriction enzyme and the linearized plasmid

purified using QIAquick PCR purification kit according to the manufacturer's instructions. The concentration of both the PCR fragment and backbone DNA were then quantified on an agarose gel. Approximately 50ng of the PCR-amplified fragment was mixed with 50ng of linearized purified pUC19_RV plasmid, and InFusion reactions were carried out with InFusion HD enzyme (Takara Clontech) for 15 minutes at 50ºC as per manufacturer's instructions. The InFusion reaction was transformed into 50µl Stellar competent cells (Clontech) and cultured overnight at 37ºC on LB agar plates containing 50µg/ml ampicillin (amp) (Sigma).

Overnight miniprep cultures were set up in amp-supplemented 2xLB for screening the resultant InFusion plasmids. Plasmids were extracted and diagnostic restriction digests were performed to confirm the presence of the correct InFusion clones by agarose gel electrophoresis. For clones showing the correct-sized bands, sequencing reactions were performed with pUC19 forward and reverse sequencing primers (listed in Appendix, Table A8), ethanol precipitated and sent out for capillary sequencing.

Construction of targeting donor vector for knockout of exon 2 of *AAAS* gene

The donor vector for targeting the *AAAS* gene was constructed in two steps by first introducing a Gateway cassette- Zeo/PheS (zeocin/phenylalanine tRNA synthetase; Skarnes et al. 2011) into the InFusion clone by recombineering (schematic diagram in Figure 2.1) and then introducing a drug selection cassette- pL1L2 EF1α-puro-polyA (Koutsourakis & Skarnes, unpublished; see Appendix A11) by performing a Gateway exchange reaction (schematic diagram in Figure 2.2).

Recombineering

I. Amplification of Zeo/PheS (Z/P) gateway cassette fragment

Initially, 70-mer recombineering primers (U5 and D3; see schematic diagram below in Figure 2.1) were designed, each consisting of 20 bases homologous to the Z/P Gateway element and 50 bases homologous to sequences flanking exon 2 of *AAAS* (listed in Appendix, Table A5). These 70-mer primers were used for amplifying the Z/P gateway cassette fragment (program in Appendix, Table A3) which was then subsequently inserted into the AAAS InFusion plasmid by homologous recombination in bacteria. The R1R2 flanked Z/P cassette in the recombineered AAAS-Z/P plasmid would serve as an intermediate substrate for the LR Clonase Gateway exchange reaction with pL1L2 EF1α-puro-polyA, forming the resultant AAAS-EF1α-puro-polyA targeting donor plasmid.

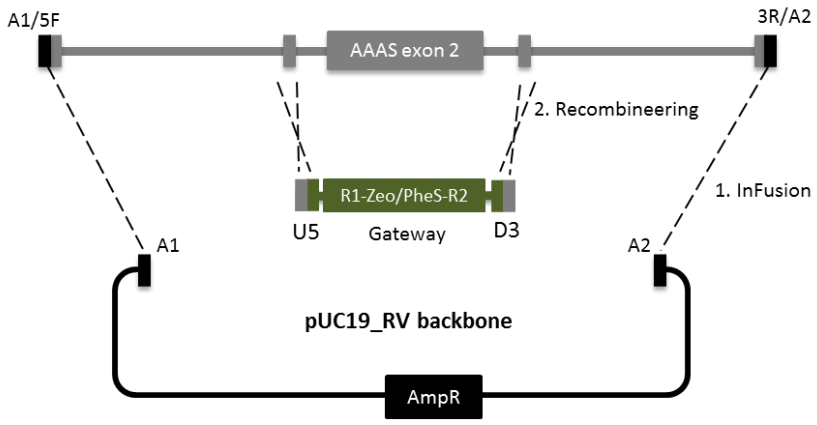

Figure 2.1: Construction of AAAS-Z/P plasmid by InFusion and recombineering for generating the final targeting donor vector for knockout of *AAAS* exon 2. 5F and 3R are InFusion PCR primers for amplification of *AAAS* exon 2.

For the amplification of Z/P cassette fragment, 10µM of each of the primers were used on 1ng of Z/P template using PrimeSTAR Max (Takara Clontech) according to the manufacturer's instructions. The PCR product fragment was then purified of any remaining primers using the Nucleospin PCR clean-up and gel extraction kit (Takara Clontech) following the manufacturer's instructions, and run on an agarose gel to estimate its concentration.

II. Transformation of recombineering-ready bacteria with the AAAS InFusion plasmid

The pBAD-gbaA plasmid (Wang et al. 2014) was transformed into DH10B bacteria and selected in tetracycline-containing 1xLB media and grown at 30^{o}C. pBAD-gbaA expresses the recombineering enzymes of the lambda phage red operon under the control of an arabinose-inducible promoter. The backbone, derived from pSC101, contains a temperature-sensitive origin of replication, and thus is lost from bacteria when grown at 37^{o}C.

a. Preparation of competent cells –

pBAD-gbaA bacterial cells were grown overnight at 30^{o}C in low salt LB (Sanger media kitchen) containing 5µg/ml tetracycline (tet) (Sigma). To prepare competent cells, the overnight culture was diluted 1:50 in 1ml low salt LB on the following day, and a 2.5-hour culture started in the same conditions as above. The culture was grown to mid-log phase (absorbance of the culture at OD_{600} = 0.2-0.4 and then incubated in ice/water slurry for 5 minutes. The cells were pelleted at 4000rpm for 5 minute at 4^{o}C and electrocompetent bacteria were made by washing the cells three times with ice cold HPLC-pure water.

b. Electroporation of AAAS InFusion plasmid

The electrocompetent cells were resuspended in 40µl water containing approximately 50ng AAAS InFusion miniprep plasmid and transferred to a 96-well cuvette placed on ice. The cells

were then electroporated at 2400V, 700Ω, and 25µF (time constants around 10msec) in a electroporator (BTX Harvard apparatus) and 50µl of 2xLB recovery media (containing 0.2% glucose without antibiotic) was added promptly into the cuvette well, while placed on ice. The media was mixed with the cell/DNA mixture by pipetting 4-5 times, and then transferred into a tube containing 500µl of recovery media (containing 0.2% glucose) without any antibiotic. The cells were then grown in 30^{o}C shaking incubator for 1 hour.

250µl of the above recovery culture was inoculated into 750µl of low salt LB containing 50µg/µl ampicillin (amp) and 5µg/µl tet, and incubated overnight in 30^{o}C shaker. On the following day, the overnight culture was diluted 1:50 in 1ml of low salt LB supplemented with tet and amp, and cultured for 2.5 hours in 30^{o}C shaking incubator.

c. Induction of the red operon with arabinose –

When the culture reached mid-log phase (absorbance at OD_{600} = 0.2-0.4), L-(+)-Arabinose 99% solution (99%; Sigma), to a final concentration of 0.2%, was added and left for 3-5 minutes in a 37^{o}C water bath. The culture was then transferred to a 37^{o}C shaking incubator and incubated for another 35 minutes. Arabinose addition induces the expression of recombineering proteins, red gamma, beta, and recA to allow subsequent homologous recombination to occur.

III. Transformation of arabinose induced bacterial cells with Z/P Gateway fragment

Electrocompetent cells were prepared from the arabinose induced cells as described above in section II (a). The pelleted competent cells were resuspended in 40µl water containing a total of 100-150ng of amplified Z/P Gateway fragment (amplified as described in section I) and electroporated as described in section II (b). However, these transformed cells were recovered at 37^{o}C shaking incubator for an hour.

250µl of the above recovery culture was inoculated into 750µl of low salt LB containing 50µg/µl amp and 6.5µg/µl zeocin (zeo) (Invitrogen), and incubated overnight in a 37^{o}C shaker. At this point, the amplified Z/P Gateway fragment is expected to have recombined with the InFusion plasmid using the 50-bp homology regions to result in the AAAS-Z/P Gateway (pAAAS-ZP) plasmid. The recombineered clones should be resistant to both zeo and amp. Plasmids were extracted from the culture using QIAprep spin miniprep and were re-transformed into DH5α and Stellar competent cells and cultured overnight at 37^{o}C on LB agar plates containing amp (50µg/µl) and zeo (6.5µg/µl); this step was performed to obtain pure colonies of cells that contain only the desired pAAAS-ZP plasmid. Miniprep cultures from single colonies were set up and plasmids were extracted. Diagnostic restriction digests with

different enzymes were performed on the extracted plasmid DNA to identify the correct pAAAS-ZP clones showing the expected sized bands on an agarose gel.

Gateway exchange reaction to produce the final AAAS donor vector

LR Clonase II enzyme (Invitrogen), containing bacteriophage lambda recombination proteins Integrase (Int) and Excisionase (Xis), catalyzes the *in vitro* recombination between *att*L-flanked EF1α-puro-polyA plasmid and *att*R-flanked Z/P cassette in the pAAAS-Z/P vector. This recombination results in the final *att*B-containing donor vector, AAAS-EF1α-puro-polyA (see Figure 2.2 below). The Gateway reaction can be represented as:

*att*L1–EF1α-puro-polyA–*att*L2 x *att*R1–Z/P–*att*R2 ⇔ *att*B1–EF1α-puro-polyA–*att*B2 x *att*P1–Z/P–*att*P2 (by product)

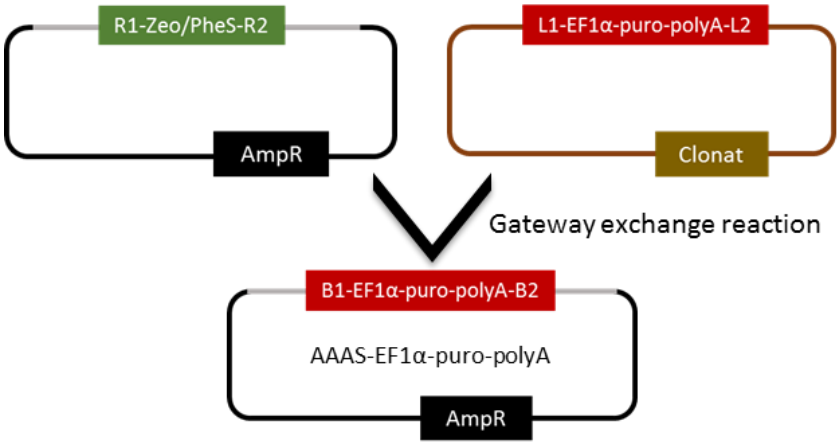

Figure 2.2: Construction of AAAS-EF1α-puro-polyA targeting donor vector by introduction of a drug selection cassette- pL1L2 EF1α-puro-polyA into AAAS-Z/P recombineered clone by LRII clonase Gateway exchange reaction

Gateway recombination reaction was set up with LR Clonase II enzyme (Invitrogen) by adding 100-200ng of AAAS-Z/P gateway plasmid DNA to 70ng of pL1L2 EF1α-puro-polyA plasmid in a total reaction volume of 10µl, and incubating at 25°C overnight as recommended by the manufacturer. After the overnight incubation, 1µl of proteinase K was added to the reaction and incubated for 10 minutes at 55°C to inactivate the Clonase enzyme. 2µl of the LR reaction mixture was then transformed into 35µl of DH10B competent cells (NEB) and cultured on YEG (yeast extract glucose plus P-chlorophenylalanine) agar plates (Sanger media kitchen) containing 50µg/ml amp. PheS (phenylalanyl tRNA synthetase) expression in *E. coli* is important in this selection in that, a mutant derivative of PheS is sensitive to p-chlorophenylalanine (Kast 1994). Thus, the mutants can be selected against in

YEG medium to kill PheS positive cells including the initial AAAS-Z/P Gateway plasmid and the by-product of LR Clonase II reaction.

From colonies on YEG-amp plate, overnight miniprep cultures were set up in 2xLB with 50µg/ml amp and plasmids extracted on the following day. Diagnostic restriction digests were set up to confirm the construction of the correct donor vectors that show the expected-sized bands on an agarose gel. The full annotated sequence of the final AAAS-EF1α-puro-polyA donor plasmid is shown in Appendix A12.

Construction of targeting donor vector for inserting extended Q-repeat in HTT gene

The HTT donor vector was constructed in two steps by InFusion cloning of exon 1 of the HTT gene and then cloning in a synthetic fragment containing an expanded Q-repeat domain.

InFusion cloning of HTT exon 1

InFusion cloning of HTT exon 1 into pUC19_RV plasmid (Figure 2.3) was performed in a similar manner as described earlier in InFusion cloning.

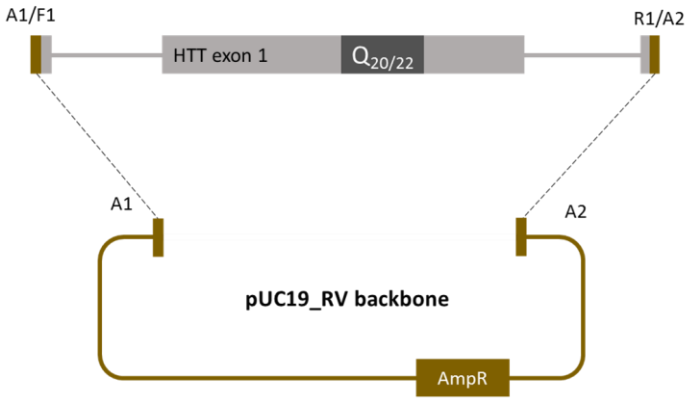

Figure 2.3: Construction of HTT InFusion clone, pHTT_exon 1

Design and synthesis of an extended Q-repeat fragment

A synthetic fragment containing 67-Q repeats in exon 1 of the HTT gene was synthesized and cloned into a kan-resistant plasmid backbone (GeneArt; refer Appendix A13). This extended repeat region was then cloned into the HTT InFusion plasmid as a BlpI/BsgI fragment (refer to Figure 2.4 below). This fragment contained mutations in PAM sites of the two CRISPRs used for the targeting experiment, so that Cas9-RNP did not cleave the donor plasmid itself, neither re-cleaved genomic DNA once the plasmid had been introduced successfully by homologous recombination.

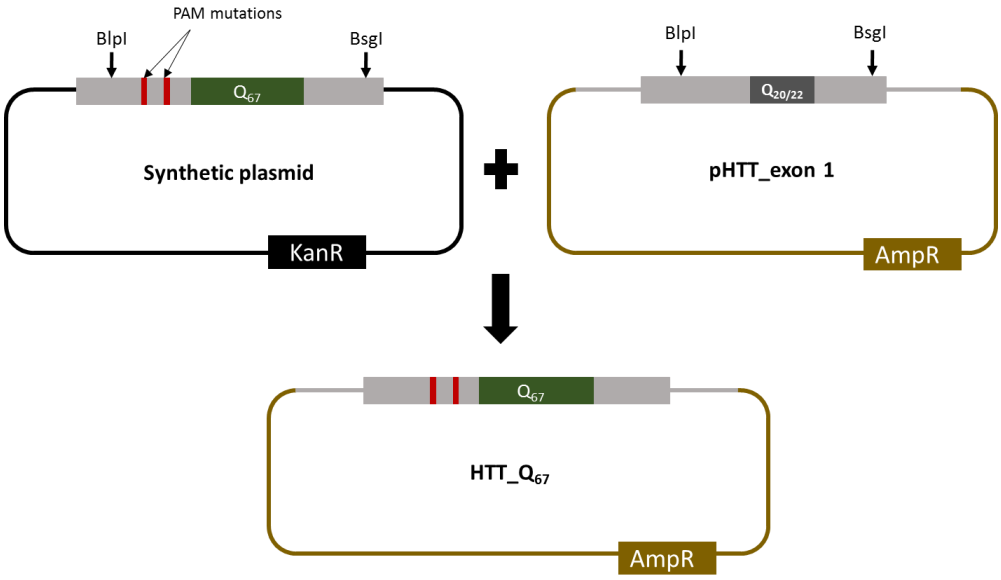

Figure 2.4: Construction of HTT_Q-67 donor plasmid by cloning in of a BlpI/BsgI digested Q67 synthetic fragment into pHTT_exon 1.

Cloning the extended Q-repeat fragment

Both synthetic and InFusion plasmids were first digested with the two restriction enzymes BlpI and BsgI. 2.5µg of HTT InFusion clone was digested in a total reaction volume of 50µl at 37°C for 3-4 hours. The backbone was then purified of the digested smaller fragment using QIAquick PCR purification kit following the manufacturer's instructions. Likewise, 5µg of the synthetic plasmid was digested in a total volume of 100µl under the same reaction conditions, and then run on 1.5% agarose gel. The extended fragment was then cut off from the gel under a UV box using a scalpel and extracted using QIAquick Gel Extraction Kit (QIAGEN) according to the instructions provided by the manufacturer. The concentrations of the fragments were then estimated on an agarose gel. Equal amounts of the synthetic fragment and the InFusion backbone were ligated together using Takara ligase/buffer mix (ClonTech) in a total reaction volume of 10µl at RT for 2 hours. This ligation reaction was then transformed into DH5α competent cells and cultured on ampicillin LB plates overnight at 37°C.

Plasmids were extracted from overnight miniprep cultures and restriction digested to diagnose the correct targeting donor plasmids from their bands on an agarose gel before they were sequenced with HTT-specific sequencing primers (listed in Appendix, Table A7). The plasmid DNA containing the perfectly matched sequences were then prepared from maxiprep cultures and extracted using QIAGEN Plasmid Maxi Kit as per the manufacturer's instructions. This donor plasmid was then used for subsequent targeting experiments in KOLF2-C1 cells.

In vitro Cas9 nuclease assay

Equimolar amounts of crRNA and tracrRNA (IDT) were annealed by heating them together at 58°C for 10 minutes and cooling down slowly to RT. The annealed cr:tracrRNA was diluted to 300nm, and added with 1µM Cas9 Nuclease NLS (NEB), both to a final concentration of 30nM in 1x Cas9 buffer. After incubating this reaction at RT for 10 minutes, InFusion plasmid DNA was added to a final concentration of 3nM and incubated at 37°C for an hour. Following this, the reaction was run on agarose gel for determination of synthetic gRNA activity.

T7 Endonuclease (T7E1) assay

T7E1 assay is often used to detect Cas9 activity in experiments. The T7E1 enzyme recognizes and cleaves non-perfectly matched DNA and heteroduplexes which are formed as a result of Cas9 activity. The cleaved products can then be run on a gel and detection of multiple bands would indicate that T7E1 cleaved the heteroduplexes which were formed due to Cas9 activity. For this assay, PCR reactions were set up with 100ng genomic DNA from Cas9-modified and wild-type cells using 20pmol of the gene-specific primer pairs (Table 2.2). The PCR amplicons were purified using QIAquick PCR purification kit and run on agarose gel to estimate their concentration. 200ng of the DNA in 19 µl NEBuffer 2 (NEB) was heated for 10 minutes at 95°C, then slowly cooled down to RT inside the block. To the reaction, 1µl of T7E1 enzyme (NEB) was added and incubated at 37°C for 15 minutes, after which the reaction was stopped by adding 0.75µl of 0.5M EDTA. The reaction was then run on an agarose gel to estimate the Cas9 activity.

PCR genotyping of human iPS cell lysates

For genotyping of cell lysates, PCR primers were first tested on wild-type KOLF2-C1 cells in order to find out the best primer combination that gave only the expected amplicon and no non-specific bands on an agarose gel.

PCR amplification of lysates

The cells in the wells of a confluent 96-well plate (BD Biosciences) were washed with 200µl DPBS per well. Discarding DPBS, 100µl of yolk sac lysis buffer (50mM KCl/ 10mM Tris-HCl pH 8.3/ 2mM $MgCl_2$/ 0.45% IGEPAL CA-630 (Sigma)/ 0.45% Tween-20 (Sigma)) with 1-1.5mg/ml of freshly added proteinase K was added per well. The plate was sealed and incubated in 58°C incubator for 4 hours to overnight, after which it was heated at 95°C on a heating block for 10 minutes to inactivate proteinase K. The crude lysates were then diluted 1:10 or 1:20 in

10mM Tris-HCl, pH 8, depending on their confluency. PCR reaction mix (Table 2.2) was used to set up a 10µl PCR reaction with 2.5µM of PCR primers and approximately 100ng of DNA. Short-range or mid-range PCR (see Appendix, Table A2 for program) was used to amplify the DNA depending on the length of the amplicon.

Table 2.2: PCR reaction mix for genotyping human iPS cell lysates

Component	**Volume (µl) for 1x reaction**
H_2O	2.1
5x LongAmp Taq buffer (NEB)	2
10mM DNTP (Thermoscientific)	0.3
100% DMSO (Sigma Aldrich)	0.2
LongAmp Taq DNA polymerase (NEB)	0.4
Total	**5 µl**

Purification of PCR amplicons

The PCR amplicons were purified with exonuclease I (NEB) and phosphatase (NEB) in successive reactions to remove the nucleotides and phosphate groups from the ends of DNA strands. In this reaction, 10µl of the PCR reaction was diluted 1:2.5 in H_2O and half of the diluted PCR amplicons was added with exonuclease reaction mix (Table 2.3). This mixture was then heated at 37ºC for 30 minutes followed by 80ºC for 15 minutes. Phosphatase reaction mix (Table 2.3) was then added to the mixture and heated again in the same manner as with exonuclease I. The resulting single-stranded DNA molecules were then diluted 1:4 in H_2O and used in subsequent sequencing reactions.

Table 2.3: Exonuclease and phosphatase reaction mixes for purifying PCR amplicons

Component	**Volume (µl) for 1x exo reaction**	**Volume (µl) for 1x phos reaction**
H_2O	3.2	2.85
10x exo-phos buffer	1.5	2
Exonuclease I (NEB)	0.3	–
Phosphatase (NEB)	–	0.15
Total	**5 µl**	**5 µl**

* 10x exonuclease-phosphatase (exo-phos) buffer (100mM $MgCl_2$/ 200mM Tris-HCl, pH 8)

Sequencing and ethanol precipitation

For the sequencing reaction, equal volumes of exonuclease-phosphatase (exo-phos) purified DNA and sequencing reaction master mix (Table 2.1) were added together in processes similar to those described earlier in the section describing sequencing. Ethanol precipitation was then

used to precipitate the samples (described in section ‘Sequencing and ethanol precipitation of samples’) prior to sending them for capillary sequencing.

Western blot

Protein lysis

The cells in a well of a 6-well plate were first washed with 3ml DPBS and the plate was placed on ice. 0.3ml of ice cold protein lysis buffer (50mM Tris, pH 7.5/ 0.5M NaCl/ 1% IGEPAL CA-630/ 1% sodium deoxycholate (Sigma Aldrich)/ 0.1% SDS (Ambion)/ 2mM EDTA/ Complete protease inhibitors (Roche)) was then added per well and incubated on ice for 5 minutes with occasional shaking. The lysate was then scraped using a cell lifter (Corning) and transferred into a 1.5ml microfuge tube to shear the DNA present in it in successive processes or stored in –70^{o}C freezer.

Shearing DNA in protein extracts

Lysates, thawed on ice, were spun through QIAshredder column (QIAGEN) at the maximum speed for 2 minutes at 4^{o}C. The flow-through was transferred into a 1.5ml tube and spun for another 10 minutes at 13000rpm and 4^{o}C. The resulting supernatant was transferred carefully into another tube, avoiding any DNA pellet at the bottom of tube, and stored on ice or at -70^{o}C until further use.

Protein quantitation

I. Quantitation of BSA (Bovin serum albumin) standards

BSA (Sigma Aldrich) with stock concentration of 1mg/ml was diluted by 1:2 in 100µl of protein lysis buffer and continued to obtain a series of dilutions, each time diluting 100µl of the preceding standard with another 100µl protein lysis buffer in the tube following it. 100µl of these serially diluted BSA standards (and a control containing 100µl protein lysis buffer only) were each mixed with 2ml Bicinchoninic Acid (BCA; Sigma Aldrich) made with BCA reagent A to reagent B ratio of 25:1. BCA contain copper (II) ions which are reduced to copper (I) ions by proteins, in a concentration dependant manner. The resulting copper (I) complex has an absorbance maximum at 562nm which is directly proportional to the concentration of the solution. Thus, absorbance of proteins is an indicator of the total protein content in a solution. The mixture was heated for 30 minutes at 37^{o}C and then cooled to RT. The OD_{562nm} of the solutions were then measured in a UV-spectrophotometer. From the absorbance values

and their corresponding concentrations, a graph was constructed, the best-fitted line of which was used for determination of concentration of the sample protein extracts.

II. Quantitation of sample protein extracts

The protein extracts were diluted 1:8 in protein lysis buffer, and 100µl of the diluted extract was mixed with 2ml BCA (like the standards) and heated likewise at 37^{o}C for 30 minutes. The samples were cooled and their absorbance at OD_{562nm} then measured in a UV-spectrophotometer. Using the graph constructed from the BSA standards, the concentrations of the sample protein extracts were determined.

SDS-PAGE

Equal amounts of each of the protein samples were measured out. Sample loading buffer (Invitrogen), equal in volume to that of the protein sample in a tube, was added. The mixture was heated in a heating block at 85^{o}C for 5 minutes, shortly spun, and loaded on a pre-cast NuPAGE^{TM} Novex^{TM} 4-12% bis-tris polyacrylamide gel (Invitrogen), along with 6µl of a protein standard (BIORAD). The gel was run in NuPAGE^{TM} Novex^{TM} SDS-PAGE gel system (Invitrogen) initially at 85V for 15 minutes and then at 100V until the dye front reached the bottom end of the gel.

Trans-blotting

One litre of 1x transfer buffer was prepared from 10x Tris/glycine buffer (BIORAD) with 200ml of methanol and MilliQ water. PVP membrane (Millipore) was soaked in methanol for 20 seconds followed by washing off the methanol in MilliQ water for 2 minutes. The membrane was then soaked in transfer buffer.

Cracking open the plates, the gel was taken off carefully on a wet filter paper soaked in transfer buffer and arranged in a sandwich (Figure 2.5) with the membrane facing the anode. The Mini Trans-Blot Electrophoretic Transfer Cell (BIORAD) was run at 150V for 2 hours for transfer of proteins onto the PVP (polyvinylpyrrolidone) membrane. After transfer, the membrane was peeled off the gel carefully and washed in transfer buffer and dried overnight, by placing between two pieces of semi-wet filter paper.

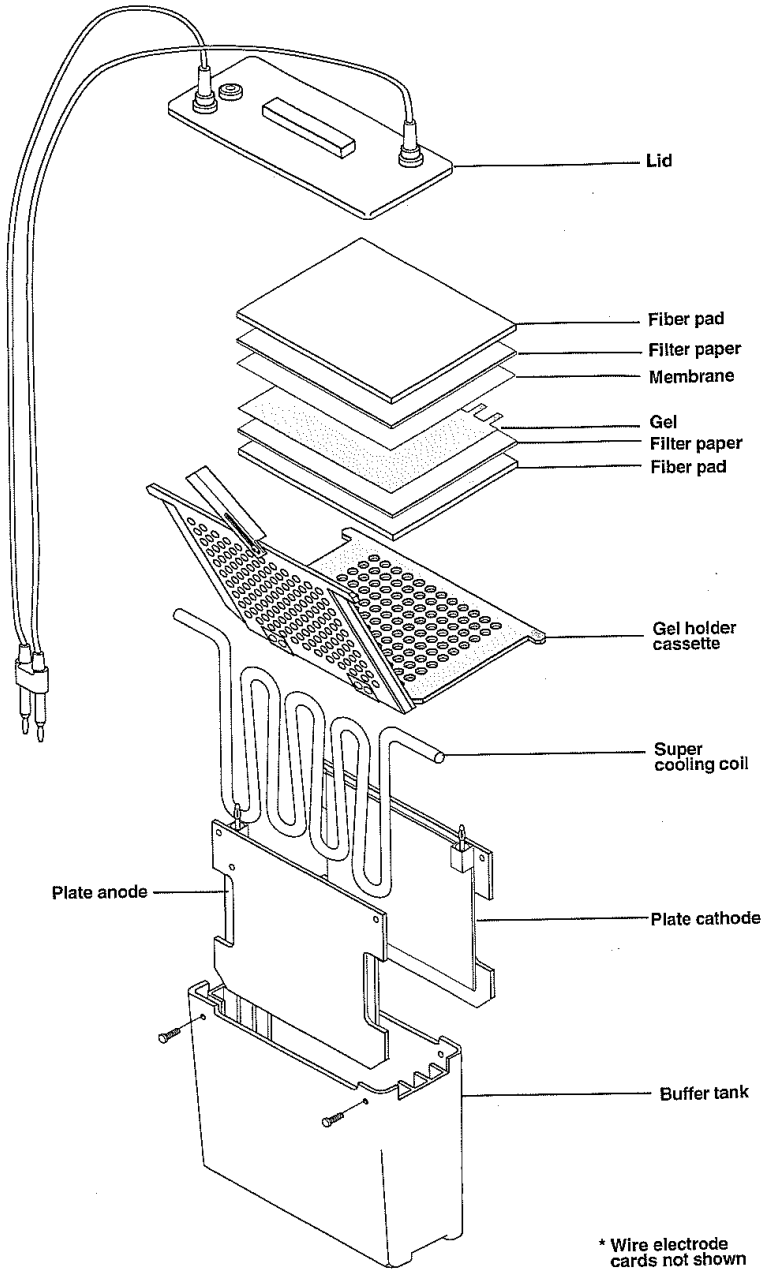

Figure 2.5: Trans-blot cell description and assembly of parts

Blocking

1xTBS (Tris buffered saline) was first prepared from 10xTBS (BIORAD) in MilliQ water. TBS-T (TBS-Tween) buffer containing 0.1% Tween-20 (Sigma) was then prepared from 1xTBS buffer. Blocking buffer was made by adding 5% w/v dried skimmed milk powder (Marvel) in TBS-T buffer and stored at 4^{o}C. The overnight dried membrane was briefly soaked in methanol and then washed in distilled water for 2 minutes. Following the wash, it was soaked

in 50ml of 1x TBS for 15 minutes and then incubated in a tray containing 30ml of 5% blocking buffer for 3-4 hours at RT on an orbital shaker.

Primary antibody binding

ALADIN polyclonal antibody, Q9NRG9 (Proteintech) was diluted 1:500 in blocking buffer from a stock concentration of ~0.5mg/ml. The membrane was then folded in a 50-ml falcon tube, and incubated in 5ml of the primary antibody solution overnight at 4^{o}C (in a cold room) on a rolling shaker. Following the incubation, the membrane was first briefly rinsed with two changes of TBS-T wash buffer. The membrane was then washed 4-6 times in approximately 25ml of TBS-T for 5-10 minutes each at RT on a rolling shaker.

Secondary antibody binding

The secondary anti-rabbit IgG antibody (Amersham Biosciences) was diluted 1:20,000 in 10ml of 5% blocking buffer and the membrane was then incubated in this antibody solution in a falcon tube for around an hour at RT on a rolling shaker. Following this secondary antibody binding, the membrane was briefly rinsed with two changes of TBS-T wash buffer, and then 4-6 times for 5-10 minutes each at RT on a rolling shaker.

Detection

Chemiluminescence detection reagent (Amersham Biosciences) was prepared by adding an equal volume of solution A, luminol enhancer to that of solution B, peroxide. The membrane was incubated in 4ml of this solution at RT for 5 minutes on a rolling shaker, then wrapped in cling film and exposed onto a chemiluminescent detection film (Roche) inside an X-ray cassette (Amersham Biosciences). The membrane was then soaked in TBS-T and stored at 4^{o}C until further use.

GAPDH loading control

The membrane was incubated in blocking buffer in a 50-ml falcon tube at RT for 2-3 hours in a rolling shaker, and the blocking buffer was then discarded. Monoclonal GAPDH-71.1 mouse primary antibody (Sigma-Aldrich; 1 mg/ml) was diluted 1:4000 in 5ml blocking buffer and the membrane was incubated in it overnight at 4^{o}C. On the following day, the membrane was briefly rinsed with two changes of TBS-T wash buffer, and then 4-6 times for 5-10 minutes each at RT on a rolling shaker.

Secondary anti-mouse IgG antibody (Amersham) was diluted 1:20,000 in 10ml of blocking buffer and the membrane was then incubated in this antibody in a falcon tube for at least an hour at RT on a rolling shaker. Following this incubation, the membrane was briefly rinsed with two changes of TBS-T wash buffer, and then 4-6 times for 5-10 minutes each at RT on a rolling shaker. The membrane was incubated in chemiluminescence detection reagents and exposed in an X-ray cassette as described in section 'Detection' above.

Tissue culture techniques

In general, gene targeting in human iPS cells was carried out using two strategies – plasmid-based and protein-based nuclease-assisted strategies. In the plasmid-based strategy, the cells were co-transfected with CRISPR gRNA expression plasmids, Cas9 nuclease expression plasmid, and targeting or donor vector plasmids. In the protein-based strategy, cells were transfected with pre-assembled Cas9-RNP and targeting plasmids or ssODN, where applicable.

Plasmid-based nuclease-assisted gene targeting of human iPS cells

Pre-nucleofection –

Plasmid preparation: The amounts of maxiprep plasmids required for nucleofection were first determined, considering the maximum amount that can be used in nucleofection to avoid significant cell death, is 12µg. 4µg of Cas9 nuclease expression plasmid (Addgene) 3µg of each of the gRNA expression plasmids, and 2µg of targeting vector plasmid (where applicable) were precipitated. NaCl was added to the plasmid mixture to a final concentration of 100mM in 100µl. 250µl of 100% ethanol was then added and incubated on ice for 5 minutes followed by centrifugation at top speed for 10 minutes. The supernatant was then carefully discarded in a tissue culture hood without disturbing the pellet. 1ml of 70% ethanol was then added to the pellet, and after spinning for 5 minutes, it was discarded; this step was repeated once more, and then the pellet was dried for 20 minutes. Finally, the semi-dried pellet was resuspended overnight in DPBS at RT at a concentration of 1µg/µl to be used subsequently for nucleofection.

Culture plates were coated with Synthemax II-SC substrate (Corning) diluted in sterile distilled water (Life Technologies) at a final concentration of 25µg/ml for 2 hours at RT. Accutase (Millipore) and DPBS were warmed to 37^{o}C in a water bath.

Nucleofection – Human iPS cells (KOLF2_C1) were grown to an almost confluent 10cm petri dish. The cells were washed with 10ml pre-warmed DPBS, and then incubated in 5ml pre-

warmed Accutase for 8-10 minutes in 37ºC/5%CO_2 to detach the cells thus obtain a single cell suspension. Following the incubation, Accutase was aspirated and 5ml of complete TeSR-E8 medium (Stem Cell Technologies) supplemented with 10µM ROCK inhibitor (Ri) (Stem Cell Technologies) was added to the plate; ROCK inhibitor enables survival of cells in single cell suspensions by preventing cell migration and dissociation-induced apoptosis. The cells were detached using a cell lifter, dissociated into a single cells by pipetting up and down 3-4 times, and collected in a universal tube (Sterilin). The cells were counted in a haemocytometer and the cell density was determined. The appropriate volume of cell suspension containing 3.5 million cells, to be used for nucleofection, was then centrifuged at 1000rpm for 3 minutes at RT to pellet the cells. Subsequently, the pellet was resuspended in 100µl of AMAXA Nucleofector Solution 2 (Solution 2 to supplement ratio of 4.5:1) (Lonza). The suspension was then mixed with plasmids, and promptly transferred into an AMAXA nucleofection cuvette (Lonza) and electroporated in the AMAXA Nucleofector 2B device (program B-016; Lonza). Using an AMAXA Pasteur pipette (Lonza), approximately 500µl of pre-equilibrated TeSR-E8 media was added to the cuvette, and finally the cell/DNA mixture was transferred immediately into a well of 6-well plate containing 3ml of pre-equilibrated TeSR-E8 media supplemented with 10µM Ri. The cells were cultured in 37ºC/5%CO_2 incubator.

Post-nucleofection – After the cells had been in ROCK inhibitor for 24-hours, the media was changed to TeSR-E8 only until the well was confluent for subsequent experiments. In case of drug selection (puromycin, as used in this study), the media was supplemented with puromycin on the 3rd day after nucleofection. In a titration experiment, 0.3µg/ml was the concentration of puromycin required to kill wild-type cells (Koutsourakis & Skarnes, unpublished data).

Protein-based nuclease-assisted gene targeting of hiPS cells

Pre-nucleofection – Pre-assembly of the annealed cr:tracrRNA to Cas9 nuclease was done in two steps:

I. Pre-annealing: crRNA and tracrRNA were added together in a 1:1 molar ratio and concentration of 2µg/µl. The mixture was then heated at 58ºC for 10 minutes and allowed to cool slowly to RT.
II. Pre-assembly: 8µg of the pre-annealed cr:tracrRNA was added with 12µg of Cas9 nuclease (Feldan) and incubated at RT for at least 15 minutes. Following the incubation, 500pmoles of ssODN or 4µg of supercoiled targeting vector plasmid,

resuspended in 2µl DPBS, was added to the pre-assembled Cas9 ribonucleoprotein complex (Cas9-RNP) and used for subsequent nucleofection.

Besides, culture plates were coated with Synthemax II-SC substrate at a final concentration of 25µg/ml for 2 hours at RT.

Nucleofection – KOLF2_C1 cell line of hiPS cells were grown to an almost confluent 10-cm dish (Thermoscientific). The cells to be used for protein nucleofection were treated in the same manner as those in the plasmid-based nucleofection described above (refer to section for plasmid-based nucleofection). However, $8x10^5$ cells were electroporated for one nucleofection which were spun at 1000rpm for 3 minutes at RT. The cell pellet was resuspended in 100µl of AMAXA Nucleofector Solution 3 (Solution 3 to supplement ratio of 4.5:1) (Lonza) and mixed with the pre-assembled Cas9-RNP and ssODN or targeting donor plasmid. The mixture was then transferred into an AMAXA nucleofection cuvette (Lonza) and electroporated using the AMAXA Nucleofector 4D device (Lonza) (program CA-137). Subsequently, using an AMAXA Pasteur pipette the transfected cells were transferred from the cuvette to a 6-well plate, pre-coated with Synthemax, containing 3ml pre-equilibrated TeSR-E8 media supplemented with 10µM Ri (TeSR-E8+Ri). The cells were grown in $37^oC/5\%CO_2$ incubator.

Post-nucleofection – The cells were fed with TeSR-E8 only, on the next day, until confluent or ready for seeding and successive experiments.

Culture and passaging of human iPS cells

The cells in a 10-cm dish or 6-well plate were first washed with DPBS (10ml for 10-cm dish; 3ml for 6-well plate) and then incubated in ReLeSR (Stem Cell Technologies), an enzyme-free reagent for gentle dissociation of cell aggregates, for 2 minutes at RT; the volume of ReLeSR used is generally 5ml in 10-cm dish and 1.5ml in a well of 6-well plate. Aspirating ReLeSR, the plate was incubated again for another 2-3 minutes and TeSR-E8 media was then added. With a cell lifter, the colonies were detached from the surface of the dish and broken into small cell clumps by triturating 2-3 times. The cell clumps were plated at the appropriate cell density onto a 10-cm dish or 6-well plate, pre-coated with 25µg/ml Synthemax II-SC substrate, and containing TeSR-E8 media. The cells were then incubated at $37^oC/5\%CO_2$, and fed until they were ready for successive experiments.

Colony picking of human iPS cells

I. Seeding single cells

The cells on a 6-well plate were first washed with 3ml of DPBS after which 1.5ml of Accutase, pre-warmed at 37ºC, was added into the well. Following incubation for 10 minutes in 37ºC/ 5% CO_2 incubator, Accutase was aspirated and the cells were collected in 3ml of TeSR-E8 medium with 10µM Ri (TeSR-E8+Ri) in a universal bottle; at this point, the cells were dissociated into a single cell suspension by triturating. The cells were then counted under a microscope, and 1500-3000 single cells were plated onto a Synthemax coated 10-cm dish containing 10ml TeSR-E8+Ri, and conditioned media to $1/10^{th}$ of the total volume. Conditioned media (CM) was collected from near-confluent cultures of KOLF2-C1 cells grown overnight and then filter sterilized. CM contains unknown secreted factors that enhance the growth and survival of iPS cells in single cell suspension (Skarnes, unpublished observation). The cells were then grown in Ri supplemented media for two days. From Day 3 onwards, the cells were fed everyday with 10ml of fresh TeSR-E8 media only, until they were ready for picking, having diameters of approximately 1mm.

II. Colony picking

For a set of 96 colonies to be picked, two flat-bottom 96-well plates were treated: one with Synthemax II-SC substrate to be used for genotyping and another with Matrigel hESC-qualified matrix (BD biosciences) for archiving the clones. 1mg/ml Synthemax was diluted 1:40 in sterile distilled water and 50µl was used to coat each well for 2 hours at RT. Matrigel was thawed on ice and diluted to a factor of approximately 1:95 in cold DMEM/F12 (Dulbecco's Modified Eagle's Medium; Life Technologies), a basal medium for iPS cell culture. 50µl of the diluted Matrigel was used to coat each well for at least an hour at RT. Matrigel was aspirated just prior to use, immediately added with 100µl of TeSR-E8+Ri per well to avoid drying out, and incubated at 37ºC/5%CO_2 until cells were plated on them. Besides, a round-bottom 96-well plate was added with 50µl TeSR-E8+Ri per well and kept in 37ºC/5%CO_2 until ready to be used in picking.

The 10-cm dish containing colonies to be picked was washed with 10ml DPBS, and 5ml of ReLeSR was added. After 5-minute incubation at RT, ReLeSR was aspirated and 10ml TeSR-E8+Ri was added to the plate. Colonies were picked in 50µl media under a stereoscope, and then transferred into the round-bottomed well plate containing 50µl media with 10µM Ri.

Following picking the required number of colonies, they were broken into smaller clumps by triturating extensively and then transferred into the Matrigel coated flat-bottomed 96-well plate containing 100µl TESR-E8+Ri per well. The cells were mixed and then 100µl media with cells was transferred into the Synthemax coated flat-bottomed 96-well plate. The cells were then fed with 200µl TeSR-E8 only until the plates were 80% confluent, and ready for archiving and genotyping. A schematic diagram for picking into each well of a 96-well plate is as follows:

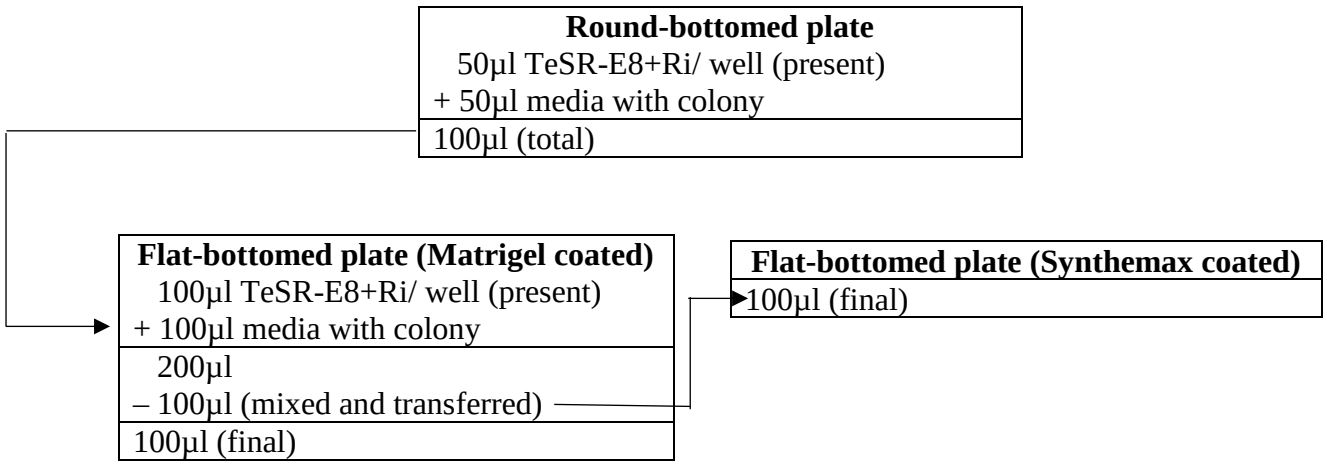

Figure 2.6: Schematic representation of a well on 96-well plate for creating plate replicas for genotyping and archiving

Colony archiving of human iPS cells into a 96-well matrix plate

The cells in the wells of a 96-well plate were first washed with 200µl of DPBS per well. 25µl of Accutase, pre-warmed at 37^{o}C, was added per well and incubated in 37^{o}C/ 5% CO_2 incubator for 10 minutes. Without aspirating Accutase, 175µl of knockout serum replacement (KSR) (Gibco Life Technologies) containing 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich) and pre-sterilized through a 0.2µm filter (Whatman), was added per well; DMSO is a cryoprotectant vitrification agent which preserves cells protecting them from mechanical injury. The cell mixture was then triturated extensively to obtain a single-cell suspension. The total volume of 200µl cell suspension was then transferred into the vials of a 96-well matrix plate (Thermoscientific) and overlaid with 100µl of mineral oil (Sigma Aldrich), pre-sterilized using a 0.2µm filter unit (Corning); the purpose of mineral oil is to prevent evaporation of medium. The plate was stored in -70^{o}C freezer and then transferred into liquid nitrogen storage after 1-2 days.

Expanding hiPS cells from 96-well matrix plate cryovials into 6-well plate

The mini cryovial containing the frozen hiPS cells was thawed in hand and the contents were transferred into a universal tube containing 5ml TeSR-E8+Ri. The cells were pelleted at 1000rpm for 3 minutes and then the pellet gently resuspended in another 4ml of TeSR-E8+Ri. The cells were plated onto a Synthemax-treated well of a 6-well plate and incubated in 37^{o}C/ 5% CO_2 incubator. They were fed with TeSR-E8 media until ready for subsequent experiments.

Archiving human iPS cells into 1ml cryotubes

The cells in a well of 6-well plate were first washed with 3ml DPBS, followed by incubation in 1.5ml pre-warmed Accutase for 10 minutes at 37^{o}C/ 5% CO_2. Aspirating the Accutase, the cells were collected in 3ml of TeSR-E8 media and then pelleted at 1000rpm for 3 minutes. The pellet was resuspended in 3-4ml of KSR containing 10% DMSO pre-sterilized using a 0.2µm filter. They were stored in 1ml cryotubes (Thermoscientific), in 0.5ml aliquots, at -70^{o}C freezer and transferred to liquid nitrogen after 1-2 days for long term storage.

Expanding hiPS cells from 1ml cryotubes into 6-well plate

The cryotube was first warmed up in hand and the thawed contents were transferred into a universal tube containing 5ml TeSR-E8+Ri. The cells were centrifuged at 1000rpm for 3 minutes after which the pellet was resuspended in 4ml TeSR-E8+Ri by triturating gently. The cells were then transferred into a well of 6-well plate pre-coated with Synthemax and cultured for subsequent experiments.

Subcloning mixed clones

Mixed clones containing cells with more than one genotype were subcloned. In this process, 1500 cells were primarily seeded on a 10-cm dish (refer to section describing seeding) and grown until they were ready for picking. The cell density was kept low in this procedure so as to obtain pure and well-separated colonies. Generally, 16 colonies were picked (refer to section describing picking) and used for subsequent processes of archiving and genotyping.