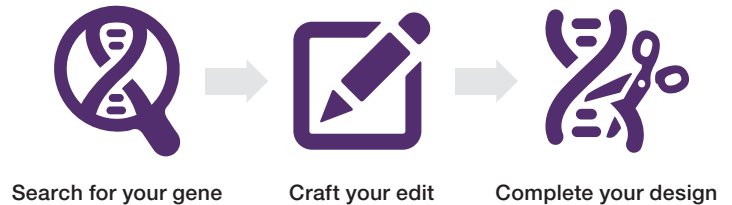


# Improving and simplifying the design of knock-in experiments using the TrueDesign Genome Editor

## Introduction

Genome editing technologies have given investigators the power to unlock a variety of new applications and experimental approaches. While gene knockouts have been achievable for several years, successful gene knock-ins have remained elusive due to the low efficiency of homology-directed repair (HDR). Successful knock-in experiments depend on the design of the donor DNA, the effectiveness of the single guide RNA (sgRNA) and Cas9 ribonucleoprotein (RNP) complex, and the efficiency of delivering materials into the target cells. Here we show how our researchers have optimized these steps and demonstrate how to introduce single-nucleotide polymorphisms (SNPs) and fluorescent tags with up to 100% editing efficiency.

To enable access to these optimized approaches to genome editing, we have created the Invitrogen™ TrueDesign™ Genome Editor, a free online tool for designing and ordering materials for knock-in CRISPR-Cas9–based editing. This application note describes two use cases: (1) introduction of a SNP change in the *LRRK2* gene (G2019S) in induced pluripotent stem cells (iPSCs) and (2) tagging of beta-actin with GFP. For SNP changes, a recommended design for a single-stranded oligo deoxyribonucleotide (ssODN) donor is automatically generated through the online tool. For fusion proteins, primer recommendations are automatically generated for production of double-stranded donor DNA using the **Invitrogen™ TrueTag™ Donor DNA Kit**. Donor DNA is then cotransfected with **Invitrogen™ TrueCut™ Cas9 Protein v2** and **TrueGuide™ Modified Synthetic sgRNA**. A detailed protocol for this transfection process is also described in this application note. Once delivered into cells, the donor DNA will be integrated into the genome of your target cells in less than a day.



## TrueDesign Genome Editor

The TrueDesign Genome Editor ([thermofisher.com/truedesign](http://thermofisher.com/truedesign)) enables scientists of all experience levels to easily design materials for accurate and successful knock-in experiments. The tool has an intuitive point-and-type interface that allows users to easily find and edit a gene by streamlining the process in four steps: search, edit, design, and review.

The simple step-by-step program allows you to:

- Generate a complete knock-in design in minutes
- Edit up to 30 bases in any human gene using CRISPR-Cas9 or TALEN technology
- Design all necessary tools for precise SNP or amino acid changes
- Design the tools to add a GFP, RFP, or luciferase tag to a target gene without the need for cloning
- Download or order your comprehensive design and recommended reagents with a single click

## Use case 1: Designing *LRRK2* G2019S SNP change

The discovery that human somatic cells can be reprogrammed to become pluripotent stem cells (PSCs) that can differentiate into neurons and cardiomyocytes has enabled scientists to use patient-derived cells for *in-vitro* disease modeling studies. Moreover, the availability of genome editing tools like CRISPR-Cas9, allows scientists to introduce or correct known disease-related genetic aberrations to study their contributions to disease phenotypes. Here we describe how the TrueDesign Genome Editor can be used to design materials for knocking in the G2019S mutation in the *LRRK2* gene, which is a well-established mutation underlying Parkinson's disease and described in greater detail at [thermofisher.com/diseasemodeling](https://thermofisher.com/diseasemodeling).

To begin your design, select SNP Edit as your experiment type and Human as your species, then search for the gene of interest (e.g., human *LRRK2*) in the TrueDesign Genome Editor (Figure 1) using the gene symbol or Entrez gene ID. Alternatively, you may enter a chromosome position or paste in 150–300 nucleotides of any human gene.

For the Parkinson's disease gene example, we will make a SNP change in exon 41 of *LRRK2* that converts the glycine (G) at position 2019 to serine (S). Exon 41 is identified from the Topology map, and the appropriate amino acid is chosen from the Sequence Viewer. To make the SNP change, select the G in the first codon position and change it to an A in the choices presented in the interface (Figure 2). Your edit will appear in the Sequence Viewer window in real time.

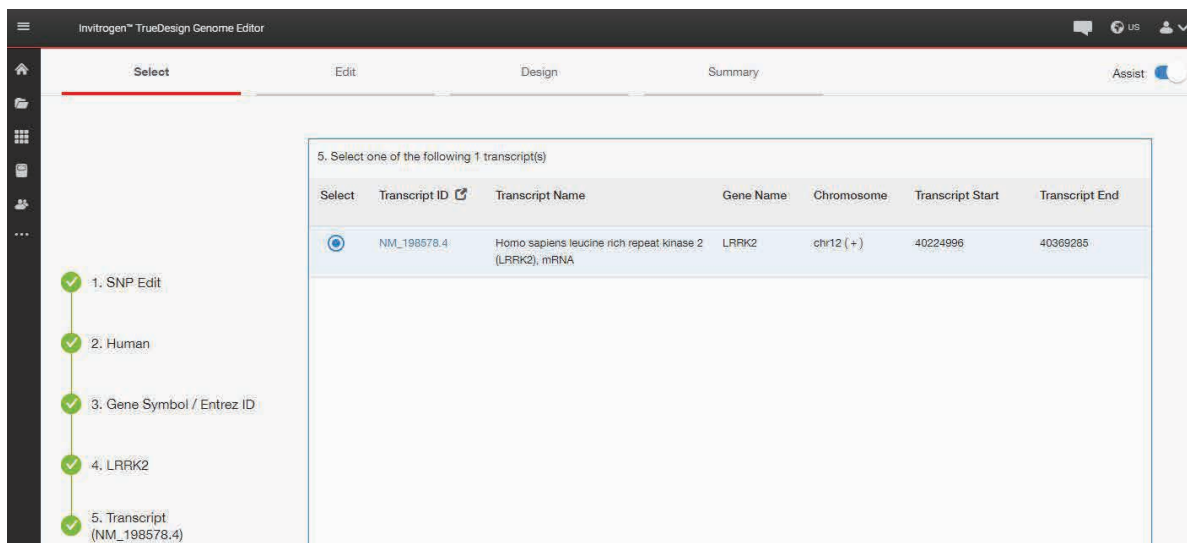


Figure 1. Identification of the human *LRRK2* gene. Users may search for genes by providing the gene symbol or Entrez gene ID.

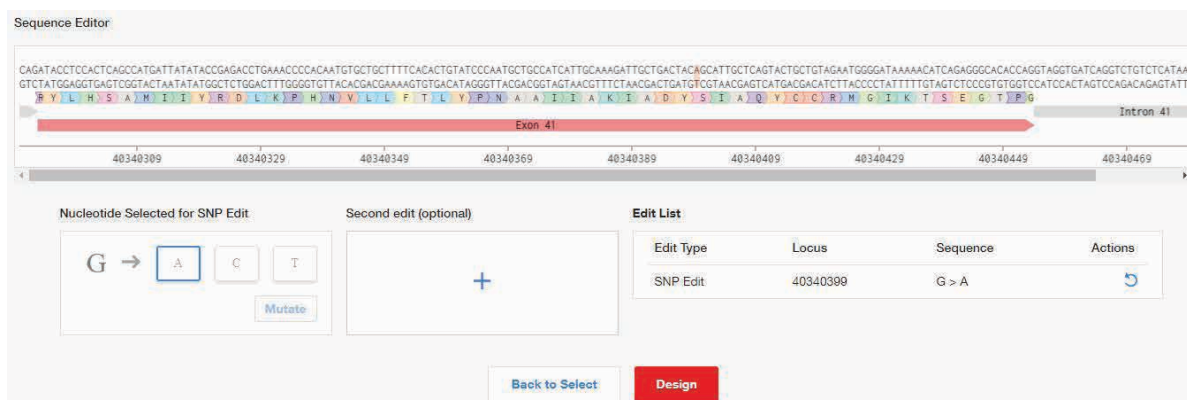


Figure 2. Creation of a SNP edit. Users can identify the specific nucleotide or amino acid of interest and change the corresponding base(s) to introduce the SNP using the tool. Two SNPs can be introduced in one editing step as long as they are less than 30 bases apart. A summary of your edits will be displayed in the Edit Summary Table.



After isolation of the desired edited PSC clones, neuronal differentiation of the edited PSCs, and characterization of the resulting neurons, investigators can use the cells to better understand the impact of *LRRK2* on Parkinson's disease progression. More information about this disease model and stem cell genome engineering can be found at [thermofisher.com/diseasemodeling](http://thermofisher.com/diseasemodeling).

## Use case 2: Designing GFP tagging of beta-actin (ACTB)

Actin is a ubiquitously expressed cytoskeletal protein involved in cell structure, integrity, and motility. Actin polymerizes to produce filaments that form crosslinked networks in cytoplasm. Fluorescent tagging of actin provides a persistent cytosolic counterstain that is similar to labeled phalloidin, and it can be used for cell cycle studies.

The same approach can be applied to any number of characterized or uncharacterized proteins to produce cell models for high-content imaging, screening assays, or intracellular trafficking studies.

For this design, choose Fluorescent Tagging, your species, and search for the *ACTB* gene in the TrueDesign Genome Editor. After choosing the appropriate transcript, select Edit to move to the next stage.

Choose the position where you would like to place the tag (N- or C-terminus), the reporter gene (RFP or GFP), and the selectable marker (blasticidin or puromycin). Once your selection has been made, click Add Tag, and your selection will be visible in the Edit Summary Table and the Sequence Editor (Figure 5).

The screenshot shows the TrueDesign Sequence Editor interface. At the top, the DNA sequence is displayed with a red arrow indicating the insertion site in Exon 2. Below the sequence, there are three selection panels:

- 1. Select a Tag:** Options include Amino-(N-) Terminus and Carboxy-(C-) Terminus.
- 2. Select a Reporter Gene:** Options include RFP and GFP.
- 3. Select a Selection Marker:** Options include Blasticidin and Puromycin.

At the bottom, there are buttons for "Back to Select", "Add Tag", and "Design". To the right, an "Edit List" table shows the selected configuration:

Edit Type	Locus	Sequence	Actions
Fluorescent Tag	5529654	Blast-GFP	

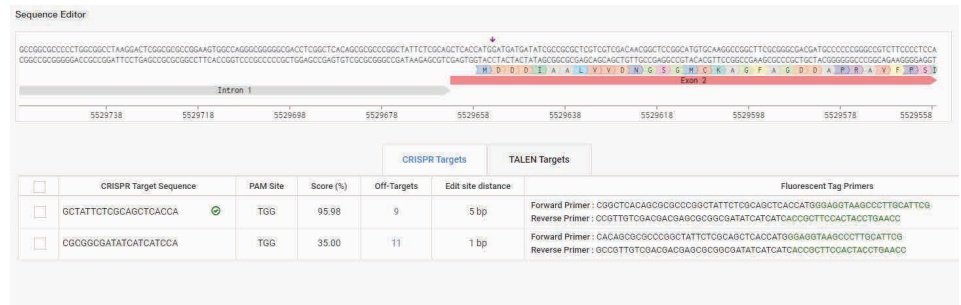
**Figure 5. Creation of a TrueTag GFP reporter.** The user can choose a GFP or RFP tag to place at the N- or C-terminus of the protein encoded by the selected gene.

After you complete your edit, click Design, which will prompt the TrueDesign tool to identify all the possible CRISPR-Cas9 gRNA binding sites and design the matching PCR primers required to configure the Puro-EmGFP donor template provided in the TrueTag Donor DNA Kit (Cat. No. A42992). The recommended CRISPR-Cas9 gRNA (Figure 6) and TALEN designs (Figure 7) will be displayed in tables with matching PCR primers for configuring the donor template.

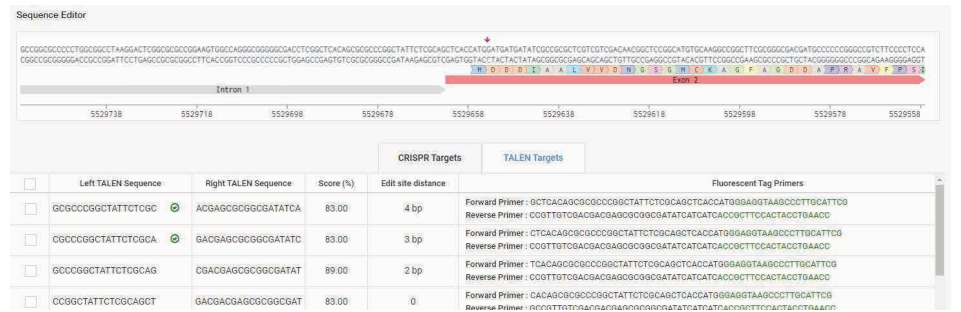
Once you have selected designs, proceed to the final Review page that displays the matching PCR primers.

This example is for the recommended gRNA design, GCTATTCTCGCAGCTCACCA (PAM TGG). To create a knock-in using TrueTag donor DNA, use the displayed forward and reverse primers with the materials provided in the TrueTag Donor DNA Kit to amplify a functional donor template.

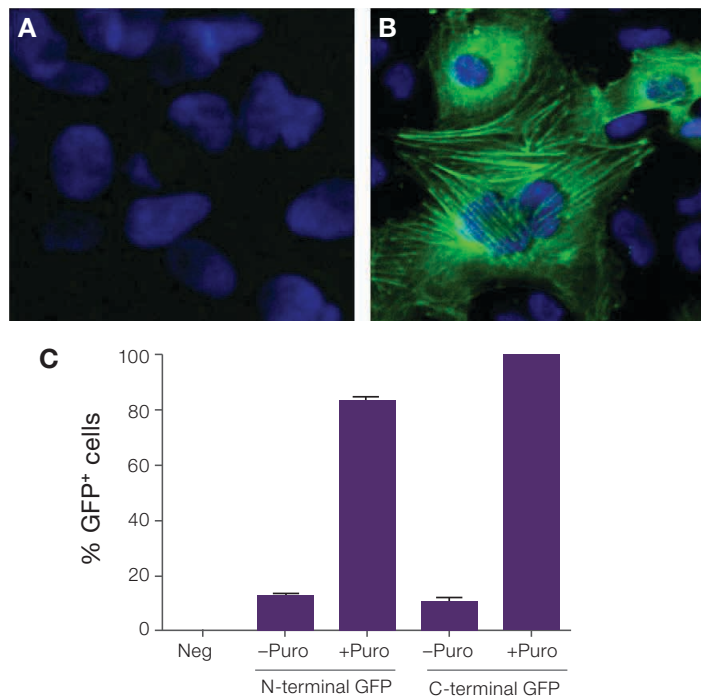
After successful amplification and purification of the donor DNA, cotransfect it into cells with the gRNA and Cas9 protein. Figure 8 shows U2OS cells (green) counterstained with Hoechst™ dye (blue) that are expressing GFP-tagged *ACTB*. When puromycin selective pressure is applied to these cell pools, the cell population can be driven to be up to 100% GFP-positive as quantified with flow cytometry. A detailed workflow is described in the TrueTag Donor DNA Kit user guide, which can be found at [thermofisher.com/truetag](http://thermofisher.com/truetag).



**Figure 6. Generation of CRISPR targets.** Up to three targets will be recommended for users. There will be a mix of CRISPR-Cas9 and TALEN designs depending on the context sequence and predicted specificity and effectiveness. The matching PCR primers required for configuring the donor template are also displayed in each row.



**Figure 7. Generation of TALEN targets.** Up to three targets will be recommended for users. There will be a mix of CRISPR-Cas9 and TALEN designs depending on the context sequence and predicted specificity and effectiveness. The matching PCR primers required for configuring the donor template are also displayed in each row.



**Figure 8. *ACTB*-tagged cells (green) counterstained with Hoechst dye (blue). (A)** Negative control and **(B)** edited cells showing clear actin filament formation with GFP-*ACTB* fusion proteins. **(C)** Summary of three experiments in which puromycin selection was applied to drive the cell populations to be >80% GFP-positive and >99% positive for the C-terminal constructs. Images were captured on the Invitrogen™ EVOS™ FL Color Imaging System.

## Successful delivery of the genome editing payload into your cells

### Cas9 protein and gRNA

For CRISPR-Cas9 editing with donor DNA designed with the TrueDesign Genome Editor, we recommend using TrueCut Cas9 Protein v2 (Cat. No. A36498, 100 µg). This is the *Streptococcus pyogenes* Cas9 protein with optimized N- and C-terminal nuclear localization signals. Cas9 protein is complexed with TrueGuide Modified Synthetic sgRNA (Cat. No. A35534, 1.5 nmol), which is a chemically modified and ready-to-use one-piece sgRNA, to drive maximum editing efficiency with valuable cells that are difficult to edit, such as primary cells, immune cells, and stem cells.

### Transfection

The following protocol is provided as a starting point for transfecting wild-type cells with TrueCut Cas9 Protein v2, TrueGuide sgRNA, and either a ssODN or TrueTag donor DNA using the Invitrogen™ Neon™ Transfection System. To optimize the electroporation conditions for a particular cell line, follow the 24-well optimization protocol for the Neon Transfection System as described in the user guide. Each of the 24 conditions are varied in pulse voltage, pulse width, and the number of pulses to determine which condition works best with your cell line.

For electroporation in a 24-well format, we recommend using 1,250 ng of TrueCut Cas9 Protein v2, 240 ng of TrueGuide sgRNA, and 500 ng of TrueTag donor DNA in a 10 µL reaction.

### Day 0: Seed cells

- 1–2 days before transfection, transfer your adherent cells to a new flask with fresh growth medium so that they are 70–90% confluent on the day of transfection.

### Day 1: Transfect cells

2. Prepare a 24-well plate with 1,000 µL of cell type-specific growth medium in each well and place it in the 37°C incubator to prewarm.
3. Prepare tube 1: RNP complex and donor DNA in Resuspension Buffer R.
  - a. Mix TrueCut Cas9 Protein v2, TrueGuide sgRNA, donor DNA, and Resuspension Buffer R in a fresh, RNase-free microcentrifuge tube. For an ssODN knock-in, follow Table 1. For a TrueTag donor DNA knock-in, follow Table 2. Mix well.

Table 1. Preparation of tube 1 for ssODN knock-in.

Reagent	Amount per well of 24-well plate
TrueCut Cas9 Protein v2	1,250 ng (7.5 pmol)
TrueGuide sgRNA	240 ng (7.5 pmol)
ssODN	10 pmol
Resuspension Buffer R	To 5 µL

Table 2. Preparation of tube 1 for TrueTag donor DNA knock-in.

Reagent	Amount per well of 24-well plate
TrueCut Cas9 Protein v2	1,250 ng (7.5 pmol)
TrueGuide sgRNA	240 ng (7.5 pmol)
TrueTag donor DNA	500 ng (0.5 pmol)
Resuspension Buffer R	To 5 µL

**Important:** Maintain TrueCut Cas9 Protein v2 and TrueGuide sgRNA at a 1:1 molar ratio. Use high-concentration TrueCut Cas9 Protein v2, and ensure that the total volume of the RNP complex (Cas9 protein + sgRNA) does not exceed 1/10 of the total reaction volume (e.g., 0.5 µL of Cas9 protein + gRNA in 5 µL total reaction volume).

- b. Incubate the mixture at room temperature for 5–20 min.
4. Prepare tube 2: cells in Resuspension Buffer R.

**Note:** Prepare an extra amount of cells (2x what is needed).

    - a. If you are using suspension cells, remove an aliquot and determine the viable cell count. If you are using adherent cells, detach the cells from the culture flask using Gibco™ TrypLE™ Express Enzyme, resuspend the cells in an appropriate volume of growth medium, then determine the viable cell count.
    - b. Transfer the appropriate number of cells into a 15 mL centrifuge tube, then pellet the cells by centrifugation at 100–400 x g for 5 min at room temperature.
    - c. Wash the cells with PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup> using the same volume as the original cell volume, then pellet the cells by centrifugation at 100–400 x g for 5 min at room temperature.
    - d. Aspirate the PBS and resuspend the cell pellet in Resuspension Buffer R at the desired concentration. For example, to use 100,000 cells/reaction, resuspend the cells at 2.0 × 10<sup>7</sup> cells/mL, then use 5 µL of the resuspended cells per reaction. Gently pipet the cells to obtain a single-cell suspension.

5. Add cells from tube 2 to tube 1.
  - a. Pipet the cells in Resuspension Buffer R (from step 4) up and down to resuspend any cells that might have settled at the bottom of the tube.
  - b. Add 5  $\mu\text{L}$  of the cell suspension to tube 1 (from step 3).
6. Electroporate on the Neon Transfection System using the cell type-specific condition.
  - a. Using the 10  $\mu\text{L}$  Neon tip, aspirate 10  $\mu\text{L}$  of the mixture from step 5, then electroporate using your cell type-specific condition.

**Important:** Avoid creating bubbles that can hinder electroporation.

- b. After electroporation, transfer the contents of the Neon tip immediately into one well of the 24-well culture plate containing 1,000  $\mu\text{L}$  of prewarmed growth medium (from step 2).
- c. Incubate the cells in a humidified 37°C incubator under 5%  $\text{CO}_2$  for 48–72 hr.

## Conclusion

In this work we show how to simplify the steps for designing knock-in experiments with the TrueDesign Genome Editor for both a disease-relevant SNP edit and a TrueTag donor DNA knock-in of a fluorescent protein. We also provide an optimized protocol for preparing a genome editing complex and donor DNA molecule for transfection using the Neon Transfection System. The suite of reagents and protocols described here provide a streamlined, end-to-end solution for improving the CRISPR-Cas9 knock-in workflow.

## Ordering information

Product	Cat. No.
TrueDesign Genome Editor	<a href="http://thermofisher.com/truedesign">thermofisher.com/truedesign</a>
TrueCut Cas9 Protein v2	A36498
TrueGuide Modified Synthetic sgRNA	A35534
TrueGuide sgRNA Negative Control, non-targeting 1	A35526
TrueTag Donor DNA Kit, GFP	A42992
Hoechst 33342 Solution (20 mM)	62249
Neon Transfection System	MPK5000
Neon Transfection System 10 $\mu\text{L}$ Kit	MPK1025
Pluripotent Stem Cell (PSC) Gene Editing Demo Kit	A43203
StemFlex Medium	A3349401
Recombinant Human Laminin-521	A29248
RevitaCell Supplement (100X)	A2644501
TrypLE Express Enzyme (1X), phenol red	12605010
DPBS, no calcium, no magnesium	14190144

Find out more at [thermofisher.com/truedesign](http://thermofisher.com/truedesign)