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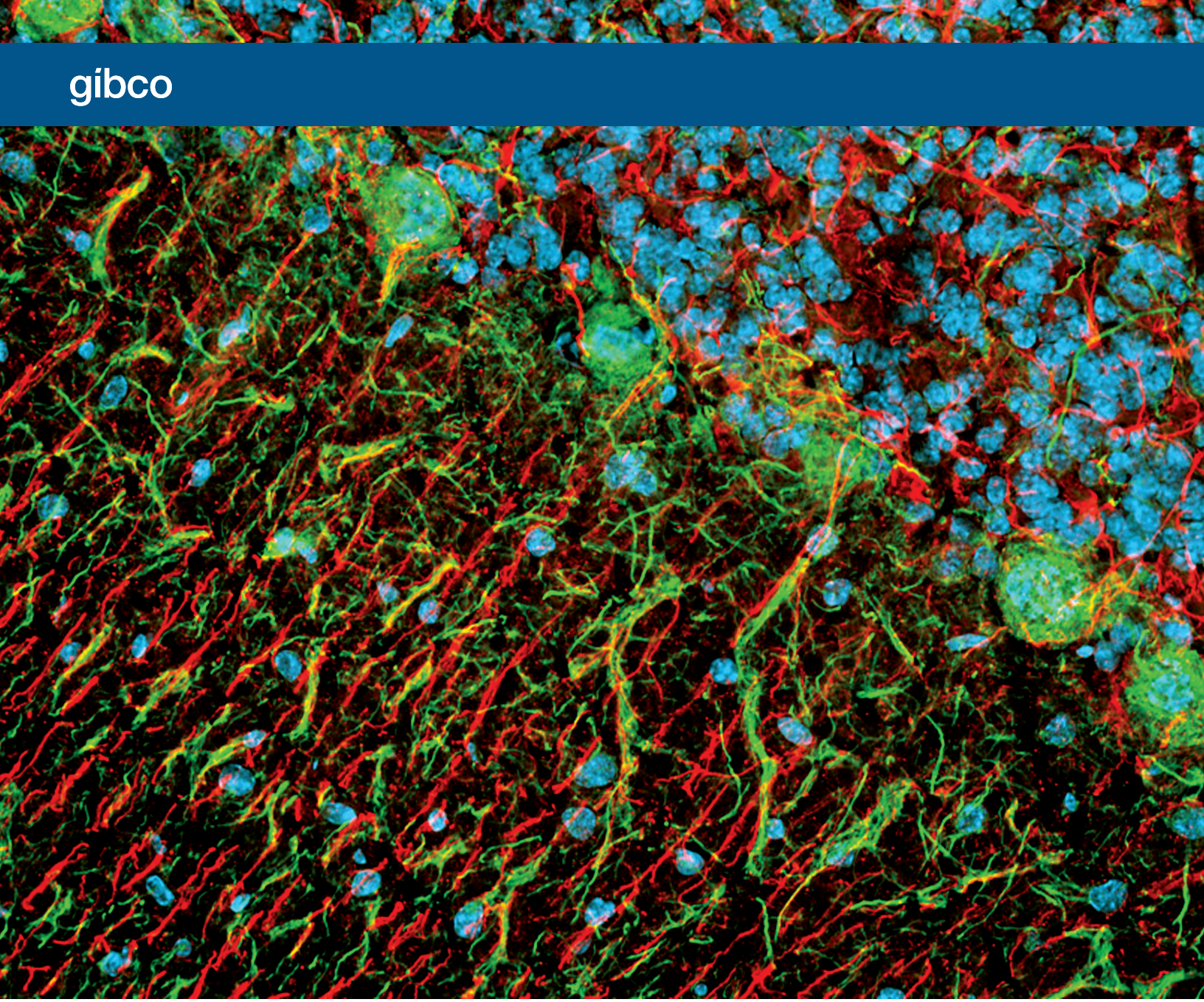


Disease model generation: 5 steps to model Parkinson's disease

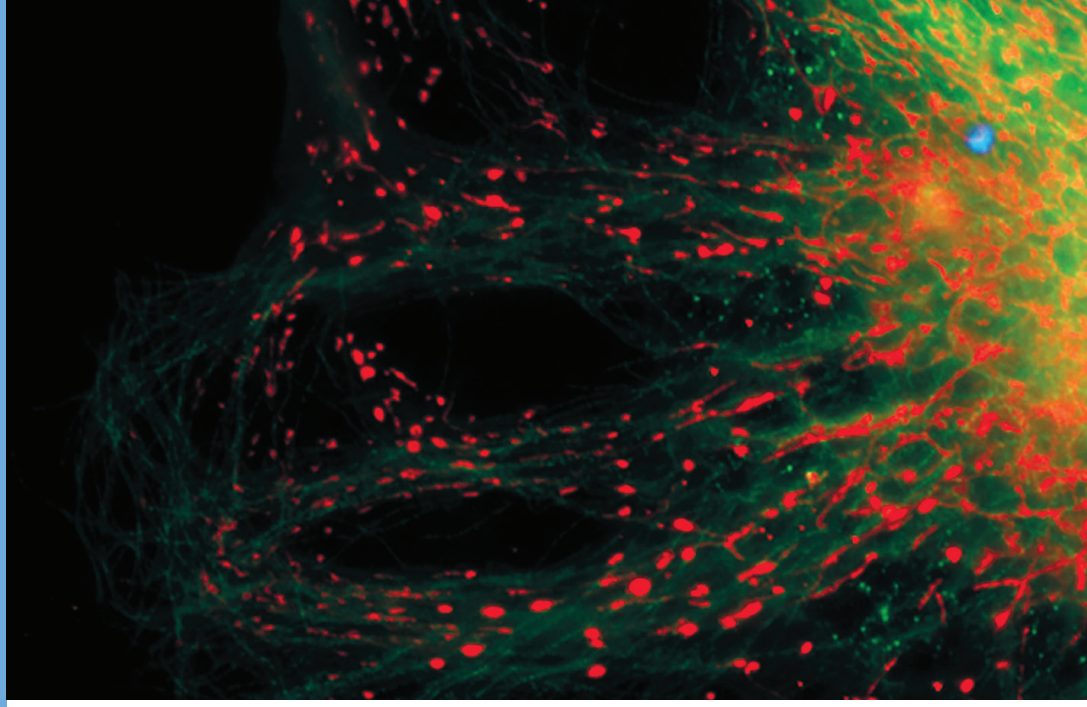
Follow this proven guide to facilitate generation of iPSC-derived disease models

ThermoFisher SCIENTIFIC

Introduction

The discovery that human somatic cells can be reprogrammed into pluripotent stem cells (PSCs) that can be further differentiated into disease-relevant cell types, such as neurons and cardiomyocytes, has enabled scientists to use patient-derived cells for *in vitro* disease modeling studies and drug discovery. Moreover, the availability of genome editing tools, such as the CRISPR-Cas9 and TALEN™ systems, now allows scientists to introduce or correct disease-related genetic aberrations, such as single-nucleotide polymorphisms (SNPs), to study their contributions to the disease phenotypes.

The generation of a disease model includes the generation of an isogenic pair (consisting of the control and mutated hiPSC lines) through genome editing, characterization of the hiPSC lines, differentiation of the hiPSC lines into relevant cell types, and visualization of disease phenotypes. The generation of a cell model for a condition such as Parkinson's disease is a significant endeavor, so consider these 5 steps to help improve your success.



1

Edit—design, order, and deliver genome editing tools

Key reagents and tools:

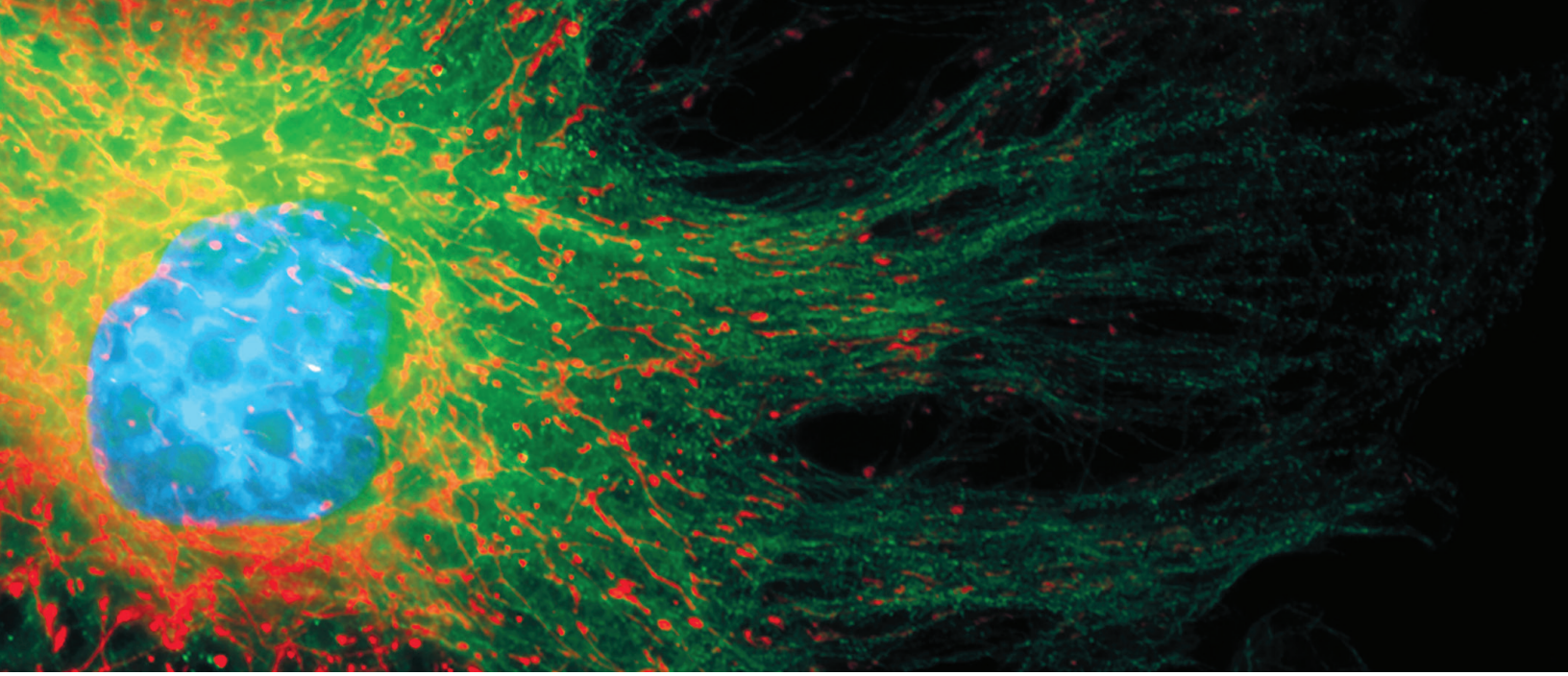
- Invitrogen™ TrueCut™ Cas9 Protein v2 and TrueGuide™ gRNA
- Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit
- Invitrogen™ PerfectMatch™ TALs
- Invitrogen™ Neon™ Transfection System
- Gibco™ StemFlex™ Medium
- Gibco™ RevitaCell™ Supplement
- Gibco™ rhLaminin-521
- Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit
- Ion GeneStudio™ S5 System and Ion AmpliSeq™ primers

2

Clone—isolate and expand clones from a pool of genome-edited hiPSCs

Key reagents and tools:

- StemFlex Medium
- RevitaCell Supplement
- rhLaminin-521
- Invitrogen™ TRA-1-60 Live Kit
- Invitrogen™ Propidium Iodide



Characterize—check pluripotency and genome stability after editing

Key reagents and tools:

- PluriTest™ services and tools
- Applied Biosystems™ KaryoStat™ services and tools
- Applied Biosystems™ TaqMan® hPSC Scorecard services and tools
- Ion GeneStudio S5 System and Ion AmpliSeq primers
- Invitrogen™ Attune™ NxT Flow Cytometer
- Thermo Scientific™ CellInsight™ CX5 and CX7 High-Content Analysis (HCA) Platforms
- Invitrogen™ EVOS™ Imaging Systems
- Invitrogen™ Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit

Differentiate—differentiate iPSCs into disease-relevant cell types

Key reagents and tools:

- Invitrogen™ PSC Dopaminergic Neuron Differentiation Kit
- Invitrogen™ antibody kits (e.g., Human Dopaminergic Neuron Immunocytochemistry Kit)

Measure—perform disease-relevant assays in iPSC-derived cell types

Key reagents and tools:

- Invitrogen™ PrestoBlue™ Cell Viability Reagent
- Invitrogen™ CellEvent™ Caspase-3/7 reagents
- Invitrogen™ Neurite Outgrowth Staining Kit
- Applied Biosystems™ TaqMan® qPCR probes and reagents
- CellInsight CX5 and CX7 HCA Platforms
- Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader

Step 1: Edit

Design, order, and deliver genome editing tools

To improve the genome editing workflow in hiPSCs, editing efficiency should be maximized to facilitate clonal isolation downstream. Aside from locus-specific differences, genome editing efficiency in hiPSCs mainly depends on the editing tools used and the method implemented to deliver those tools. Genome editing can be achieved using TALEN technology or the CRISPR-Cas9 system, with the latter being easier to implement (Figure 1). With both tools, DNA breaks are introduced that may be randomly repaired by nonhomologous end joining (NHEJ), yielding a mixture of insertions and deletions (indels), or may be selectively repaired through homology-directed repair (HDR). NHEJ-generated indels may be used to create gene knockout cell lines, while HDR may be used to change single bases or introduce small or large tags (e.g., 6xHis or GFP), resulting in mutated or tagged cell lines.

Tips

- SNPs can be introduced using a 100 nt single-stranded DNA (ssDNA) oligo as a donor, which works well with CRISPR-Cas9 and TALEN-based genome editing.
- Protocols that use the Neon Transfection System may need to be optimized for genome editing efficiency and cell survival for your own hiPSC line, but in general program 7 provides good delivery of CRISPR editing tools and cell survival with maintenance of high editing efficiency.
- A complete protocol for delivery of Cas9 protein, gRNA, and oligos into hiPSCs can be found at **thermofisher.com/diseasemodeling**.

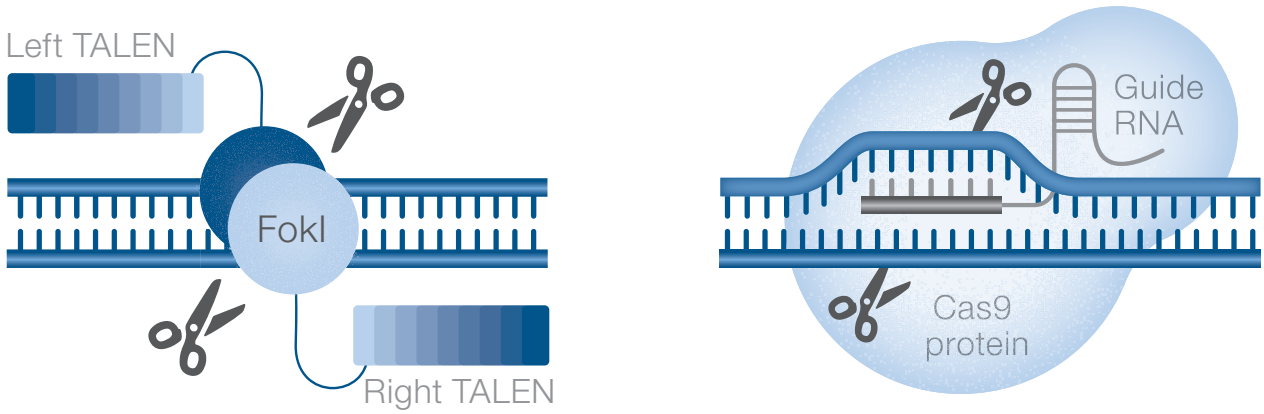


Figure 1. TALEN and CRISPR-Cas9 genome editing tools. Each editing tool binds and cleaves double-stranded DNA at specifically determined loci in the genome. Specificity is determined by the TAL or guide RNA (gRNA) sequence, which can be changed to target the desired genomic DNA sequence. While the CRISPR-Cas9 system is easy to use due to simple design and ordering of gRNAs, sequence constraints may limit the loci that can be targeted. PerfectMatch TALs can target any sequence in the human genome and may have fewer off-target effects due to the longer DNA-binding site.

Product highlights

- Neon Transfection System: Delivery of editing tools into iPSCs can be achieved using electroporation with the Neon Transfection System or lipofection with Invitrogen™ Lipofectamine™ Stem Transfection Reagent. While both methods enable successful genome editing, delivery of tools through electroporation typically provides higher editing efficiencies (Figure 2). A protocol is available at **thermofisher.com/neon**.
- Cas9 iPSCs and TrueCut Cas9 Protein v2: CRISPR-Cas9 editing in hiPSCs can be achieved by transfecting gRNA into a stable, Cas9-expressing hiPSC line such as Invitrogen™ Cas9 hiPSCs. Alternatively, a highly efficient Cas9 nuclease such as TrueCut Cas9 Protein v2 can be delivered into cells along with gRNA (Figure 3). These methods provide superior editing efficiencies compared to plasmid- or RNA-based methods. Explore our CRISPR products and services at **thermofisher.com/crispr**.
- GeneArt Precision gRNA Synthesis Kit: Make your own IVT gRNAs that are compatible with the available editing and delivery tools. To obtain gRNA sequences, use our Invitrogen™ GeneArt™ CRISPR Search and Design Tool at **thermofisher.com/crisprdesign**.
- PerfectMatch TALs: PerfectMatch TALs provide locus-specific localization of the FokI nuclease. Unlike other TAL effector proteins, PerfectMatch TALs are engineered to remove the 5´ base constraint and thus can be designed to target any desired sequence in the genome. Explore our TALEN products and services at **thermofisher.com/tals**.
- Custom genome editing services: Rely on our trusted products and expertise to have genome editing done for you. To send us a project inquiry, go to **thermofisher.com/cellmodels**.

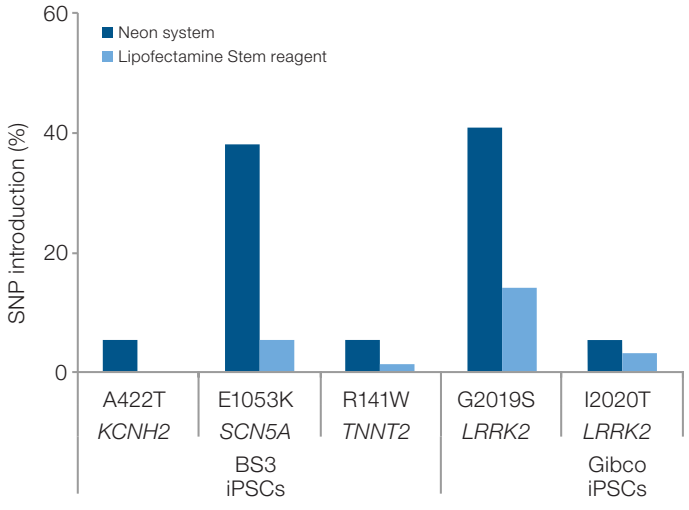


Figure 2. A comparison between delivery of editing tools using the Neon Transfection System and Lipofectamine Stem Transfection Reagent. Gibco™ Episomal and BS3 iPSC lines were edited at the indicated genomic loci using TrueCut Cas9 Protein v2, *in vitro* transcribed (IVT) gRNAs, and single-stranded oligo donors.

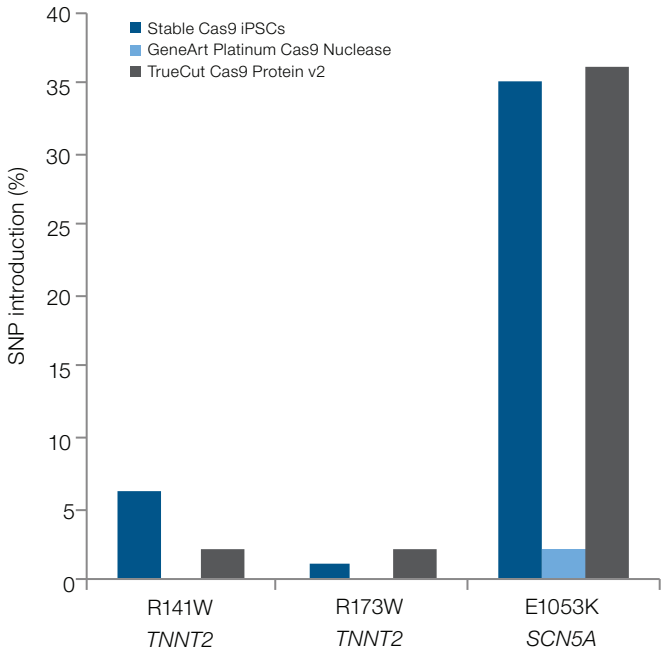


Figure 3. TrueCut Cas9 Protein v2 can induce SNP formation at frequencies equivalent to stable Cas9 iPSCs. HDR-mediated genome editing with gRNA and single-stranded oligo donor was performed using stable Cas9 hiPSCs or Cas9 protein variants, using the Neon device for delivery of the tools.

Step 2: Clone

Isolate and expand clones from a pool of genome-edited hiPSCs

After genome editing is achieved, the next step involves isolation of clonal hiPSC populations. Manual isolation of clones emerging after sparse plating of singularized cells is tedious and often requires multiple rounds of subcloning to isolate a fully clonal population. Single-cell isolation through cell sorting–based methods is a viable alternative, which had been challenging with hiPSC lines until recently. With the availability of state-of-the-art hiPSC culture reagents that are optimized for single-cell dissociation and survival of hiPSCs, we can now reliably isolate single-cell clones using cell sorting, with clonal recovery rates of 20–60% depending on the hiPSC line. The right extracellular matrix, growth medium, and survival supplements are key to a high recovery rate. In addition, stringent settings for cell sorting are required to reliably isolate single, viable hiPSCs and can be achieved through gating for cells that are negative for propidium iodide (PI) and positive for the pluripotency marker TRA-1-60 (Figure 4A). About 2 weeks after sorting, single-cell clones can then be easily visualized and further expanded for genetic analysis to identify clones with the edit of interest (Figure 4B). A protocol for clonal hiPSC isolation and expansion is available at **thermofisher.com/diseasemodeling**.

Tips

- Reagents developed for single-cell recovery dramatically improve cell survival of hiPSCs after sorting.
- Medium should not be changed for 3 days after single-cell sorting, to avoid aspiration of the single cells that are not fully attached.
- When using StemFlex Medium, medium changes every 3 days are sufficient for expansion of clones.
- Single-cell sorting into 96-well plates allows automation of downstream clone processing.

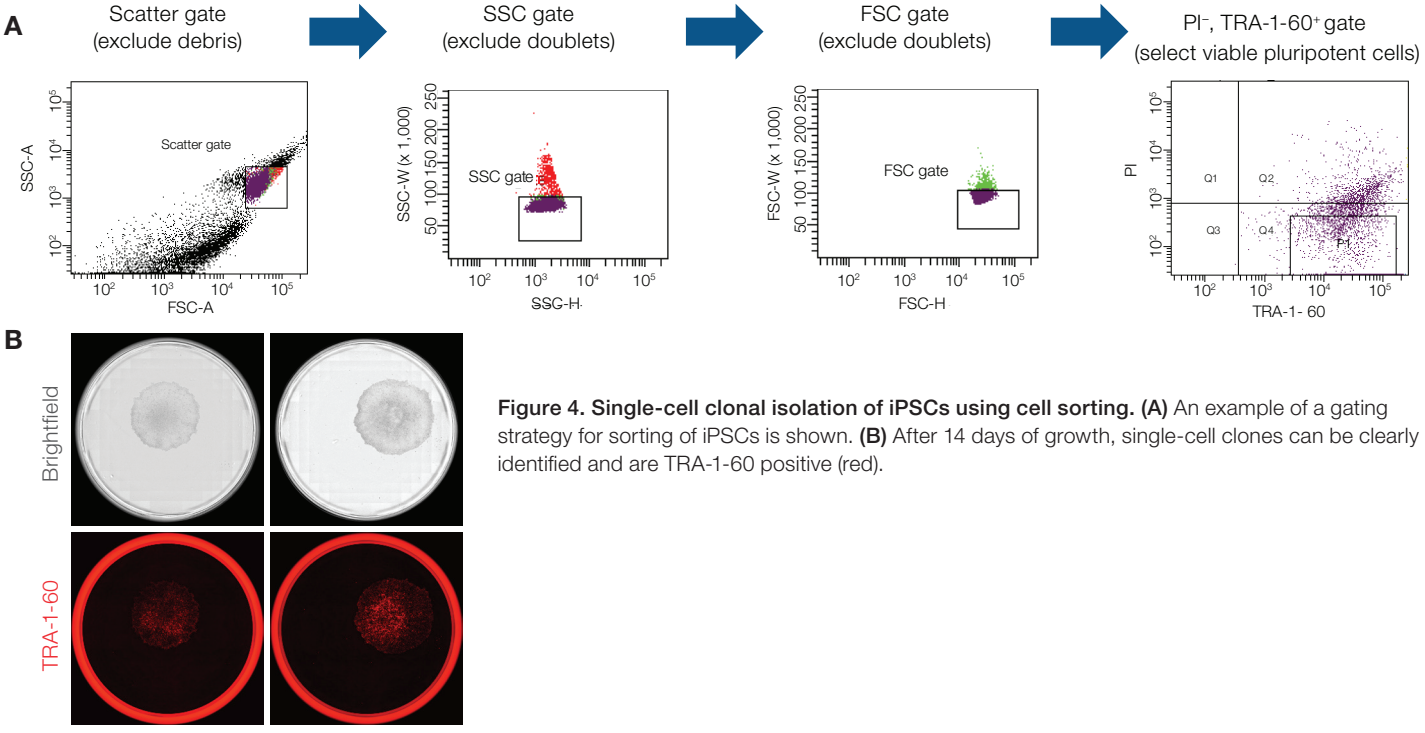


Figure 4. Single-cell clonal isolation of iPSCs using cell sorting. (A) An example of a gating strategy for sorting of iPSCs is shown. **(B)** After 14 days of growth, single-cell clones can be clearly identified and are TRA-1-60 positive (red).

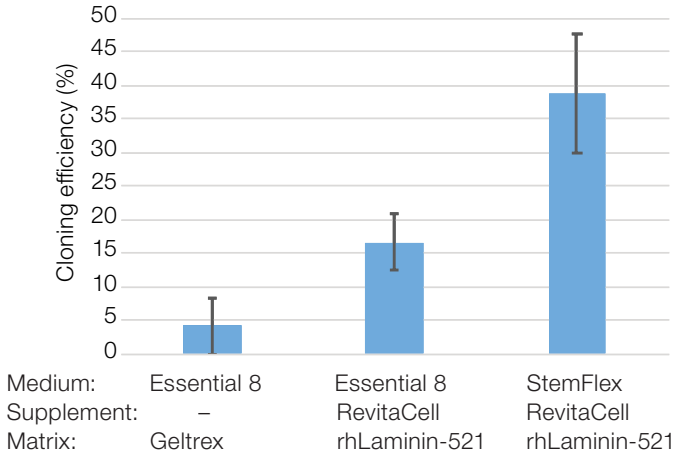


Figure 5. StemFlex Medium enhances cloning efficiency after sorting. StemFlex Medium, RevitaCell Supplement, and rhLaminin-521 are optimized for challenging applications such as single-cell passaging and clonal isolation.

Product highlights

- StemFlex Medium: Single-cell sorting of hiPSCs requires a robust medium that allows culture of single hiPSCs and extended culture of hiPSCs without the need for daily medium changes (Figures 5 and 6). Go to **thermofisher.com/stemflex** for protocols and additional application data.
- rhLaminin-521: Attachment and recovery of single hiPSCs is the key for effective clonal recovery after sorting. Our rhLaminin-521 provides superior single-cell attachment and survival of hiPSCs compared to other matrices such as vitronectin.
- RevitaCell Supplement: Cell survival cocktails greatly enhance the recovery of hiPSCs after singularization. In addition, the presence of such cocktails in the medium after sorting provides an additional boost to clonal recovery.

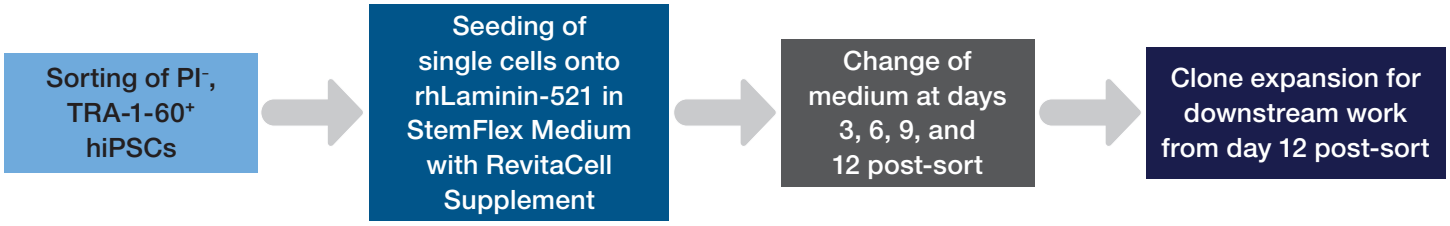


Figure 6. Workflow for isolation and recovery of single-cell hiPSC clones using cell sorting.

Step 3: Characterize

Check pluripotency and genomic stability after editing

Genome editing and clonal isolation of hiPSCs can be very stressful to the cells, and while in general no adverse effects are observed, it is important to characterize the hiPSCs before and after genome editing and clonal isolation experiments. Several options are available to easily assess the pluripotency status of hiPSCs at the protein or mRNA level, including systems that correlate your hiPSC line with a large reference set of commonly used PSC lines (Figure 7). Genomic stability is generally measured using traditional G-band karyotyping or through more objective digital karyotyping methods where DNA probes across the genome are used to map chromosomal gains and losses. To explore these options and more, go to **thermofisher.com/stemcellanalysis**.

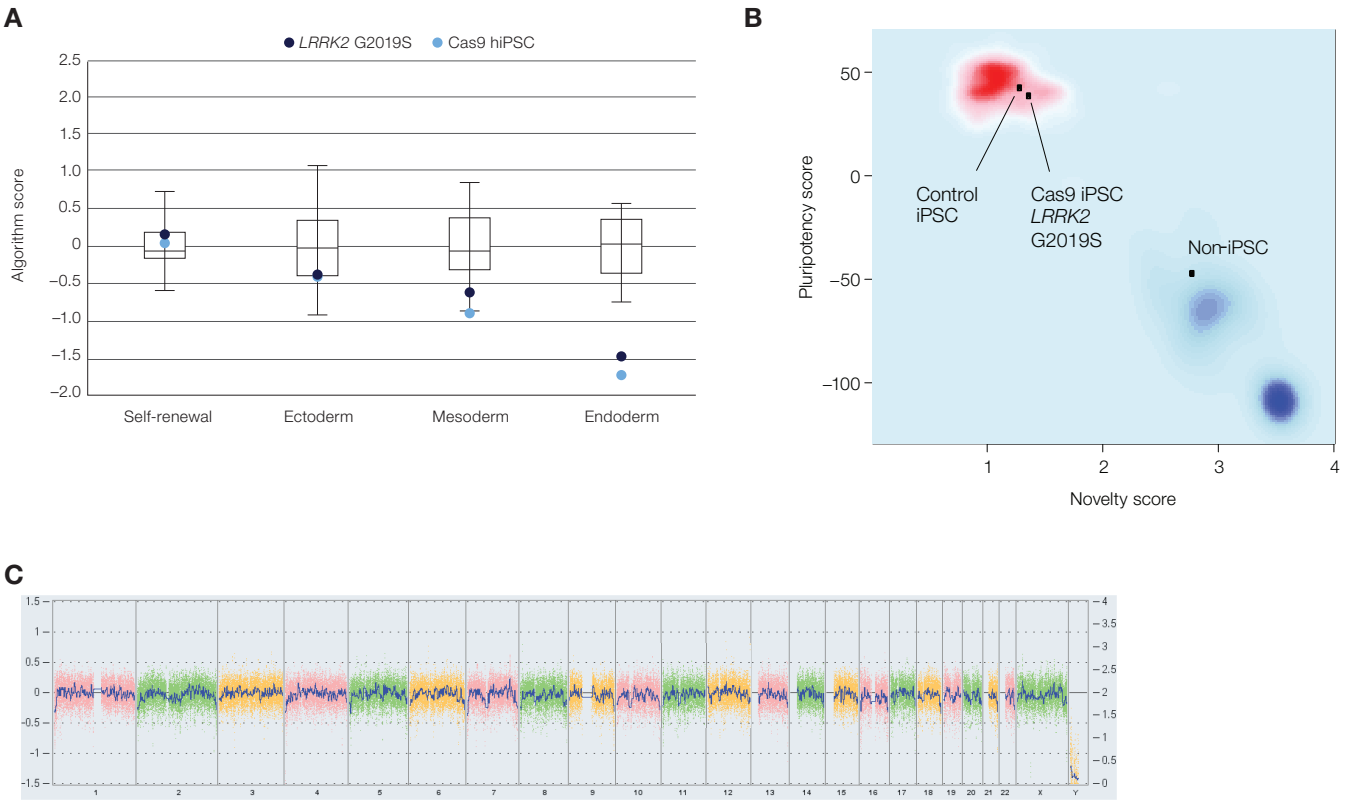


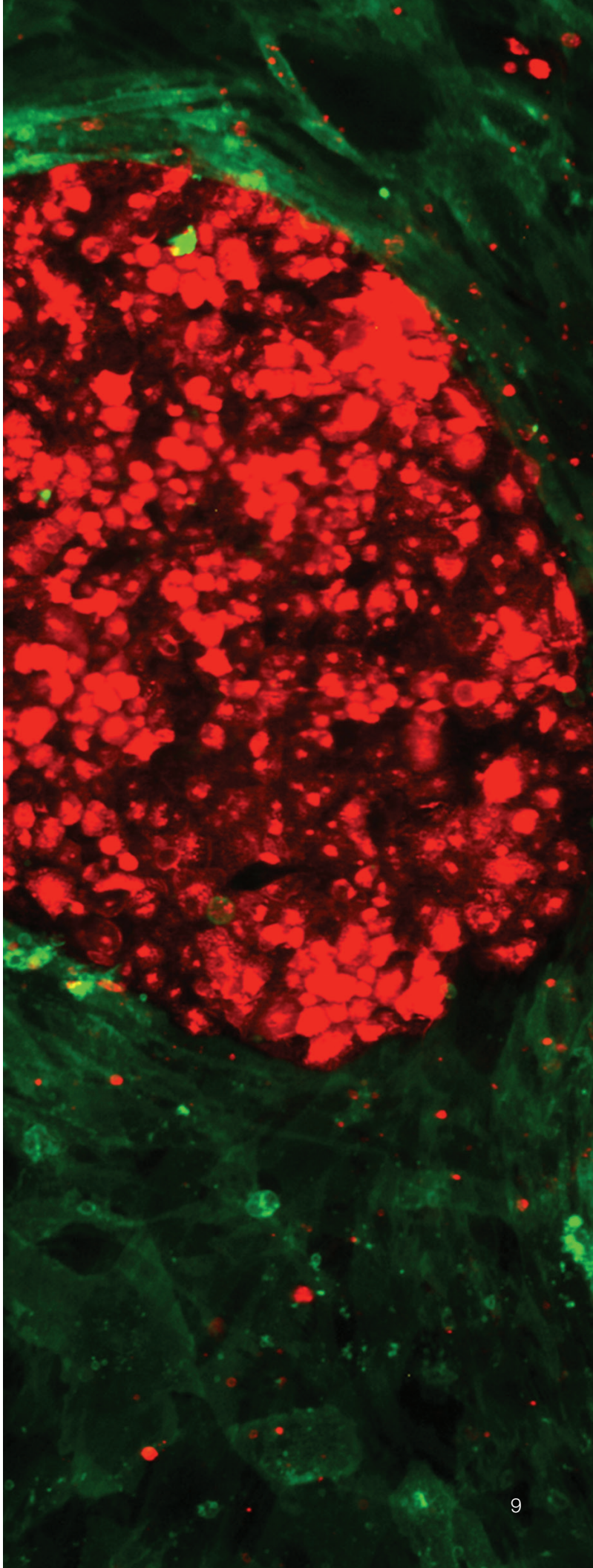
Figure 7. Characterization of a cell model for Parkinson's disease research. The *LRRK2* G2019S mutation was introduced by gene editing of Cas9 hiPSCs. A combination of approaches showed that pluripotency and karyotype were unaffected by genome editing. **(A)** The TaqMan hPSC Scorecard Panel compares the gene expression profile of the sample to that of a reference set (colored dots and gray box plots, respectively). This assay uses over 90 genes and a static database of 13 PSCs for the comparison. **(B)** The PluriTest Assay uses microarray data to confirm pluripotency marker expression via a pluripotency score (reflecting degree of pluripotency) and novelty score (reflecting degree of differentiation). This assay uses more than 36,000 transcripts and a fluid reference set of over 450 cell and tissue types for the comparison. **(C)** The KaryoStat Assay offers whole-genome coverage for accurate detection of copy number changes and genomic aberrations.

Product highlights

- TaqMan hPSC Scorecard Panel: Teratoma formation is one of the methods used to demonstrate true pluripotency of an hiPSC line, but it is a lengthy process that requires the use of animals. Using mRNA isolated from hiPSCs and embryoid bodies (an unbiased way to generate all three germ layers from hiPSCs) in gene expression analysis, pluripotency can be confirmed through the presence of pluripotency genes in the hiPSCs and germ layer genes in the embryoid bodies [1,2].

- PluriTest Assay: RT-qPCR methods allow pluripotency assessment through mRNA expression analysis of a selected panel of well-established genes. However, unbiased methodologies based on mRNA from the entire genome allow a more accurate comparison against the mRNA profile of a collection of well-characterized hiPSCs [2,3]. The PluriTest Assay is a bioinformatics-based assay that compares the transcriptional profile obtained by microarray analysis of a sample to an extensive reference set of hESC lines, iPSC lines, somatic cells, and tissues.

- KaryoStat Assay: Genomic stability of hiPSCs should be checked regularly since hiPSCs are known to have an unstable genome. Traditionally, the karyotypes of hiPSCs have been analyzed using G-band–based karyotyping to reveal chromosomal aberrations. Alternatively, a molecular approach can be taken where the genomic DNA of the hiPSC line is surveyed on a microarray containing DNA probes across the genome. Probe intensities are then used to obtain a detailed view of the copy number and aberrations in DNA regions across the genome.



Step 4: Differentiate

Differentiate iPSCs into disease-relevant cell types

Disease phenotypes generally manifest themselves in specific cell types, so we must differentiate the genome-edited hiPSC line into a mature cell type that is relevant to the disease. For Parkinson's disease, dopaminergic (DA) neurons in the midbrain, specifically in the substantia nigra, become affected and die as the disease progresses. Differentiation typically occurs in multiple stages that can take up to 2 months, depending on the cell type. Cardiomyocytes can be obtained in less than 2 weeks, while generation of DA neurons generally takes about 2 months. Due to the lengthy differentiation process, it is important to monitor the cells during differentiation through immunocytochemistry for markers that are specific for the fate of interest (Figure 8).

Tips

- Large cryobanks of progenitor cells can be made to shorten the time spent generating DA neurons.
- Tracking of stage-specific markers is important to ensure high-quality differentiation.
- DA neurons can be generated in bulk and replated for downstream assays.

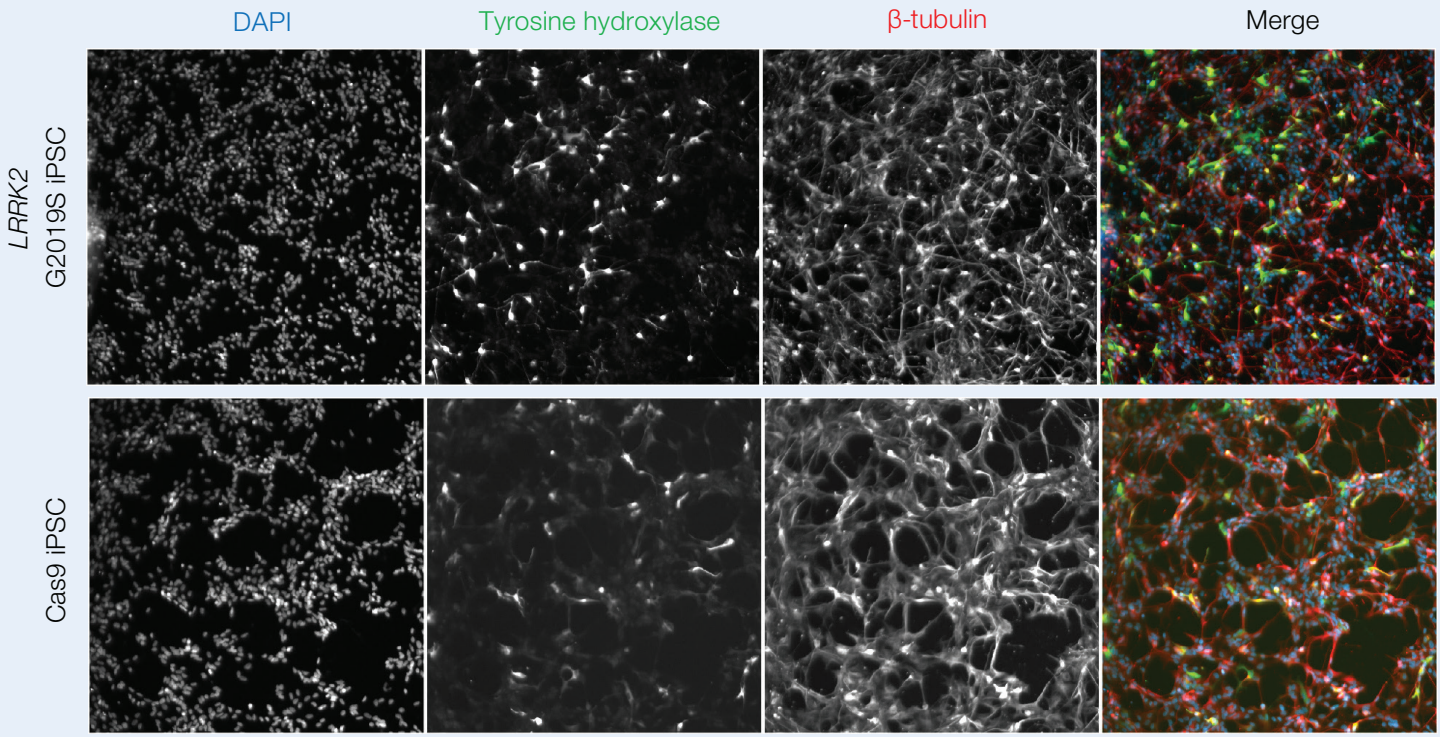
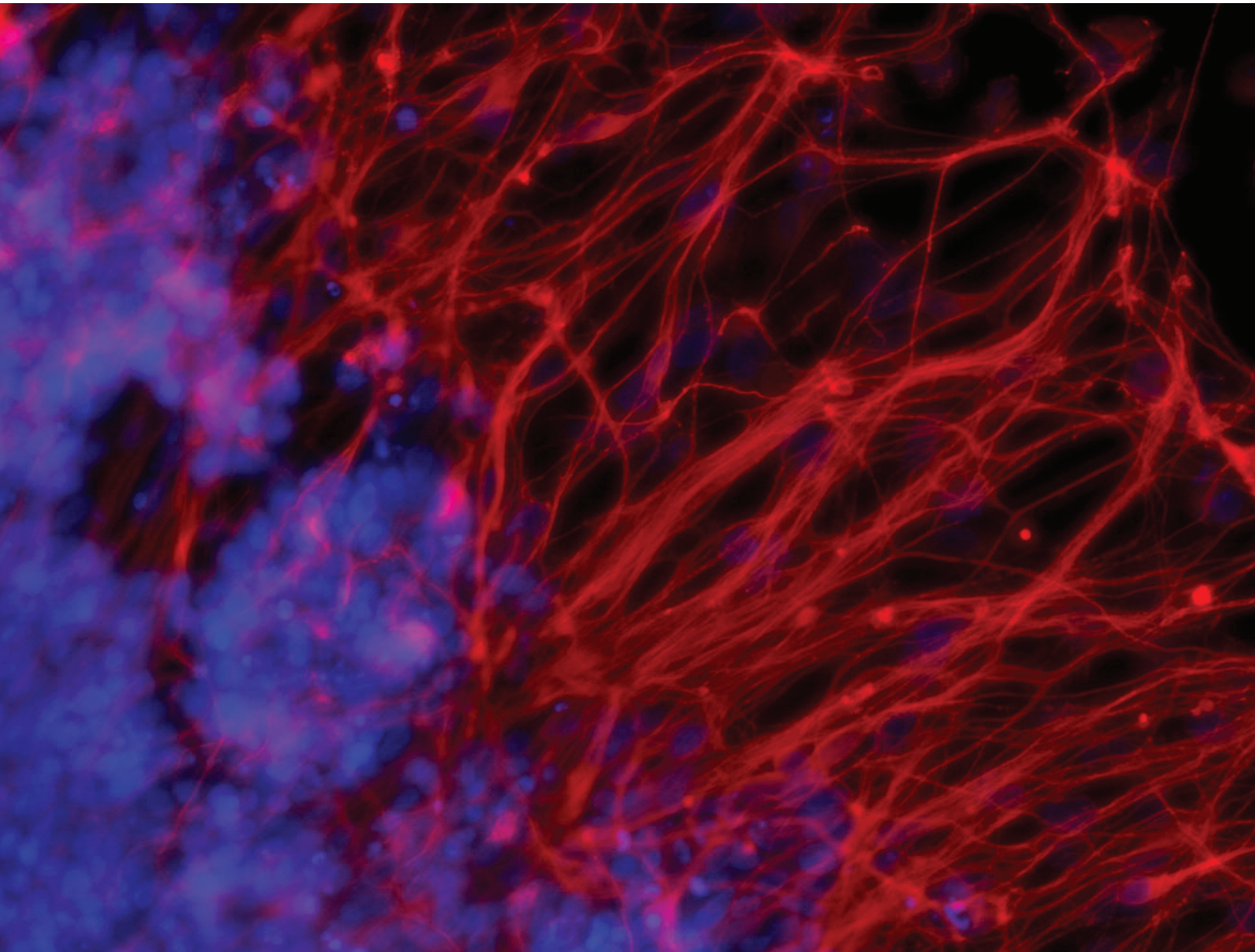


Figure 8. DA neurons derived from iPSCs with the *LRRK2* G2019S mutation. Cells were stained for tyrosine hydroxylase, an enzyme required for dopamine biosynthesis and a key marker of differentiation to dopaminergic neurons.

Product highlights

- PSC Dopaminergic Neuron Differentiation Kit: Differentiation to specialized cells such as DA neurons can be challenging due to complicated and lengthy processes. Kits that simplify hiPSC differentiation in the lab are now available, relying on a small number of media that are used to specify progenitors and ultimately the mature cell type. Protocols and data for this kit are available at **thermofisher.com/differentiation**.
- Human Dopaminergic Neuron Immunocytochemistry Kit: Throughout the differentiation process, efficiency during the different stages can be followed through protein expression analysis of fate-specific markers such as tyrosine hydroxylase.



Step 5: Measure

Perform disease-relevant assays in iPSC-derived cell types

After differentiation of edited and isogenic control hiPSCs into the cell type of interest, disease-relevant phenotypes can be studied. A wide variety of assays can be used, such as live tracking of cells, endpoint assays such as Invitrogen™ MitoTracker™ or CellEvent™ assays, and gene expression assays. In Parkinson's disease, it has been established that neurite length is affected, and DA neurons are much more sensitive to cell death compared to normal DA neurons [4] (Figures 9–11).

Tips

- Cell behavior and health can be measured over time using an HCA instrument that has an onstage incubator such as the CellInsight CX7 HCA Platform.
- Unwanted progenitor cells in a culture can be excluded from analysis using cell-specific markers as a gating strategy during image analysis.
- Cell health can be visualized in live cultures using PrestoBlue or CellEvent reagents.
- RT-qPCR or whole-genome mRNA analysis can reveal new molecular signatures of disease.

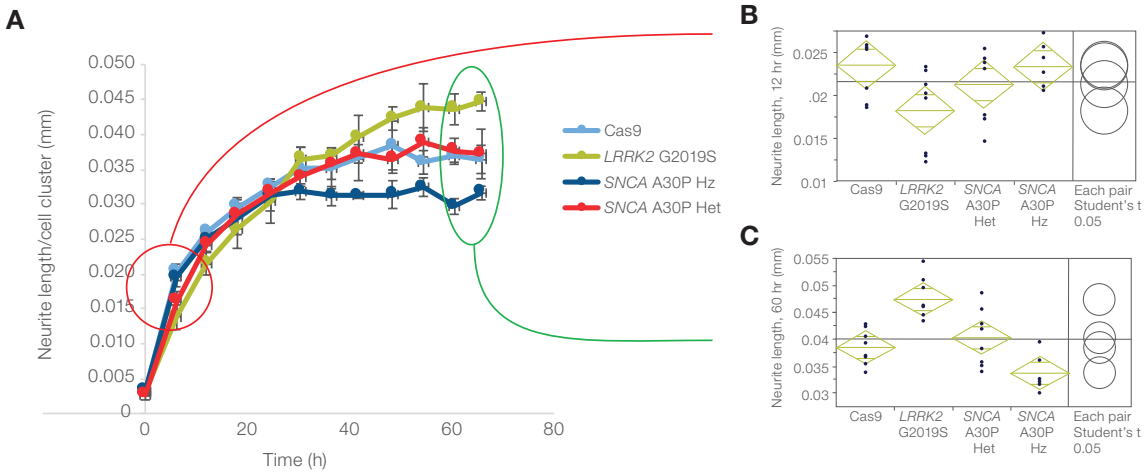


Figure 9. The Parkinson's disease–related *LRRK2* G2019S mutation impacts neurite outgrowth. (A) Live-cell HCA was used to measure DA neurite length over time. **(B)** *LRRK2* G2019S DA neurons show a neurite outgrowth delay compared to control Cas9 iPSC–derived neurons and other neurons carrying Parkinson's disease–related mutations, including *SNCA* A30P homozygous (Hz) and heterozygous (Het) mutations. **(C)** Once neurites have grown out, *LRRK2* G2019S DA neurons have longer neurites while *SNCA* A30P Hz neurons have shorter neurites.

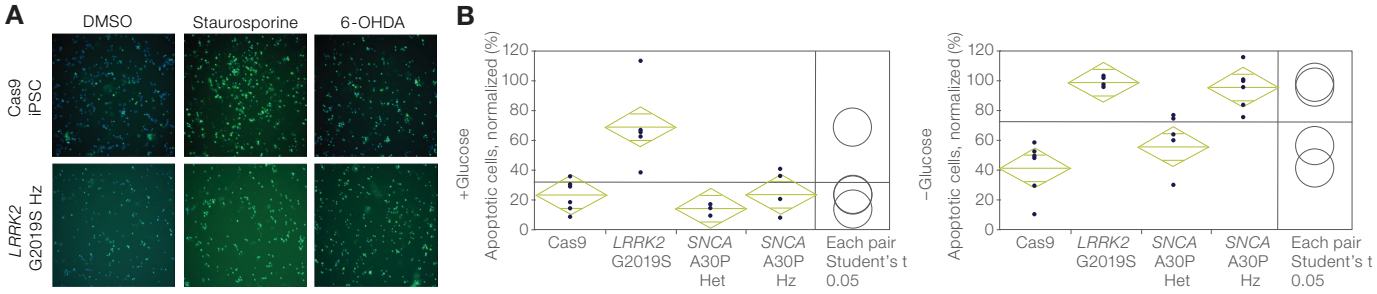
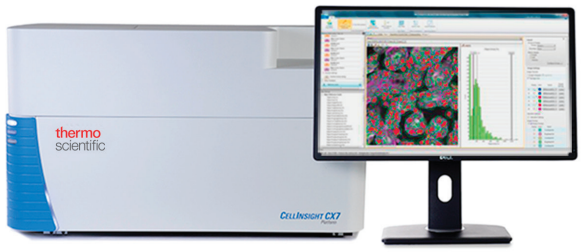


Figure 10. Neurons with the *LRRK2* G2019S mutation are more sensitive to 6-hydroxydopamine (6-OHDA). The CellEvent Caspase-3/7 Green Detection Reagent was used to measure apoptosis of neurons with the *LRRK2* G2019S mutation under different conditions. **(A)** Representative images are shown of *LRRK2* G2019S and control cells after treatment with staurosporine or 6-OHDA (green signal indicates ongoing apoptosis). **(B)** HCA reveals greater sensitivity of *LRRK2* G2019S DA neurons to 6-OHDA than control Cas9 cells, under normal glucose conditions [4]. When cells are further stressed through the removal of glucose, the effects of 6-OHDA are more pronounced and now also manifest in the *SNCA* A30P Hz neurons.



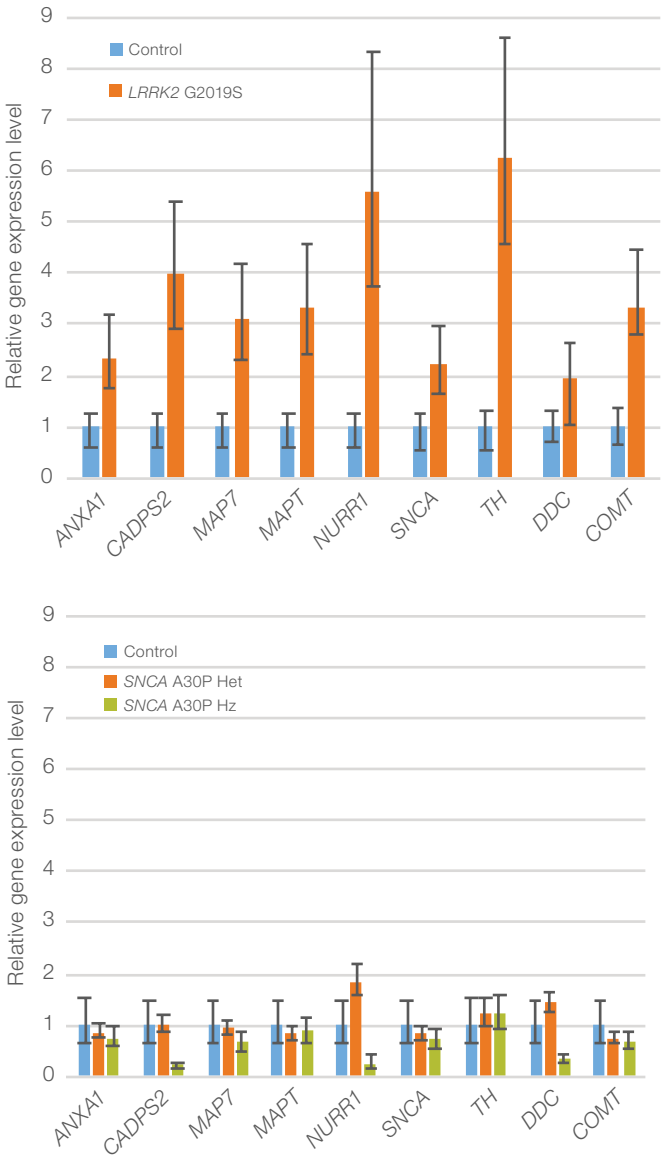


Figure 11. Measurement of gene expression changes with TaqMan Assays. Genes associated with *LRRK2* mutants are exclusively upregulated in *LRRK2* G2019S DA neurons and are largely unaffected in neurons derived from hiPSCs carrying other Parkinson's disease–related mutations.

Product highlights

- CellInsight HCA platforms with onstage incubator: Assays for disease models require precise control of temperature, humidity, and CO_2 levels to observe and measure biological activity and changes over time. Data gathered from longer-term imaging studies are the basis of quantitative analysis studies, which implement image analysis software to generate meaningful data. Find out more about live-cell HCA at **thermofisher.com/hca**.
- CellEvent Caspase-3/7 detection reagents: Cellular processes can be visualized using one of many available dyes to unravel disease-specific phenotypes. Reagents designed to measure cell division, mitochondrial health, cell health, and many other processes are widely available and are compatible with most plate readers and HCA instruments.
- QuantStudio 12K Flex Real-Time PCR System and TaqMan Assays: Disease is often related to changes in gene expression. Through careful selection of TaqMan Assays to measure these changes, disease-specific mechanisms in cell lines can be uncovered. To find assays for your cell model, go to **thermofisher.com/taqman**.

Notes

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References

1. Bock C, Kiskinis E, Verstappen G et al. (2011) Reference maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* 144:439–452.
2. Bouma MJ, van Iterson M, Janssen B et al. (2017) Differentiation-defective human induced pluripotent stem cells reveal strengths and limitations of the teratoma assay and *in vitro* pluripotency assays. *Stem Cell Reports* 8:1340–1353.
3. Müller FJ, Schuldt BM, Williams R et al. (2011) A bioinformatic assay for pluripotency in human cells. *Nat Methods* 8:315–317.
4. Reinhardt P, Schmid B, Burbulla LF et al. (2013) Genetic correction of a *LRRK2* mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. *Cell Stem Cell* 12:354–367.

Find out more at **thermofisher.com/diseasemodeling**

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