

Genome editing of induced pluripotent stem cells from Parkinson's disease and multiple system atrophy patients

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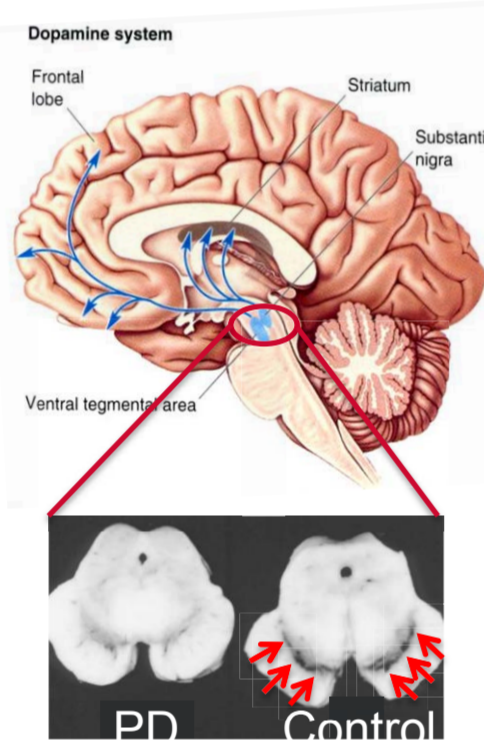
ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects 1-2% of the population over age 65. PD is marked by a loss of dopaminergic neurons in the midbrain substantia nigra pars compacta region. Because of lack of access to such tissue, or availability of good animal models of PD, iPSC-derived neurons hold promise in the development of model systems to study PD. We have generated iPSCs from patients harboring mutations in the PARKIN and LRRK2 genes, as well as a rare case with mutations in both the LRRK2 and GBA genes, and a patient with Multiple System Atrophy (MSA). In order to discern subtle, disease-relevant phenotypes in midbrain dopaminergic neurons derived from iPSCs, or to understand the impact a specific gene or mutation on those phenotypes, we generated a set of isogenic iPSC lines using the Transcription Activator-Like (TAL) effector nuclease technology. We deleted the alpha-synuclein (SNCA) gene (both heterozygous and homozygous alleles) in the MSA iPSC line via non-homologous end-joining repair mechanism, and reverted the LRRK2 and GBA mutations back to wild type in a PD iPSC line via homologous recombination to better understand any synergies between these mutations. These lines are currently being differentiated into midbrain dopaminergic neurons, and disease-relevant assays are being developed. In this presentation, we will describe the optimized iPSC TAL-editing workflow, the genomic analysis methods we have developed for colony screening to identify edited clones and the approach we took to screen for and analyze off-target effects. We hope to share our protocols and lessons learned to enable broader research community to successfully perform TAL-based genome editing of iPSCs.

INTRODUCTION

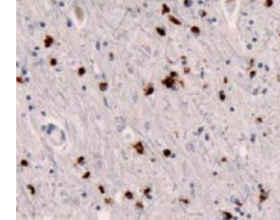
Parkinson's Disease

- Neurodegenerative disorder affecting 1-2 % of the population over age 65.
- Cardinal clinical symptoms include rest tremor, bradykinesia and muscle rigidity.
- Loss of dopaminergic neurons in the substantia nigra pars compacta (SNPC)
- Characteristic protein inclusions known as Lewy Bodies containing α -synuclein aggregates

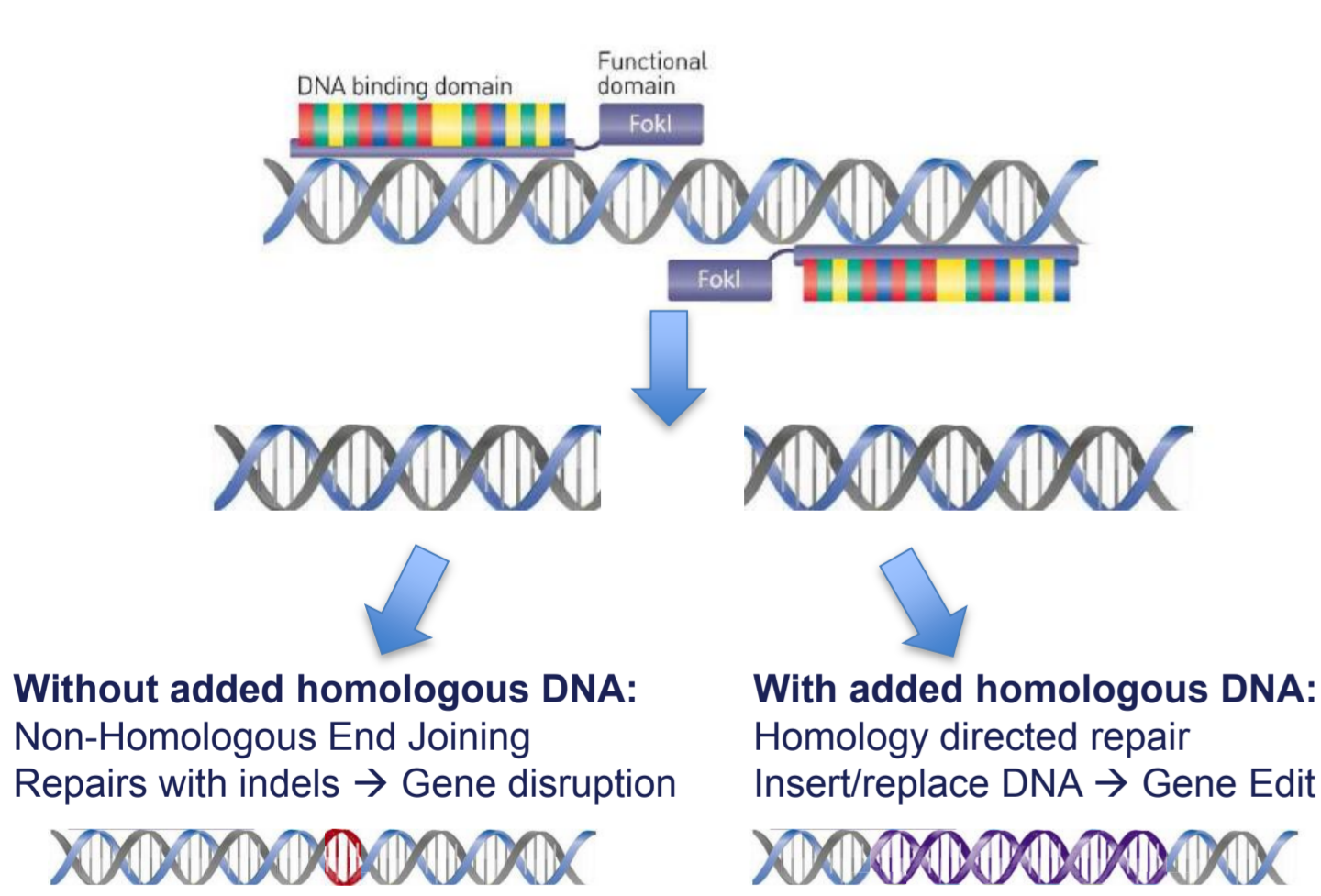


Multiple System Atrophy (MSA)

- Neurodegenerative disorder of primarily glial origin
- Clinically predominantly autonomic failure and motor impairment.
- The motor symptoms respond poorly to dopaminergic therapy.
- The presence of glial (oligodendrocytes) cytoplasmic inclusion bodies, containing α -synuclein aggregates.



Transcription Activator Like (TAL) Effector Nuclease



METHODS

Workflow of Gene Editing in iPSCs

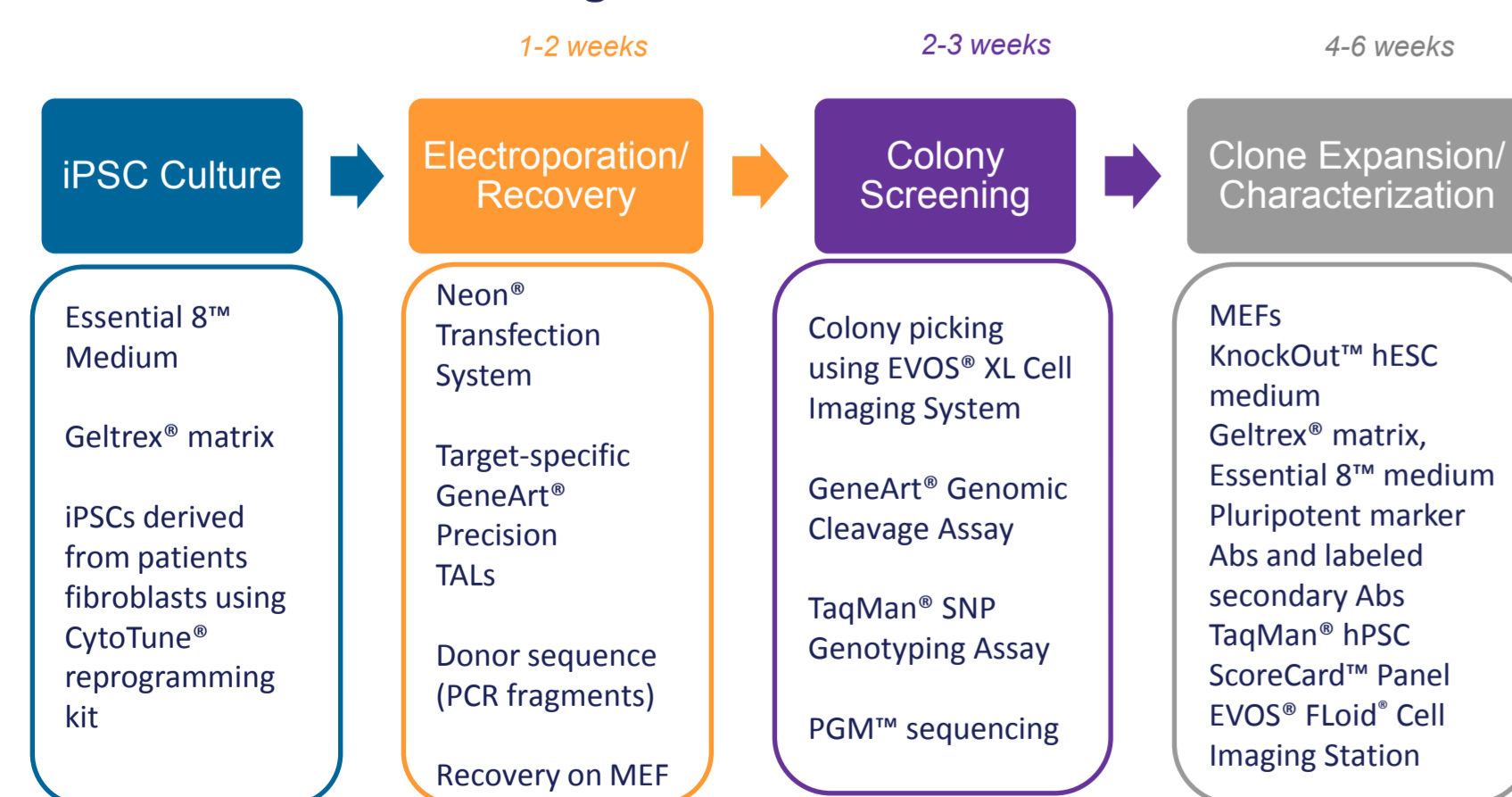


Figure 1 Genome editing of iPSC workflow

TAL Expression Constructs and Transfection/Electroporation

SNCA, LRRK2 and GBA TAL pairs were cloned into an EF1 α expression vector. iPSCs grown on feeder-free GeltrexTM coated plates in Essential 8TM medium were dissociated into single cells with StemPro[®] Accutase[®] reagent. About 0.4X10⁶ cells were electroporated with 2.5 μ g each of SNCA forward and SNCA reverse TAL expression constructs using the Neon[®] Transfection System (system settings: 1400V, 10ms, 2 pulses). After electroporation, cells were plated onto MEF culture dishes (200,000 cells/cm²) in MEF conditioned KSR hiPS medium supplemented with 10 ng/mL bFGF.

Workflow for iPSC Colony Screening

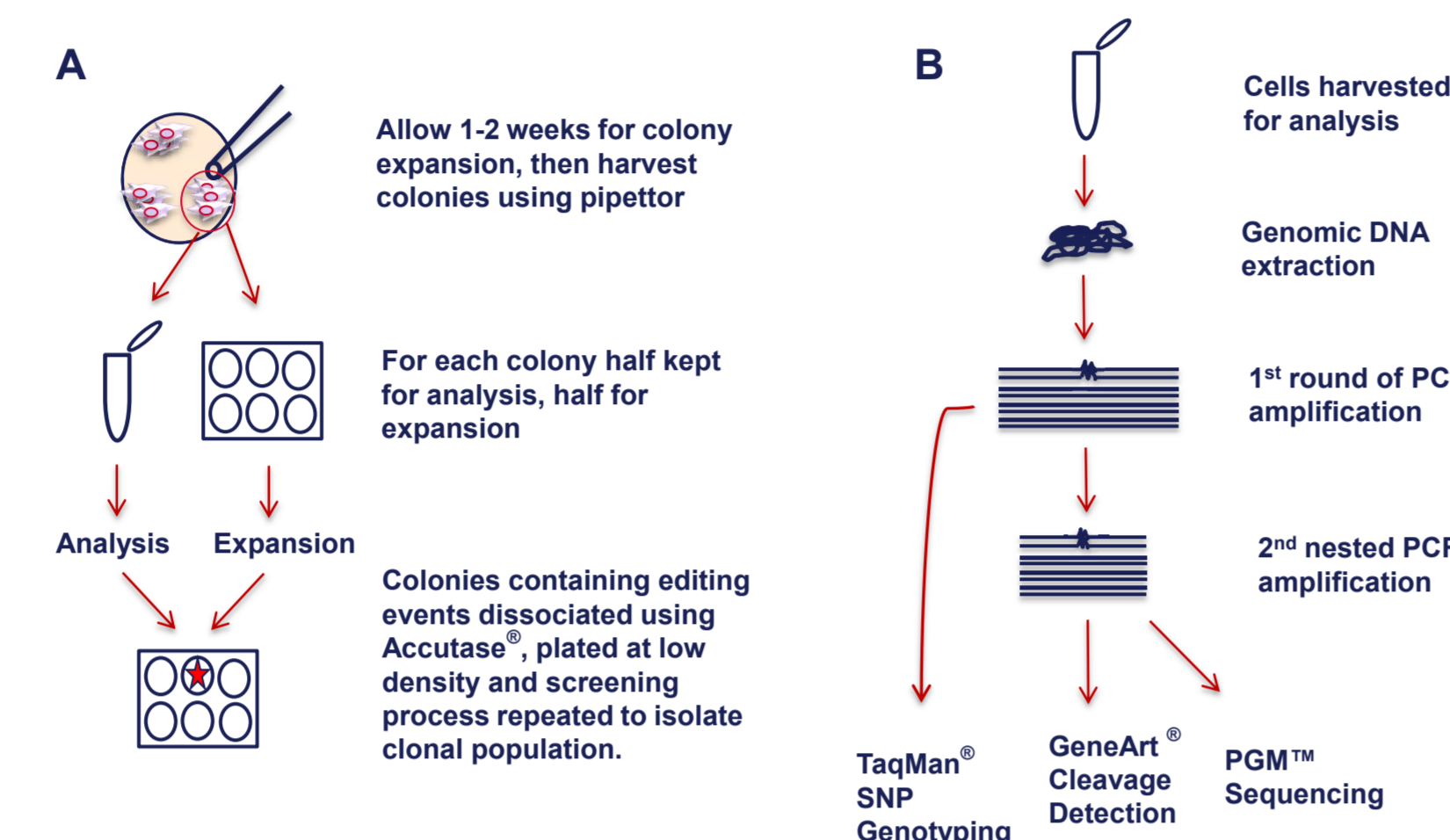
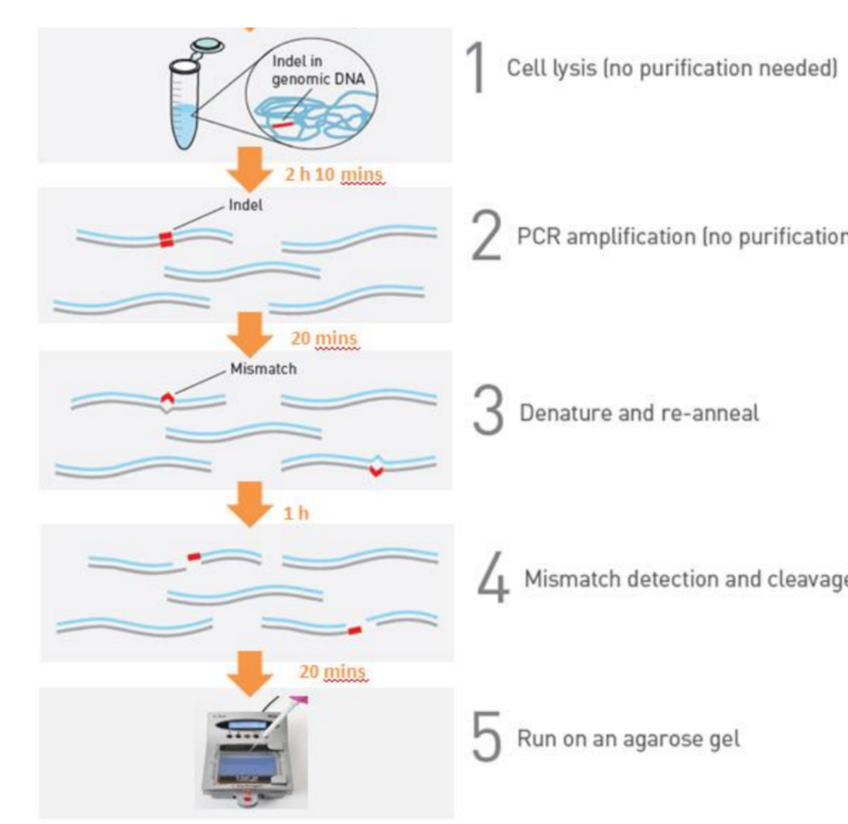


Figure 2 Workflow for colony screening. (A) Colonies were manually divided in an half, with half the cells analyzed and the other half cultured until analysis was complete. Colonies containing editing events are expanded and assays repeated until a clonal population is achieved. (B) PCR amplification of genomic DNA was performed to obtain the sensitivity needed to monitor changes at the target location. These resulting amplicons were then analyzed using the three screening methodologies shown.

GeneArt[®] Cleavage Detection Assay

Figure 3 GeneArt[®] Genomic Cleavage Detection assay. To detect either an indel or a mutation within a specific sequence of DNA, the region is first amplified using primers specific for that region. After heating the sample and re-annealing the PCR products, amplicons containing indels or other changes in sequence will result in the formation of heteroduplexes with amplicons containing non-modified sequences. When these heteroduplexes are treated with an endonuclease that only cleaves in the presence of a mismatch, two pieces of DNA of known size are generated, which can be detected by agarose gel electrophoresis.



TaqMan[®] SNP Genotyping Assays

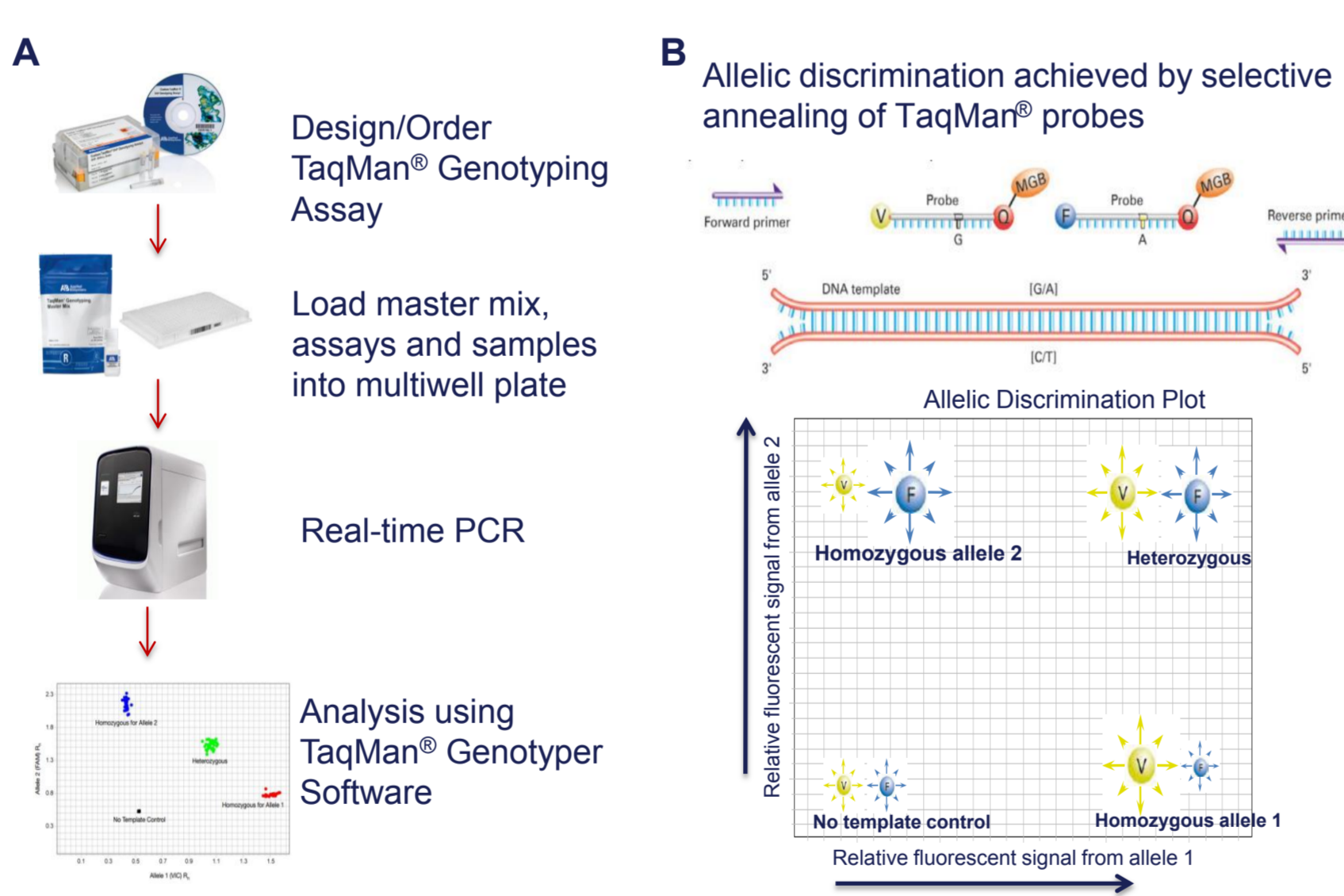


Figure 4 TaqMan[®] SNP Genotyping Assays. (A) Workflow. TaqMan[®] SNP Genotyping assays can be used to rapidly screen multiple samples for the presence of editing events. (B) Selective annealing of TaqMan[®] probes during Real-time PCR allows for the discrimination of allele states. This method can be used to detect changes in a heterogeneous population and monitor progress towards a clonal population.

Ion Personal Genome Machine[®] (PGM[™]) Sequencing

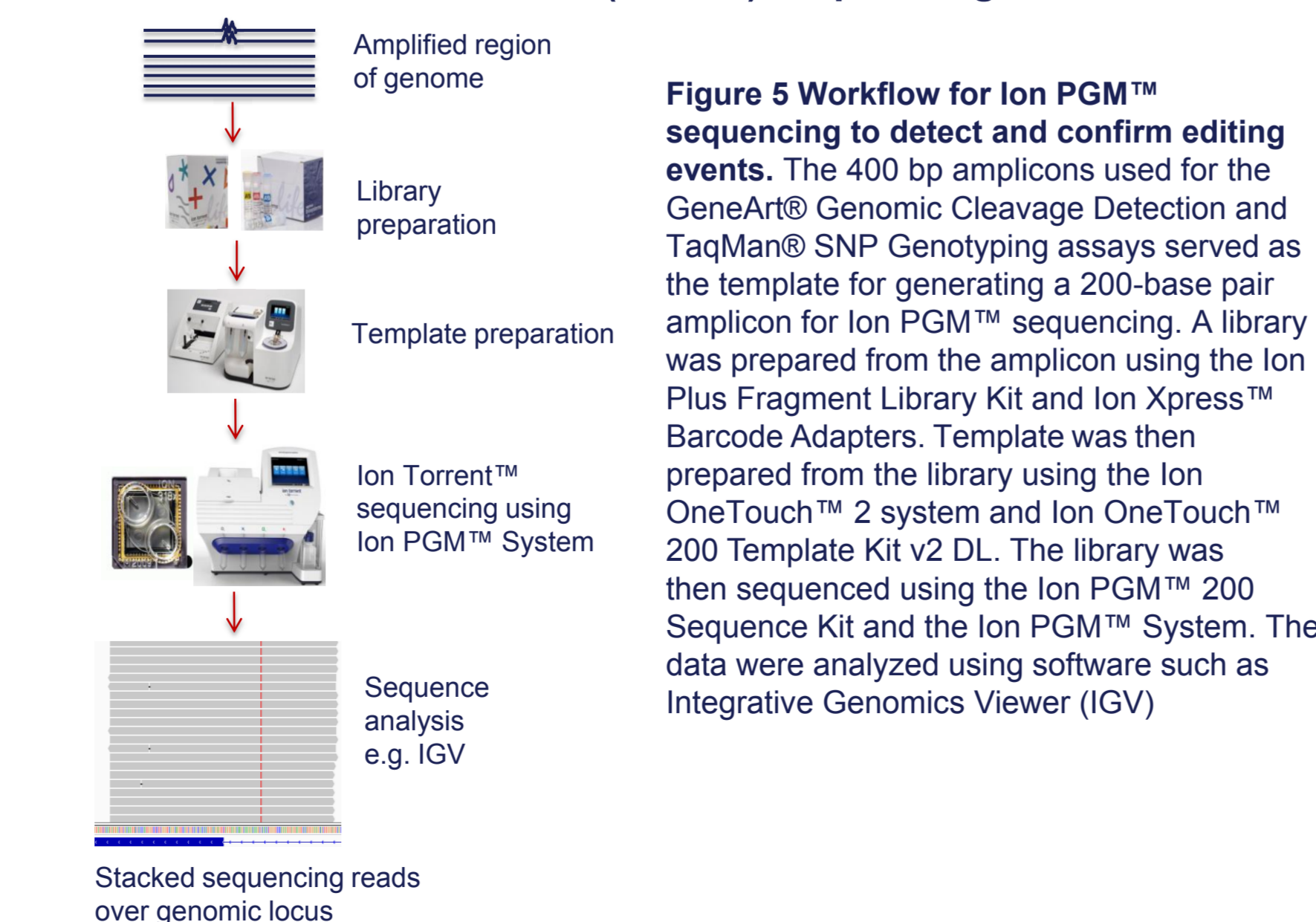


Figure 5 Workflow for Ion PGM[™] sequencing to detect and confirm editing events. The 400 bp amplicons used for the GeneArt[®] Genomic Cleavage Detection and TaqMan[®] SNP Genotyping assays served as the template for generating a 200-base pair amplicon for Ion PGM[™] sequencing. A library was prepared from the amplicon using the Ion Plus Fragment Library Kit and Ion Xpress[™] Barcode Adapters. Template was then prepared from the library using the Ion OneTouch[™] 2 system and Ion OneTouch[™] 200 Template Kit v2 DL. The library was then sequenced using the Ion PGM[™] 200 Sequence Kit and the Ion PGM[™] System. The data were analyzed using software such as Integrative Genomics Viewer (IGV)

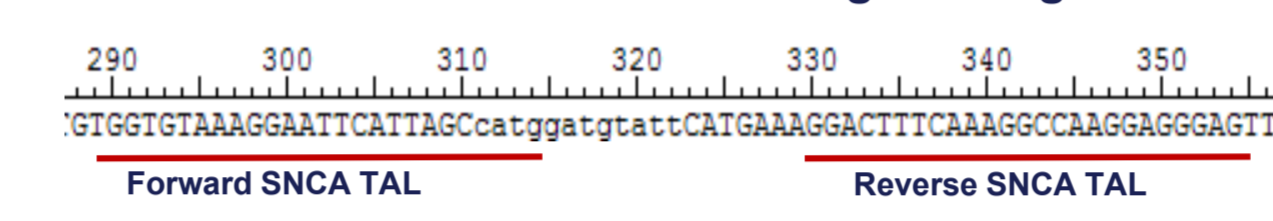
Table 1. Comparison of the three colony screening methods

Methodology	Advantages	Limitations	When to use?
GeneArt [®] Genomic Cleavage Assays	<ul style="list-style-type: none"> Inexpensive Can detect small changes in homozygous state of DNA – HDR and NHEJ editing 	<ul style="list-style-type: none"> Not quantitative Limited use if editing heterozygous locus 	Triaging colonies from editing via NHEJ repair
TaqMan [®] SNP Genotyping Assay	<ul style="list-style-type: none"> Inexpensive Fast Clearly distinguishes changes in allele status 	<ul style="list-style-type: none"> Only detects changes in allele that the assay is designed for – may not detect indels from NHEJ repair 	Triaging colonies from editing via homologous recombination
PGM [™] sequencing	<ul style="list-style-type: none"> Can specifically detect all changes in population Quantitative results 	<ul style="list-style-type: none"> Higher cost compared to other assays Longer workflow 	Best used as a secondary assay – for confirmation and quantitation of editing in populations identified from primary screens

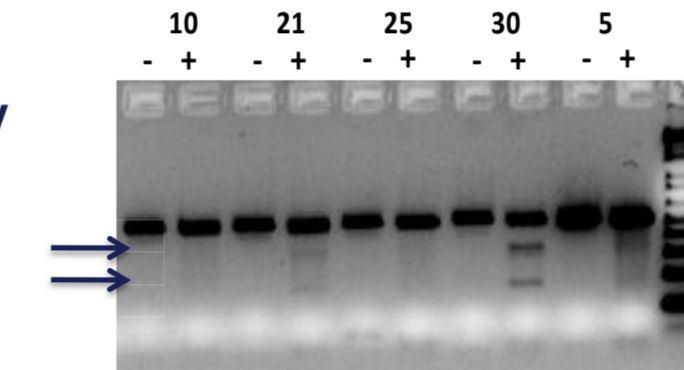
RESULTS

Generation of α -synuclein knockout lines from the MSA iPSCs

A SNCA GeneArt[®] Precision TALs design to target exon 2



B GeneArt[®] Genomic Cleavage Detection Assay



C Examples of deletions in TAL-edited MSA iPSCs



D Ion Personal Genome Machine[®] (PGM[™]) for detecting 2bp deletion

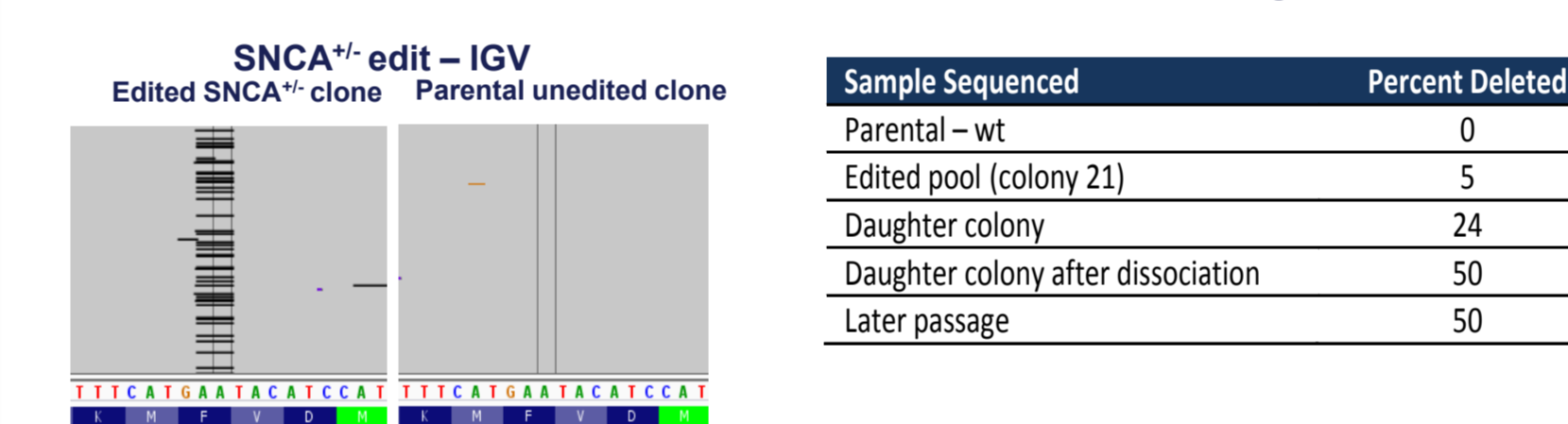
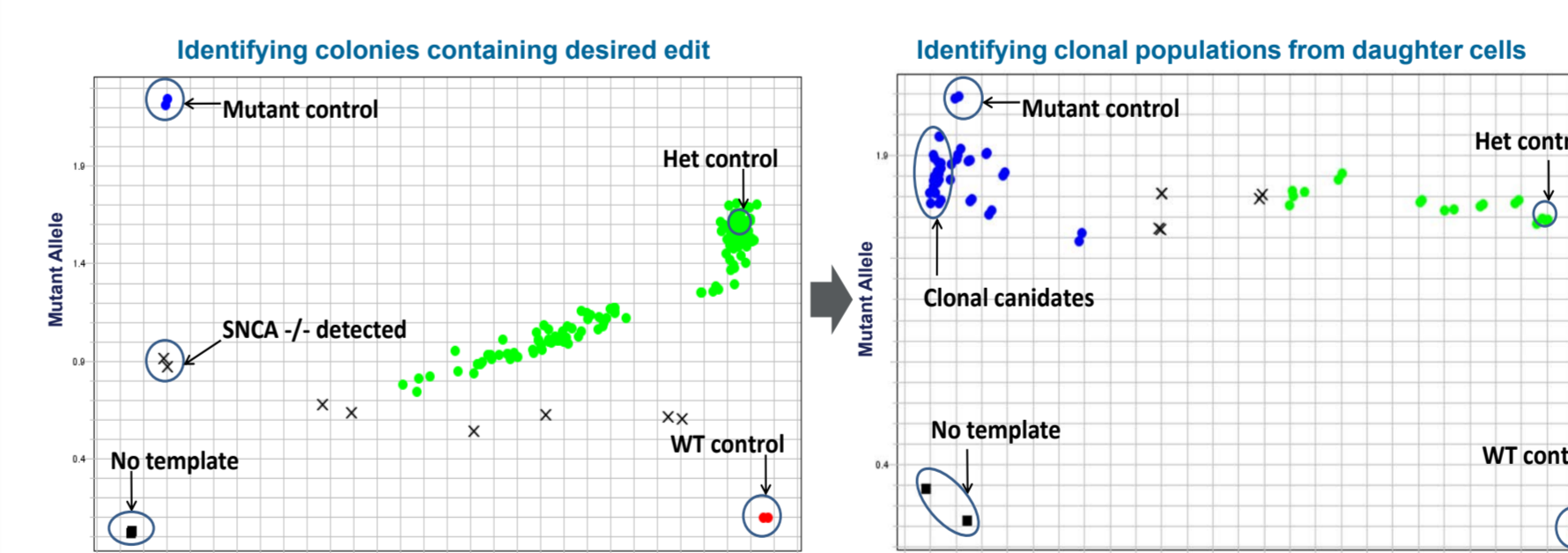


Figure 6 Generation of SNCA^{-/-} MSA iPSC line. (A) SNCA TAL pair binding sites. (B) Genomic DNA was prepared from recovered iPSC colonies, followed by a nested PCR over the SNCA region to be edited. The PCR product (~400 bp) was denatured and re-annealed. If editing occurred, heteroduplexes were formed, which were cleaved. Lanes lacking cleavage enzyme are shown with a minus symbol (-) and lanes that were treated with cleavage enzyme are shown with a plus symbol (+). Samples from colonies 21 and 30 have clearly visible cleavage products with desired sizes, indicating the presence of a mismatch as a result of a TAL editing event. (C) Amplicons from positive colonies were TOPO[®] cloned and sequenced using sanger methodology to confirm editing at the SNCA locus. (D) PGM[™] sequencing of clonal candidates confirms that a clonal population containing a heterozygous mutation in the SNCA locus was achieved. The IGV software shows stacked sequencing reads across exon 2 of SNCA. The black bars indicate a location where a deletion was present. The 2-bp deletion in the fourth amino acid of SNCA is clearly distinguished when compared to the unedited parental cell line. The percentage of bases called as deleted at the 2-bp location increased as edited clones were enriched at each round of colony screen.

A TaqMan[®] SNP Genotyping Assay for detecting double allele deletion



B PGM[™] sequencing to confirm clonal population with double allele deletion

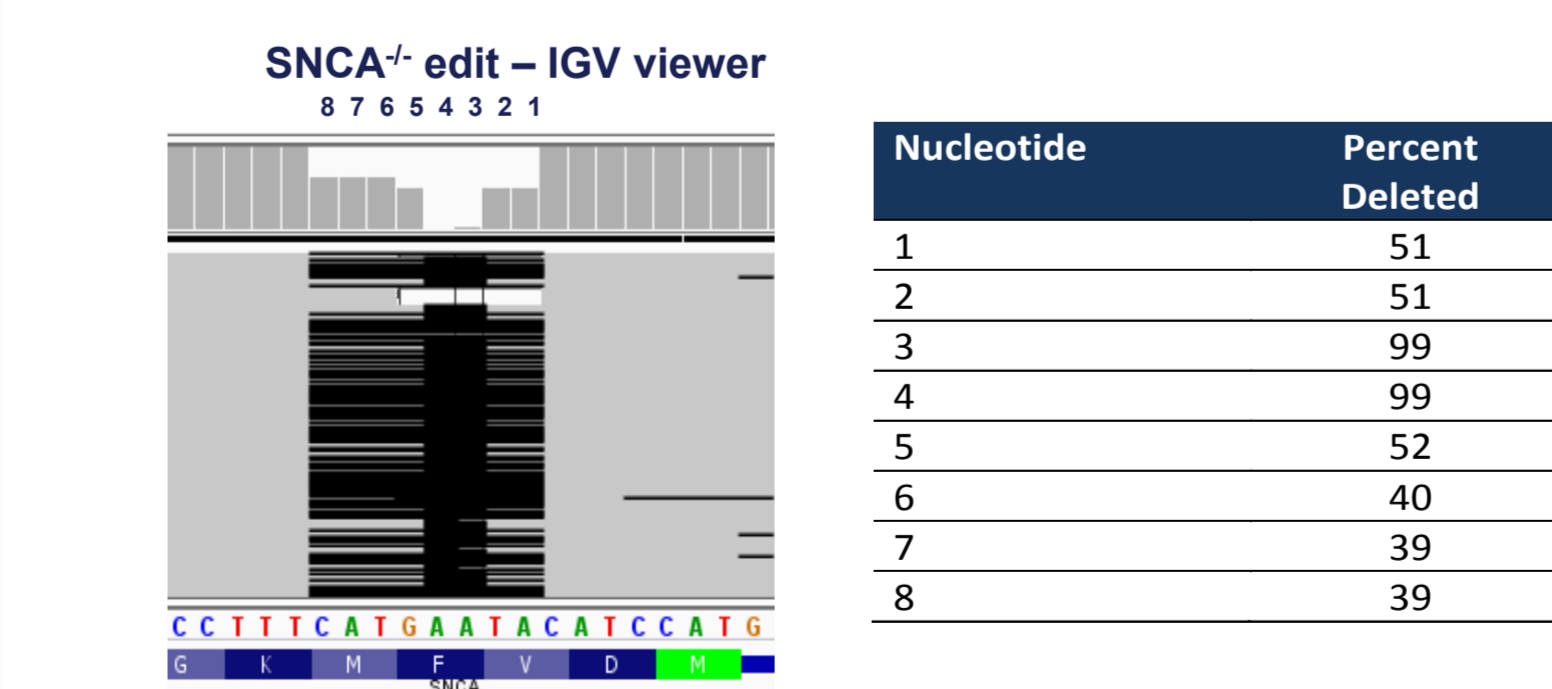


Figure 7 Screening for clones with SNCA double allele deletion. (A) TaqMan[®] SNP Genotyping assay. Allelic Discrimination Plot on the left shows first round screening result. Most colonies (green) were heterozygous, but two colonies (X's in circle) showed as mixtures of cells with heterozygous and homozygous deletions. WT control: template from the parental MSA line; Mutant control: template (from a donor plasmid) containing a 2-bp deletion in SNCA; Het control: 50% unedited template/50% mutant template; No template: TaqMan[®] reagents and water only. The plot on the right shows 2nd round screening of daughter colonies result. Candidate clonal populations with two mutant alleles are indicated as the blue dots on the left of the plot. (B) PGM[™] sequencing result. IGV of the stacked sequencing reads clearly shows an 8-bp deletion on the second allele. Sanger sequencing confirmed the 8-bp deletion in clone 32 (data not shown). The additive effect on the percent of bases called as deleted where the two deletions (nts 3 and 4) overlap confirms that the deletion has occurred in the second allele.

Characterization of the Edited MSA iPSC clones

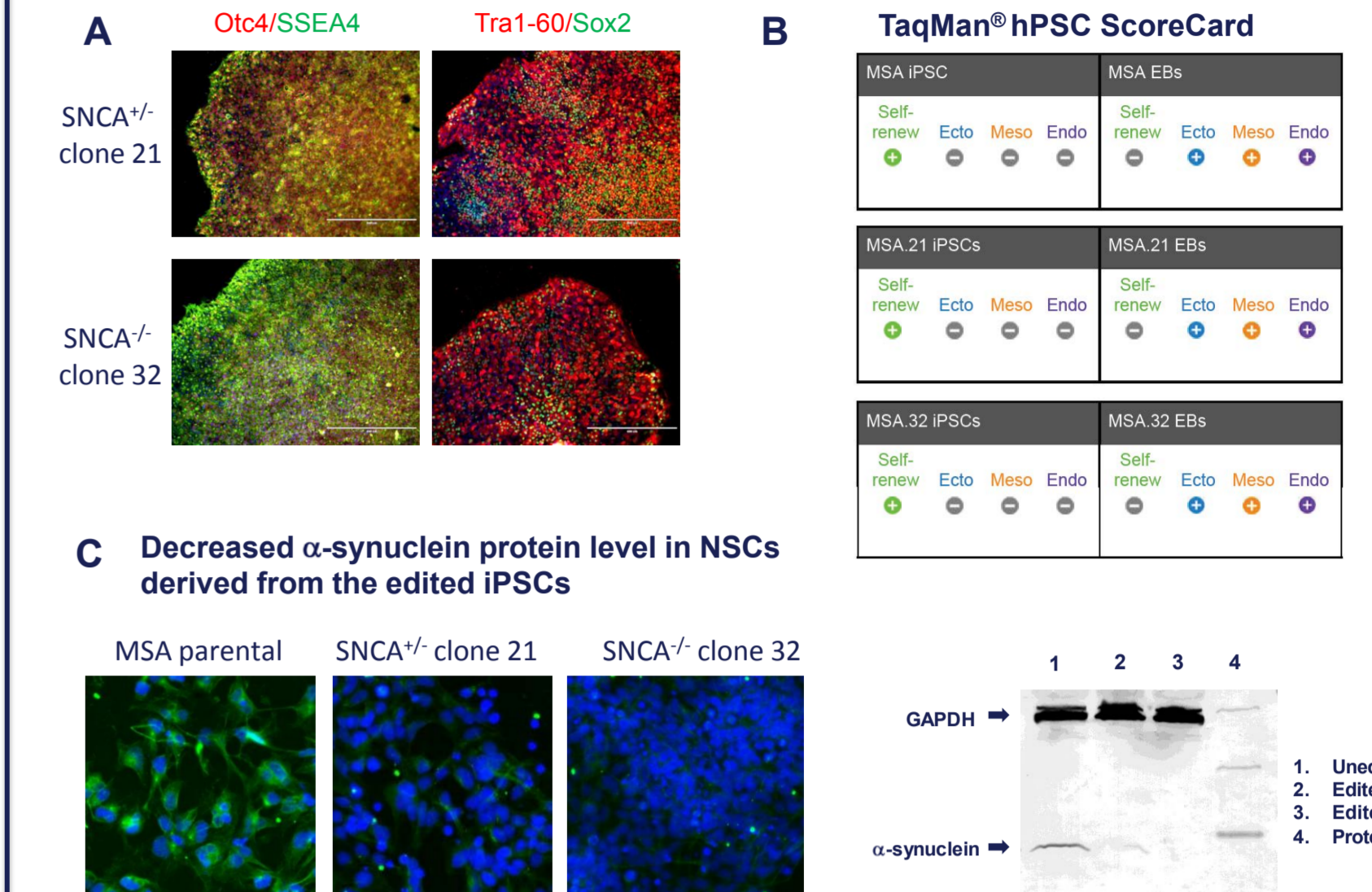
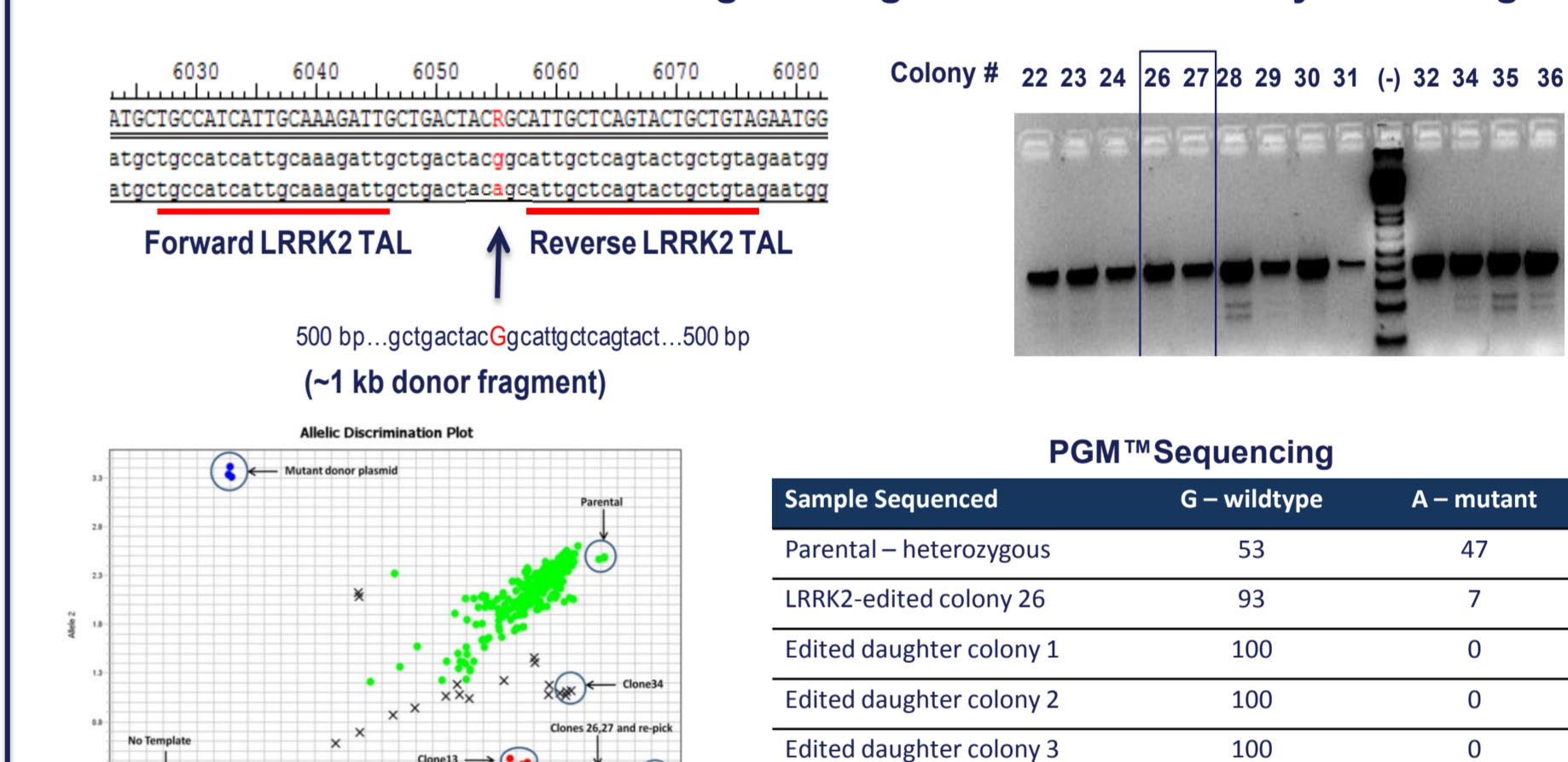


Figure 8 Characterization of TAL-edited MSA lines. (A) Feeder-free iPSCs (MSA clone 21 and MSA clone 32) in Essential 8[™] medium on Geltrex[®] matrix-coated plates were stained for Oct 4 (red), SSEA4 (green), Tra1-60 (red), and Sox2 (green) using the PSC 4-Marker Immunocytochemistry Kit with DAPI (blue) nuclear DNA staining. (B) Pluripotency analysis using TaqMan[®] hPSC Scorecard Panel. (C) Feeder-free iPSCs were differentiated to neural stem cells (NSCs) using Gibco[®] PSC Neural Induction Medium. NSCs were fixed, permeabilized, and stained for α -synuclein (green). Nuclear DNA was stained with DAPI (blue). Images were captured with the EVOS[®] Fluo[®] Cell Imaging Station with the same exposure, brightness, and contrast settings. NSC pellets were resuspended in M-PER Mammalian Protein Extraction Reagent. Western blotting was performed with primary antibodies (2 μ g/mL α -synuclein Monoclonal Antibody, mouse (clone Syn 211) and 0.25 μ g/mL GAPDH Mouse Monoclonal Antibody (clone 258)).

Correction of LRRK2 G2019S and GBA N370S mutations in the PD-3 line

A GeneArt[®] Precision TALs design to target LRRK2 and colony screening



B GeneArt[®] Precision TALs design to target GBA and colony screening

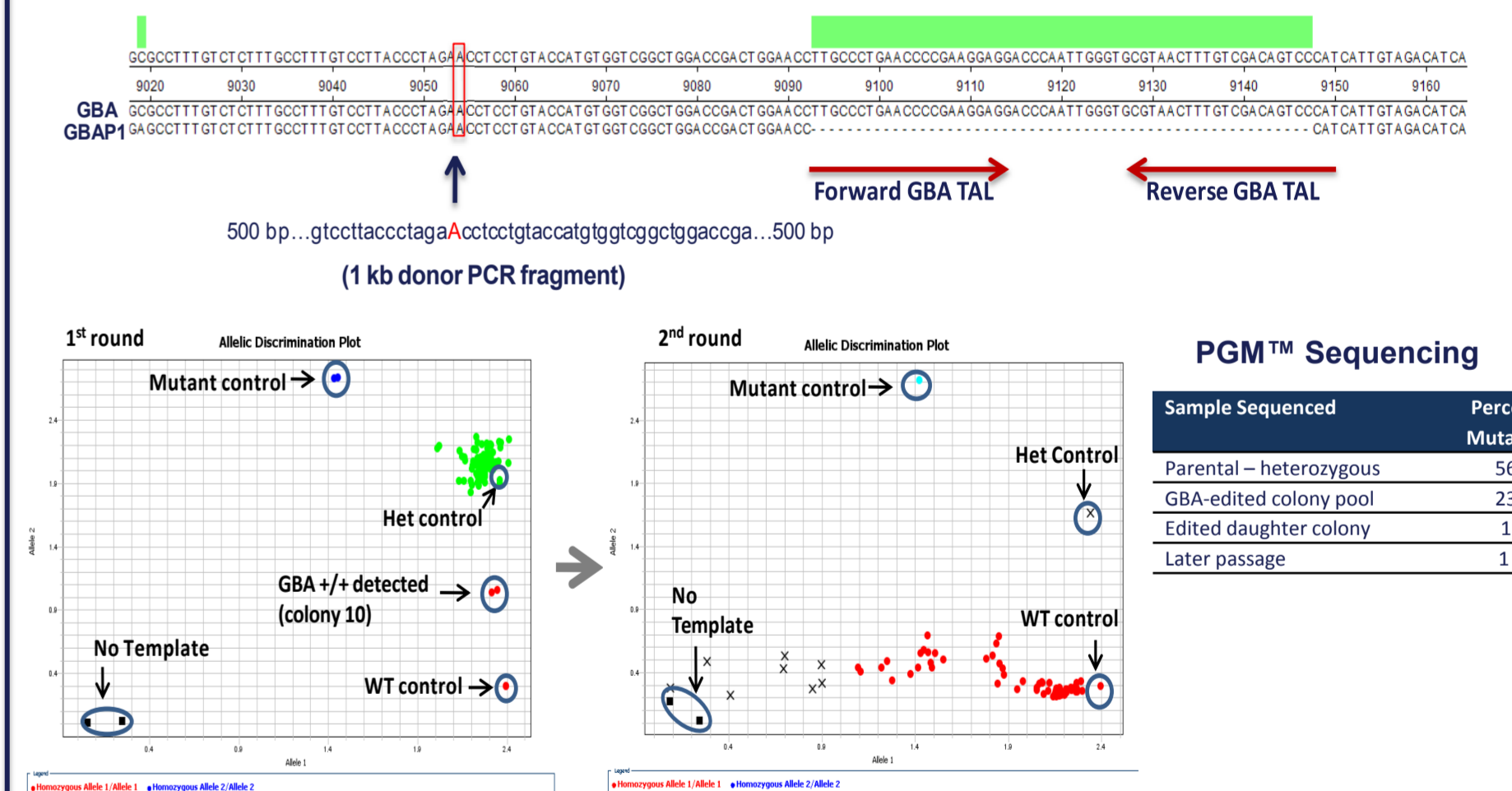


Figure 9 Correction of LRRK2 and GBA mutations in the PD-3 line. (A) Generation of PD iPSCs with LRRK2 G2019S corrected to wild type. LRRK2 TAL binding sites are underlined red. TaqMan[®] SNP Genotyping Assay and PGM[™] sequencing results are shown. (B) Generation of PD iPSCs with GBA N370S corrected to wild type. Sequence s of GBA N370S and pseudogene GBAP1, GBA TALs and 1-kb purified PCR donor fragment containing the wild type sequence and 500-bp flanking sequences. TaqMan[®] SNP Genotyping Assay and PGM[™] sequencing results are shown.

SUMMARY

We generated isogenic MSA iPSC clones with a heterozygous and homozygous deletion of α -synuclein using the GeneArt[®] Precision TAL technology.

- 16% (2/12) of the screened colonies were positive in GeneArt[®] Cleavage Detection assay for editing of SNCA allele1. 0.8% (1/120) of the screened colonies were positive in TaqMan[®] SNP Genotyping Assay for editing of the SNCA allele2.
- We developed and optimized a PGM[™] sequencing method that allows for confirmation and quantitation of sequence changes detected using GeneArt[®] Cleavage Detection Assay and TaqMan[®] SNP Genotyping Assay.
- The resulting edited iPSCs express the expected pluripotency markers and are karyotypically normal (data not shown).
- Cellular levels of alpha-synuclein reduced in both mutant lines.

We have corrected heterozygous LRRK2 and GBA mutations in the PD-3 line using the GeneArt[®] Precision TAL technology via homologous recombination with estimated editing efficiency of 1.4% and 2.1% respectively.

TRADEMARKS/LICENSING

For Research Use Only. Not for use in diagnostic procedures.