# USING CELLS FROM PATIENTS WITH MULTIPLE SYSTEMS ATROPHY OR PARKINSON'S DISEASE (PARK2, LRRK2, AND/OR GBA MUTATIONS) TO BUILD STEM CELL DERIVED DISEASE MODEL SYSTEMS

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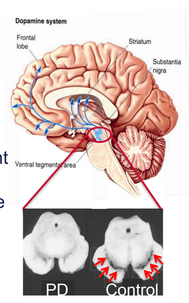
## ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects 1% of people over age 60 and more than five million people worldwide. PD research has been hindered by a lack of access to diseased tissue to study. However, with recent advances in stem cell biology, it is now possible to derive induced pluripotent stem cells (iPSCs) from control or PD patient fibroblasts and differentiate them into neurons. Concomitantly, there have been advances in gene editing technologies which now allow select mutations to be corrected or genes to be knocked out within iPSCs, resulting in pairs of cell lines with the same genetic background that differ by the presence or absence of specific disease-linked mutations or genes. Recently, Life Technologies has collaborated with the Parkinson's Institute to develop PD model systems using donor fibroblasts collected at the Institute. We created a set of six iPSC lines from three PD patients (harboring PARK2, LRRK2 and/or GBA mutations), one MSA patient, and two age-matched controls. TALEN gene editing was utilized to correct mutations/knock out genes within two of the patient derived iPSC lines. All of the iPSC lines (including the TALEN edited lines) were differentiated into neural stem cells (NSCs). These NSCs were directly utilized in functional assays to compare their response to a number of different cellular stressors and were further differentiated into dopaminergic neurons. We will complete our disease modeling by using the pairs of isogenic lines generated in this study to assess functional differences between edited and unedited cells in fully differentiated dopaminergic neurons.

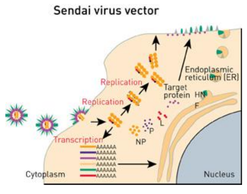
## INTRODUCTION

### Parkinson's disease (PD)

A progressive neurodegenerative disorder that affects 1% of people over age 60 and more than 5 million people worldwide.
PD results primarily from the selective loss of dopaminergic neurons in the substantia nigra. The absence of physiologically relevant cellular models for PD represents a major bottleneck for PD research. Novel models are urgently needed to accelerate the discovery of disease mechanisms and drug targets as well as for screening purposes.




### Generation of iPSCs using Sendai reprogramming vectors




- **RNA virus**. No DNA during replication, and replicates exclusively in the cytoplasm. No possibility of integration into host genome
- **Safe**. CytoTune® has Fusion (F) gene deleted and cannot produce infectious particles. Non-pathogenic to humans
- **Broad tropism**. Capable of transducing a wide range of cells with a short contact time.

### iPSC generation and live-staining

Reprogramming of fibroblasts to generate iPSCs and live-staining was performed according to the User Guide of the CytoTune® -iPS Sendai Reprogramming Kit (Life Technologies).

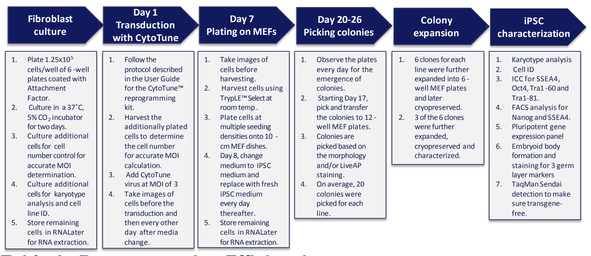



**Table 1. Reprogramming Efficiencies**

| | | Day 7 plating density (# cells plated/10-cm MEF dish) | | |
|---|---|---|---|---|
| | | 50,000 cells | 100,000 cells | 200,000 cells |
| Donor | Mutation | SSEA4[+] colonies (Efficiency) | SSEA4[+] colonies (Efficiency) | SSEA4[+] colonies (Efficiency) |
| PD-1 | Parkin | 55 (0.110%) | 132 (0.13%) | 583 (0.300%) |
| Ctrl-1 | - | 35 (0.070%) | 81 (0.08%) | 97 (0.050%) |
| PD-2 | LRRK2 | 55 (0.110%) | 63 (0.06%) | 193 (0.100%) |
| PD-3 | LRRK2/GBA | 3 (0.006%) | 7 (0.007%) | 17 (0.009%) |
| MSA | | 3 (0.006%) | 20 (0.02%) | 40 (0.020%) |
| Ctrl-2 | | 22 (0.044%) | 18 (0.02%) | 63 (0.031%) |

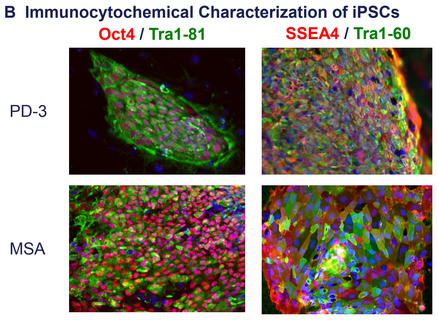
## RESULTS

**Figure 1 iPSC Characterization**

**A Cytogenetics of iPSCs**

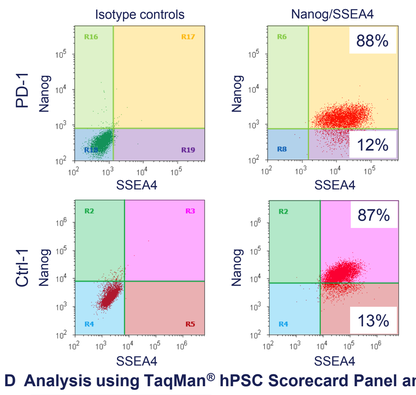
**Cytogenetic analysis of iPSCs** iPSC clones (from passage 11 to15) were cultured in KSR iPSC Medium on 6-well MEF plates for 4 days. Cells were harvested for G-banded karyotypic analysis. All clones from 6 lines tested were karyotypically normal. No clonal abnormality was detected at the G-banded level of resolution.

**B Immunocytochemical Characterization of iPSCs**



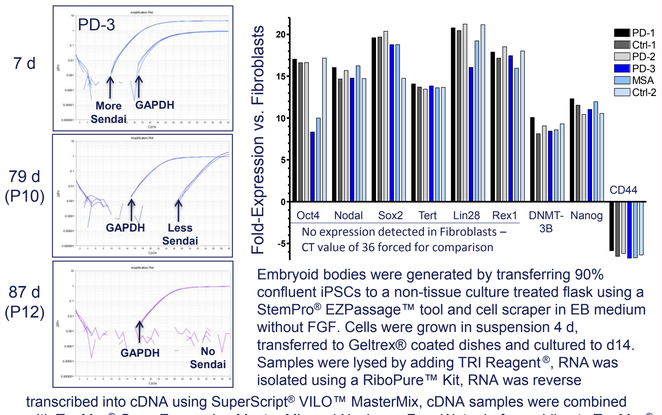

**Immunocytochemistry demonstrated that iPSCs express pluripotency markers Oct4, Tra1-81, Tra1-60 and SSEA4.** Images were captured using FLoid® Cell Imaging Station.

**C Flow Cytometry Analysis of iPSCs**




**Immuno-staining and flow cytometry analysis demonstrated that iPSCs express pluripotency markers Nanog and SSEA4.** iPSCs were cultured in KSR-supplemented iPSC Medium on MEFs for 4 days. Cells were harvested, fixed, permeabilized and co-stained with isotype control antibodies (Left) and antibodies (Right) for Nanog and SSEA4. FACS analysis was performed using the Attune® Acoustic Focusing Cytometer.

**D Analysis using TaqMan® hPSC Scorecard Panel and Sendai Detection Kit**




Embryoid bodies were generated by transferring 90% confluent iPSCs to a non-tissue culture treated flask using a StemPro® EZPassage™ tool and cell scraper in EB medium without FGF. Cells were grown in suspension 4 d, transferred to Geltrex® coated dishes and cultured to d14. Samples were lysed by adding TRI Reagent®, RNA was isolated using a RiboPure™ Kit, RNA was reverse transcribed into cDNA using SuperScript® VILO™ MasterMix, cDNA samples were combined with TaqMan® Gene Expression Master Mix and Nuclease-Free Water before adding to TaqMan® hPSC Scorecard™ Panel, TaqMan® OpenArray® Human Stem Cell Panel or Sendai Detection Kit and run on a QuantStudio™ 12K Flex Real-Time System.

**iPSC cells from all six donor lines tested positive for pluripotency (data not shown). Late passage (>p25) iPSC cells from MSA and Ctrl-2 tested positive for pluripotency and EBs showed potential for all three lineages.**

| MSA | MSA EBs | Ctrl2 | Ctrl2 EBs |
|---|---|---|---|
| Pluri | Ecto Endo Meso | Pluri | Ecto Endo Meso |
| Pluripotency markers expressed. Test differentiated cells to determine utility. | Good general purpose pluripotent cell line. | Pluripotency markers expressed. Test differentiated cells to determine utility. | Good general purpose pluripotent cell line. |

**E Embryoid Body Formation and Immuno-characterization**

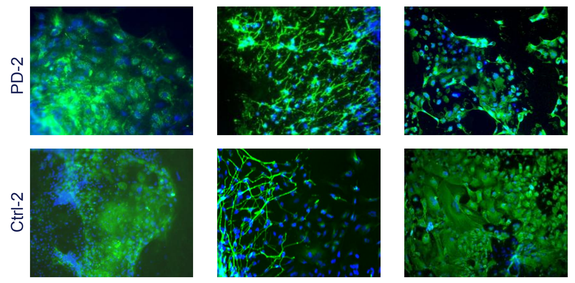
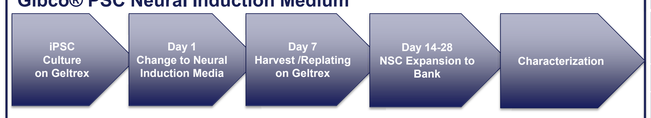
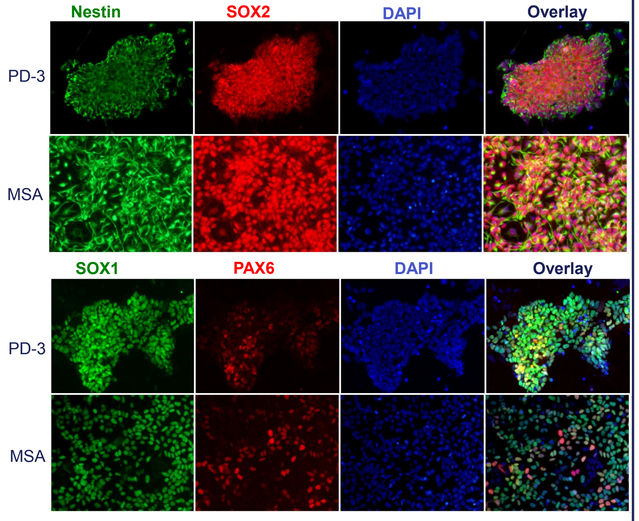



**Figure 2 Neural Induction and Expansion Timeline using Gibco® PSC Neural Induction Medium**




Timeline illustrates the rapid and efficient strategy for the generation of patient derived NSCs in just seven days followed by banking and characterization.

**A All iPSC-Derived NSCs Demonstrate Expected Neural Markers by ICC**



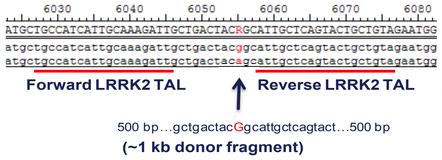

All six iPSC-derived NSC lines stained positive for known neural markers Sox1, Sox2, and Nestin. All NSCs were negative for Oct4. In addition, NSCs stained positive for Pax6, a known regulator in neurogenesis and molecular regulation of the central nervous system. ICC samples were stained with the Human Neural Stem Cell Immunocytochemistry Kit and analyzed on the **FLoid® Cell Imaging Station.**

**B All iPSC-Derived NSC Lines Exhibit Expected Neural Markers by Flow Cytometry**

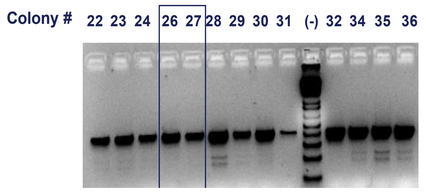
| ID | Gene | Mutation(s) | Nestin | SOX1 | SOX2 |
|---|---|---|---|---|---|
| Ctrl-1 NSCs | n/a | n/a | 99% | 77% | 87% |
| PD-1 NSCs | PARKIN | Ex2del c. 102delAG | 99% | 98% | 98% |
| Ctrl-2 NSCs | n/a | n/a | 100% | 94% | 97% |
| PD-2 NSCs | LRRK2 | G2019S, Heterozygous | 100% | 96% | 92% |
| PD-3 NSCs | LRRK2/GBA | G2019s, N370S | 100% | 90% | 87% |
| MSA NSCs | Unknown | Unknown | 100% | 89% | 93% |

**Figure 3. Generation of PD-3 iPSCs with LRRK2 G2019S corrected to wild type.**

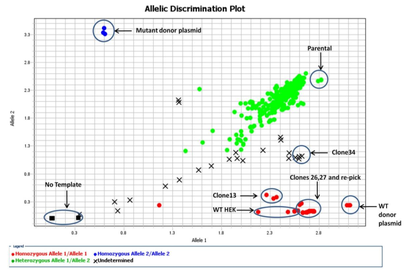
**A TAL editing of the PD-3 LRRK2 Mutation**




**B Colony screening with GeneArt® Genomic Cleavage Detection Assay.**




**C TaqMan® SNP genotyping assay**



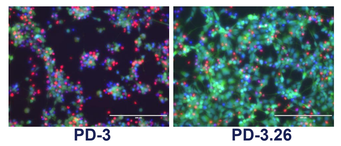

**(A)** Sequence of LRRK2 G2019S region in the PD-3 line. The binding sites for the TAL pair are underlined in red. The TALs were electroporated into the cells along with a 1-kb purified PCR donor fragment containing the wild type sequence and 500-bp flanking sequences. **(B)** Colony screening with GeneArt® Genomic Cleavage Detection assay. Out of the 140 colonies screened, colonies 26 and 27 seemed to have no detectable cleavage product caused by mismatch, indicating that the heterozygous mutation was corrected to homozygous wild type or G2019S. **(C)** TaqMan® SNP genotyping assay confirmed that clones 26, 27, and their daughter colonies contain homozygous wild type allele, as these clones (red)

**Figure 4 Summary of Gene Editing Results**

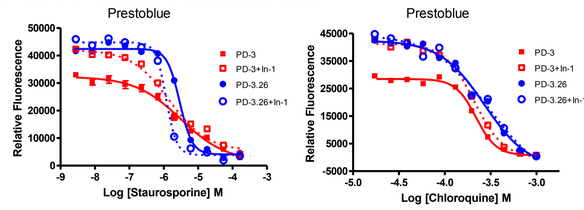
| iPSC line | Genotype | Karyotype | Pluripotent (ICC/ScoreCard) | Sequence confirmation | Off-target indels in exons | Estimated editing efficiency | Mycoplasma |
|---|---|---|---|---|---|---|---|
| MSA | SNCA $^{+/+}$ | normal | yes | yes | n/a | n/a | negative |
| MSA.21 | SNCA $^{+/-}$ | normal | yes | yes | 0 | 1.5% | negative |
| MSA.32 | SNCA $^{-/-}$ | normal | yes | yes | 0 | 0.8% | negative |
| PD-3 | LRRK2 $^{wt/G2019S}$ GBA $^{wt/N370S}$ | normal | yes | yes | n/a | n/a | negative |
| PD-3.26 | LRRK2 $^{wt/wt}$ GBA $^{wt/N370S}$ | normal | yes | yes | 0 | 1.4% | negative |

**Figure 5 Comparison of LRRK2 Edited and Unedited NSCs Under Starvation Conditions Reveals Functional Differences**

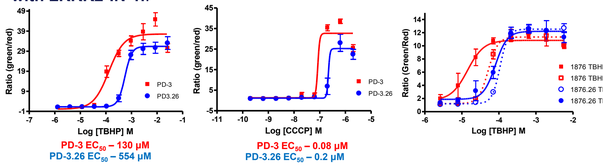
**A NSCs Harboring a LRRK2 Mutation are More Susceptible to Cell Death**




**B LRRK2 Inhibitor (LRRK2-IN-1) Reverses Metabolic Differences Observed Between LRRK2 Edited and Unedited NSCs**




**C NSCs With a LRRK2 Mutation Show Greater Sensitivity to Compounds in a Mitochondrial Membrane Potential Assay. Effect is Reversible by Treatment with LRRK2-IN-1..**




NSCs from PD-3 or PD-3.26 (G2019S LRRK2 mutation corrected) were grown overnight in Neurobasal Media with neural induction media supplement but without glucose (starvation media). **(A)** NSCs were stained with the LIVE/DEAD® Viability/Cytotoxicity Kit and counterstained with the NucBlue® dye. NSCs from PD-3 (LRRK2 mutant) are more susceptible to cell death than PD-3.26. **(B)** NSCs from PD-3 or PD3-26 were exposed to a dilution series of staurosporine or chloroquine in the presence or absence of the LRRK2-IN-1 for 24 hours. The PrestoBlue® Cell Viability Assay reveals that the metabolic difference observed between PD3 and PD-3.26 is reversed by the LRRK2-IN-1. **(C)** NSCs from PD-3 or PD-3.26 were left untreated or treated with LRRK2-IN-1 for 24 hours. NSCs were then loaded with a mitochondrial membrane potential dye (m-MPI Codex™) and exposed to a dilution series of the indicated compounds for 2 hrs. or 6 hrs. NSCs from PD-3 showed greater sensitivity to TBHP and CCCP. The greater sensitivity to TBHP was reversed by pre-treatment with LRRK2-IN-1.

## CONCLUSIONS

We have successfully generated iPSCs, NSCs and edited iPSCs from the dermal fibroblasts of 3 Parkinson's disease patients, one MSA patient, and 2 age-matched control individuals using the CytoTune®-iPS Sendai Reprogramming Kit, Gibco® PSC Neural Induction Medium, and GeneArt® Precision TALENs .

- CytoTune®-iPS Sendai reprogramming kit is easy to use with high efficiency
- The resulting iPSCs express the expected pluripotency markers and are karyotypicallty normal.
- Line to line variations were observed during the reprogramming.
- Neural stem cells were rapidly obtained from monolayer cultures using the Gibco® PSC Neural Induction and these NSCs expressed the expected neural stem cell markers
- GeneArt® Precision TALENs were used to correct the various disease mutations in order to provide genetically matched control cell model pairs
- Functional differences were observed between the LRRK2 G2019S edited and unedited NSCs in a LIVE/DEAD® Cell Viability/Cytotoxicity Assay, PrestoBlue® Cell Viablity reagent, and a mitochondrial membrane potential assay (m-MPI Codex™) under starvation conditions.

Given the efficiency, speed, and ease with which we were able to reprogram adult disease fibroblasts, it is clear that the CytoTune® -iPS Sendai Reprogramming Kit can be applied to large-scale reprogramming of multiple disease lines in an automated fashion. Similiarly, use of the Gibco® PSC Neural Induction will enable generation of NSCs, neurons and glia that may provide more relevant cell models when coupled with various assays to interrogate cell health or target-specific function. Finally, gene editing technologies, such as those enabled by TAL nuclease fusions, will allow us to address mutated genes, correct them to wild-type and generate matched cell model controls.

## ACKNOWLEDGEMENTS

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## TRADEMARKS/LICENSING

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