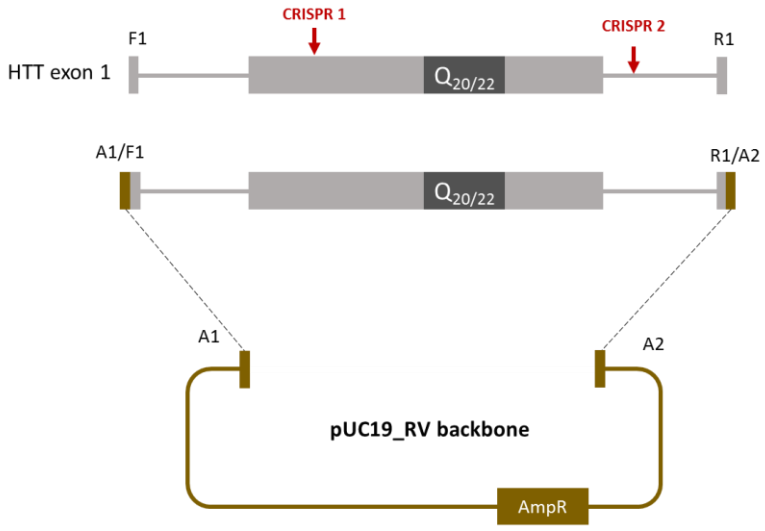
Chapter 4

Huntington’s disease

Insertion of extended Q-repeat (Q_{67}) fragment in HTT exon 1 (HTT1)

In this part of the study, the sequences of the alleles of HTT gene present in KOLF2-C1 cells were first determined. For this purpose, a genomic region containing HTT exon 1 (~1.3kb) was cloned into pUC19_RV backbone (~2.7kb) by InFusion cloning, forming an approximately 4kb plasmid, pHTT_exon 1 (Figure 4.1 (a)). Sequencing of multiple plasmids showed that there were two haplotypes for this gene with different number of Q-repeats in KOLF2_C1 cells (Figure 4.1 (b)). Compared to the reference sequence, which contains 21 Q-repeats (Ensembl), the two alleles of HTT in KOLF2-C1 cells contain 20 and 22 Q repeats, respectively.

(a)



(b)

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5′- CTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG---CAACAGCCGC Q21(ref)
5′- CTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAACAGCCGC Q22
5′- CTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG------CAACAGCCGC Q20
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Figure 4.1: **Sequencing of the HTT alleles of KOLF2-C1 cells.** (a) Construction of InFusion clone, pHTT_exon 1. (b) Sequences of alleles with 20 and 22 Q-repeats (CAG-repeats; indicated in red) in KOLF2_C1 genome aligned with the reference sequence from Ensembl.

A pair of CRISPRs were tested in KOLF2-C1 cells to initially delete a region of HTT exon 1 by NHEJ before attempting to insert the extended Q_{67} repeat fragment into the exon by homologous recombination (Figure 4.2). The deletion would not have any effect on the gene function because the gene sequence deleted along with the wild-type repeat length ($Q_{20/22}$) can be determined by sequencing; this deleted gene sequence can then be inserted into the DNA along with the extended fragment by homologous recombination with a donor plasmid. The cells were co-transfected in separate plasmid-based and protein-based experiments. In the

plasmid-based experiment, the cells were co-transfected with Cas9 nuclease and CRISPR gRNA expression plasmids. This plasmid-based experiment was also attempted using trans-puromycin selection, that is, the delivery of a puro-selection plasmid along with the CRISPR and Cas9 plasmids during transfection, to select for the desired clones. On the other hand, in the protein-based experiment, cells were transfected with Cas9-RNP, pre-assembled from crRNA, tracrRNA, and Cas9 protein purchased commercially.

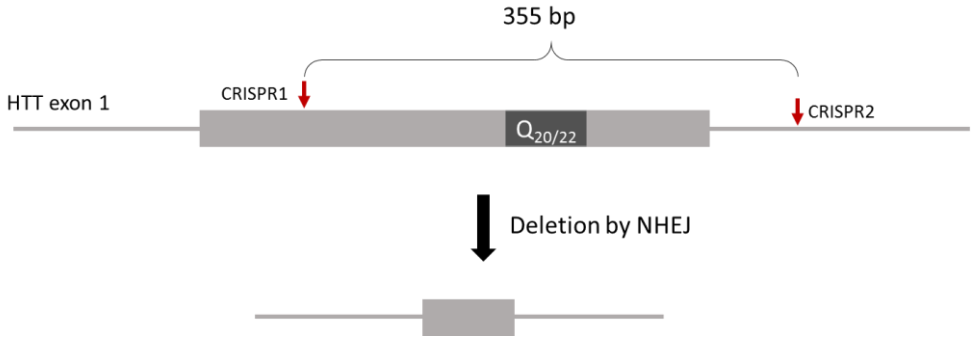
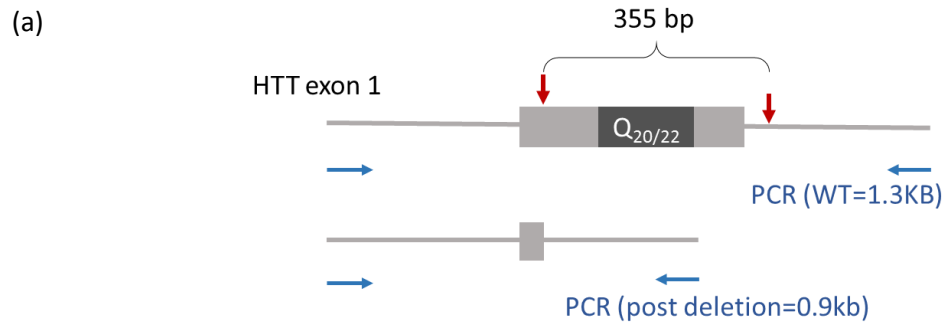


Figure 4.2: **Schematic representation of deletion of HTT exon 1 by paired CRISPRs (1 & 2)**. The deletion is for subsequent insertion of the extended Q_{67} repeat fragment

DNA extracted from transfected cells were assayed for deletion by NHEJ along with wild-type KOLF2_C1 DNA as a control. The length of the PCR amplicon from wild-type KOLF2_C1 DNA was ~1.3kb and that from the cells after deletion was expected to be 938bp (1293- 355bp). Thus, the presence of a 938bp band in a cell pool would indicate activity of both the CRISPRs and hence the 355bp deletion. However, as the *in vivo* assay demonstrates (Figure 4.3), the expected deletion did not occur in both the plasmid and protein based experiments. This implies that either or both CRISPRs were not functional in cleaving the DNA. The trans-puromycin selection also did not seem to affect in enrichment of the cells containing the deletion.



(b)

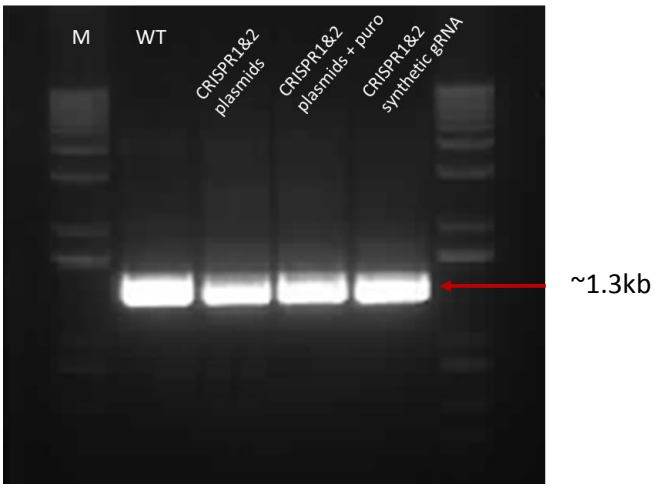
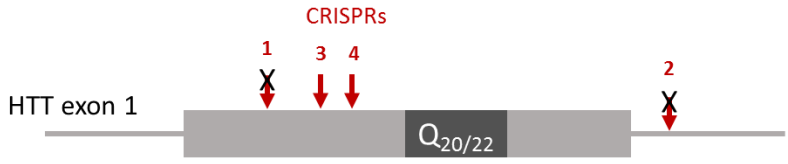


Figure 4.3: ***In vivo* assay for detection of activity of paired CRISPRs on HTT exon 1 of KOLF2_C1 genomic DNA.** (a) Schematic illustration showing the region of HTT exon 1 amplified by PCR (primer positions indicated in blue arrows) to detect for the 355bp deletion by paired CRISPRs flanking the Q-repeat region (indicated in red arrows). (b) Gel image for the assay of 355bp deletion with paired CRISPRs (1&2).

Two new CRISPRs (CRISPRs 3 and 4; Figure 4.4 (a)) were selected for introducing the extended HTT_Q_{67} repeat into the exon by homologous recombination. The activities of these CRISPRs along with the original pair were tested *in vitro* by measuring cleavage of the pHTT_exon 1 plasmid. It is worth mentioning here that *in vitro* Cas9 assay was done instead of a T7E1 assay because HTT exon 1 had two alleles in KOLF2_C1 which would have formed heteroduplexes in T7E1 assay giving misleading results. The *in vitro* Cas9 digestion assay (Figure 4.4 (b)) demonstrated greater activity for CRISPRs 3 and 4 when compared to little or no detectable activity for CRISPRs 1 and 2. This was suggested by the presence of a slower migrating band for these new CRISPR gRNAs, indicating the pHTT_exon 1 plasmid was linearized. This band was, however, very faint using the two previous CRISPR guides. Thus, the *in vitro* Cas9 assay implies that the new guides were active in Cas9 cleavage of the plasmid, and that they were suitable for transfection into iPS cells.

(a)



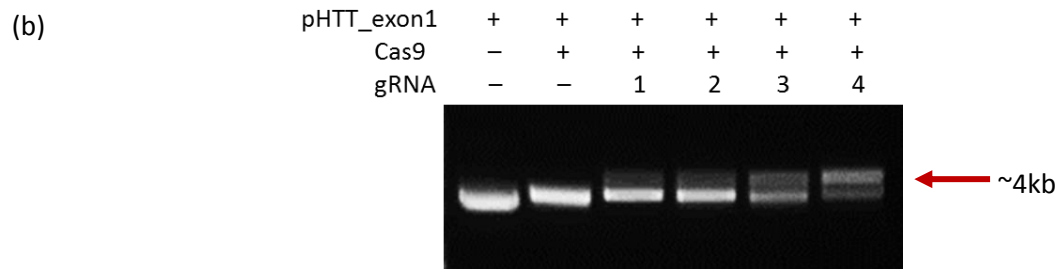


Figure 4.4: ***In vitro* Cas9 assay for detection of cleavage activity of all CRISPR gRNAs on pHTT_exon1 plasmid.** (a) CRISPR positions on the exon in pHTT_exon 1. Red arrows indicate new CRISPRs and crossed arrows indicate the paired CRISPRs used in first experiment. (b) *In vitro* Cas9 assay testing the activity of all four CRISPRs on pHTT_exon 1.

In the second targeting strategy using CRISPRs 3 and 4, the cells were co-transfected with pre-assembled Cas9-RNP and a donor plasmid containing the extended HTT_Q_{67} repeat fragment. This targeting donor plasmid was constructed by cloning in of a BlpI/BsgI digested Q_{67} synthetic fragment into the pHTT_exon 1 plasmid (Figure 4.5).

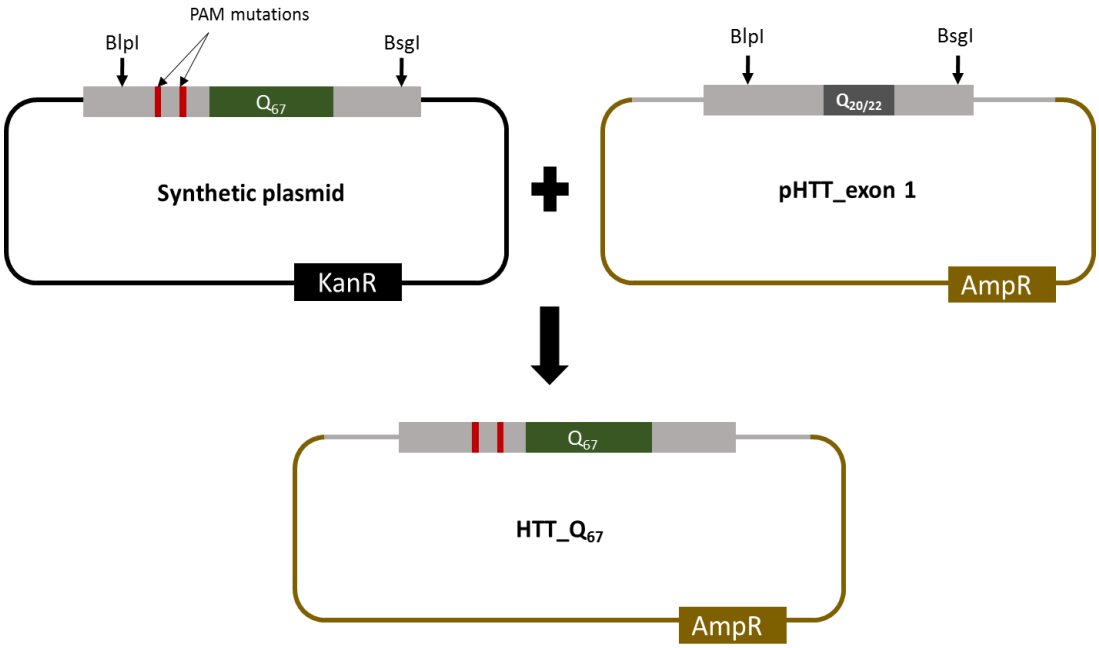


Figure 4.5: Construction of HTT_Q_{67} donor plasmid by cloning in of a BlpI/BsgI digested Q_{67} synthetic fragment into pHTT_exon 1.

The donor plasmid was expected to introduce the extended Q-repeat, by homologous recombination, into HTT exon 1 upon genomic DNA cleavage with Cas9-RNP (Figure 4.6). Two transfection experiments were conducted, one with each guide RNA.

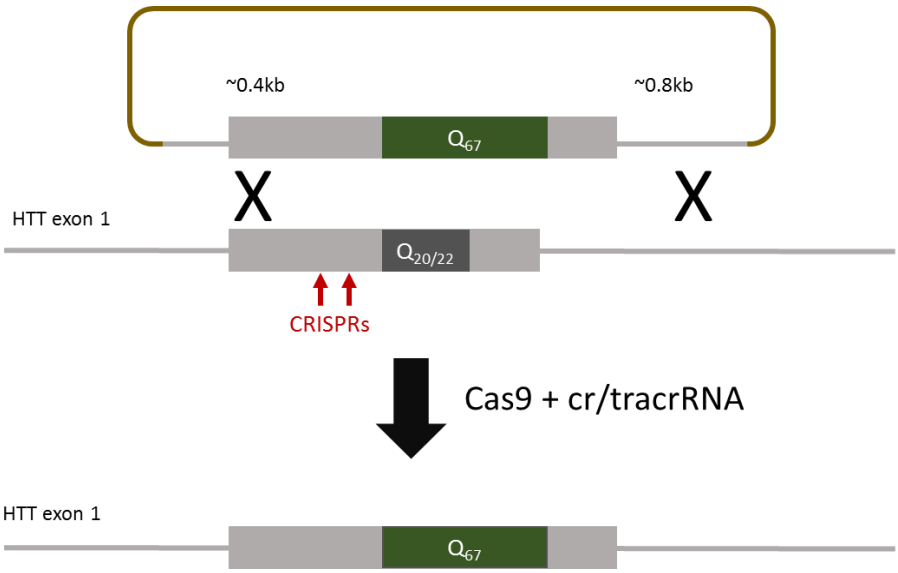


Figure 4.6: **Strategy for insertion of the extended Q_{67} repeat into HTT exon 1 in genomic DNA by homologous recombination with HTT_Q_{67} donor plasmid.** CRISPRs at which the homologous recombination occurs are indicated in red arrows. "X" denotes homologous recombination.

The genomic DNA from a pool of transfected cells was extracted and amplified with primers outside the homology arms to avoid detection of random insertions of the plasmid donor. These PCR amplicons were then amplified again with nested primers to assay for the insertion of the 67 Q-repeat fragment, a 512bp PCR amplicon (Figure 4.7 (a)). In assaying this pool of cells, it was expected that only a small fraction of the cells will contain the expanded allele. As the gel image shows (Figure 4.7 (b)), the 512bp band was not observed indicating very few or no cells contained the inserted fragment. Therefore, individual clones were picked and screened by nested PCR amplification in the same manner as described above. Detection of both the expanded (512bp) and wild-type (374bp) bands should be observed in a small subset of clones. Curiously, some colonies (for example B12, E6, and H10) exhibited only the one band approximately 500bp in size (Figure 4.7 (c)). However, sequencing these products yielded no trace. Therefore, I conclude that this band is not the expanded allele, but rather non-specific amplification or contamination which yielded a similar sized amplicon. The remaining clones amplified a wild-type band which was confirmed by sequencing. Thus, I conclude that I was unsuccessful in my attempt to engineer cells with an expanded CAG repeats.

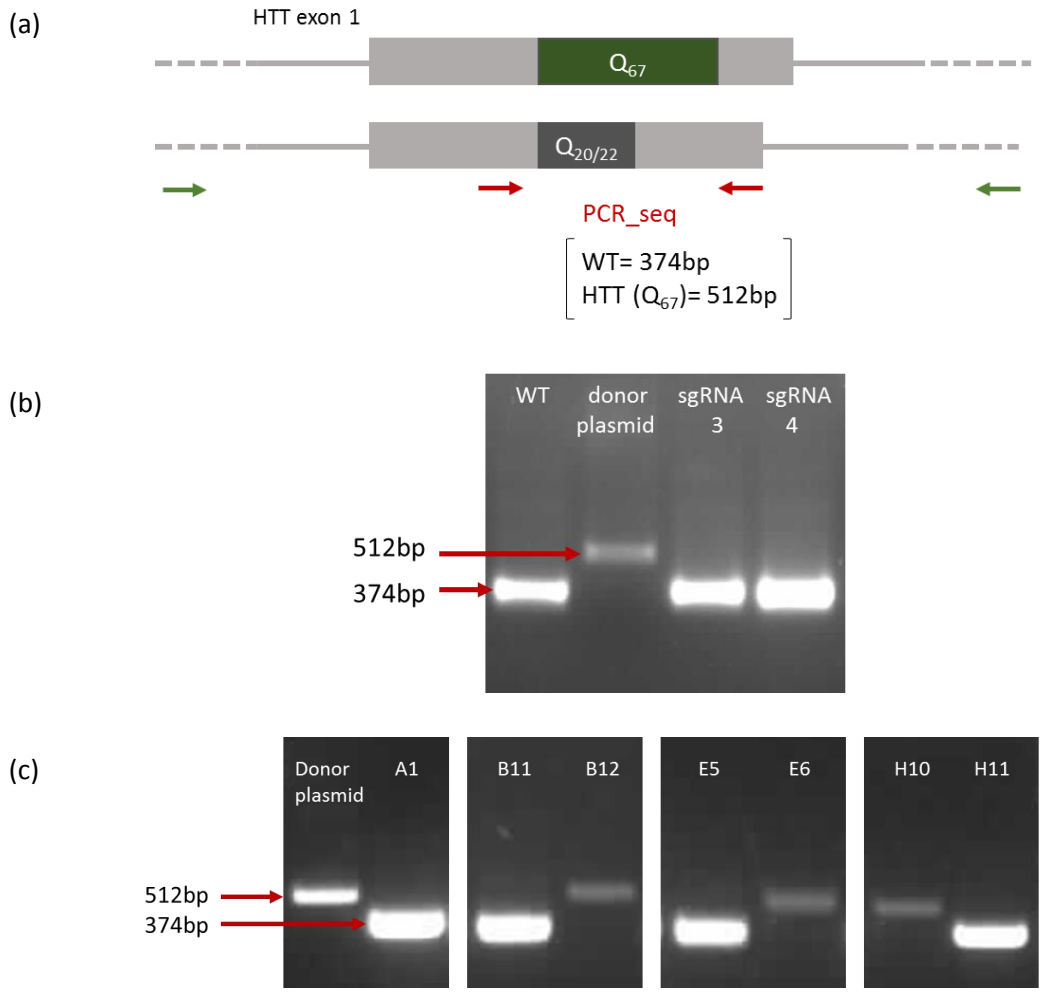


Figure 4.7: **Assay for detection of insertion of extended Q_{67} fragment into HTT exon 1 of KOLF2_C1 genomic DNA.** (a) Schematic diagram showing the region of HTT exon 1 amplified with primers outside the homology arms (shown in green arrows) and then with nested primers (shown in red arrows) to assay for the insertion. (b) Gel for detection of the 512bp band (corresponding to 67 Q-repeats) in genomic DNA of the pool of transfected cells. (c) Cropped images of gels showing the bands for individual cell colonies.