Gene editing

A rapid method for GFP tagging of genes and enrichment of edited cells

Introduction

Precise tagging of genes via the homology-directed repair (HDR) pathway is a versatile approach to study gene function. Advances in genome editing technologies such as the CRISPR-Cas9 system have allowed researchers to target nearly any gene for modification [1]. However, the efficiency of integrating large DNA molecules into the mammalian genome via HDR is inherently low. Here we describe a simple method for efficiently tagging endogenous genes using donor DNA prepared with the Invitrogen™ TrueTag™ Donor DNA Kit, GFP. The efficiency of tagging endogenous genes ranges from 50% to 100% with antibiotic selection. The method has been verified using multiple targets in many different cell lines, including human induced pluripotent stem cells (iPSCs) and hematopoietic stem cells (HSCs). This technique has broad applications in general genome engineering, protein production, and immune cell therapy.

Materials and methods

Use of the CRISPR-Cas9 system enables researchers to efficiently introduce double-strand breaks (DSBs) in genomic DNA. In this method, transfection of a complex of target-specific guide RNA (gRNA) and Cas9 protein efficiently creates a DSB in the target of interest [2]. The DSBs are then mostly repaired by either the nonhomologous end joining (NHEJ) pathway or the HDR pathway. For the HDR pathway, the cells may utilize sister chromatids or an exogenous donor DNA template to repair the DNA damage, but the efficiency is relatively low. The TrueTag Donor DNA Kit, GFP (Cat. No. A42992), enables rapid production of donor DNA that contains an antibiotic selection marker to enrich cells containing the tagged gene (Figure 1).

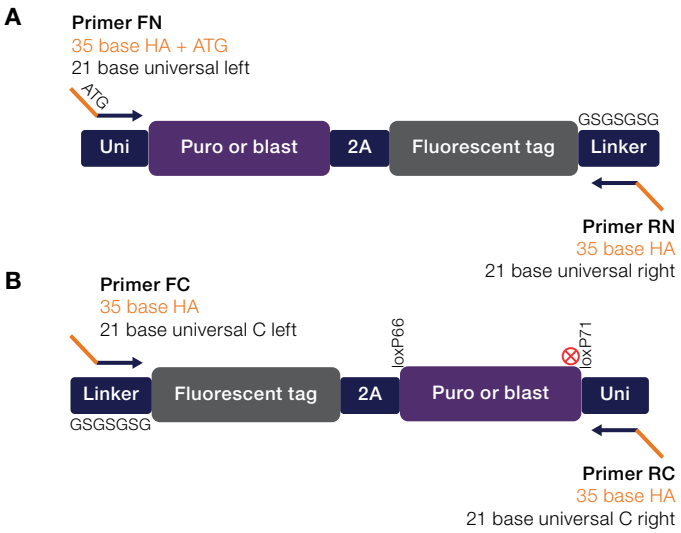

Figure 1. Donor DNA template design. The TrueTag Donor DNA Kit provides PCR templates for either **(A)** N-terminal tagging or **(B)** C-terminal tagging of a target gene. Locus-specific primers with short homology arm (HA) sequences are used for PCR amplification to generate the donor DNA molecule. After cleavage of the target site via the CRISPR-Cas9 or TALEN™ system, the donor DNA is integrated into the genome during the HDR process. The 2A self-cleaving peptide (2A) allows the selection marker (puromycin or blasticidin) and tagged gene to be expressed from the endogenous promoter. Universal priming sequences (Uni) for each template allow easy design of PCR primers.

The gRNAs were designed using the Invitrogen™ TrueDesign™ Genome Editor (Figure 2) and then synthesized using the Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit. The concentration of gRNA was determined using the Invitrogen™ Qubit™ RNA BR Assay Kit. Predesigned or custom synthetic gRNA can also be ordered directly from the TrueDesign Genome Editor software or at **thermofisher.com/trueguide**.

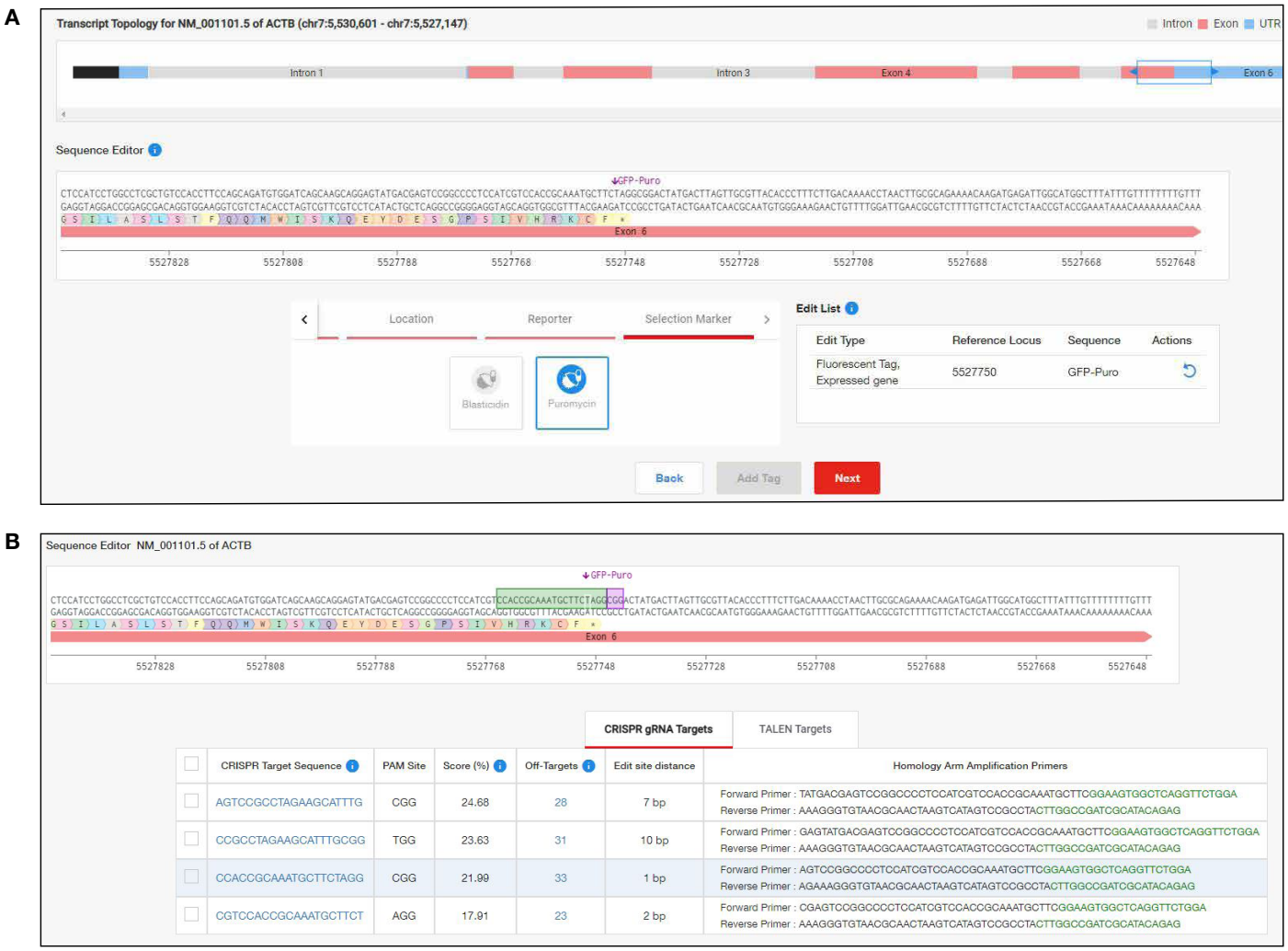

Figure 2. gRNA design and homology arm primer design. The TrueDesign Genome Editor is specially designed as a companion to the TrueTag Donor DNA Kit experimental workflow. **(A)** This free online tool walks users through selection of a gene transcript, N- or C-terminal reporter, and selection marker. **(B)** The tool then offers a choice of HDR-appropriate CRISPR gRNAs or TALEN mRNA pairs and generates designs for locus-specific homology arm primers. gRNA designs are limited to within 10 bp upstream and downstream of the insertion site to improve overall editing efficiency.

Invitrogen™ TrueCut™ Cas9 Protein v2, locus-specific gRNA, and purified donor DNA were co-delivered into cells by electroporation using the Invitrogen™ Neon™ Transfection System or by lipid-mediated transfection using Invitrogen™ Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent. 293FT cells, iPSCs, and HSCs were evaluated in separate experiments. GFP-positive cells were imaged using the Invitrogen™ EVOS™ Imaging System. Genome modification efficiency was determined using the Invitrogen™ Attune™ NxT Flow Cytometer or by junction PCR and sequencing.

Results

The TrueTag Donor DNA Kit simplifies the production of donor DNA molecules for targeting of different genes. For a given tag, the same donor template is amplified to generate donor DNA molecules with different homology arms. Six different genes that encode protein products localized at different subcellar compartments were tagged with GFP. The basal expression levels of fluorescent fusion proteins and their subcellular locations could be visualized by fluorescence microscopy (Figure 3).

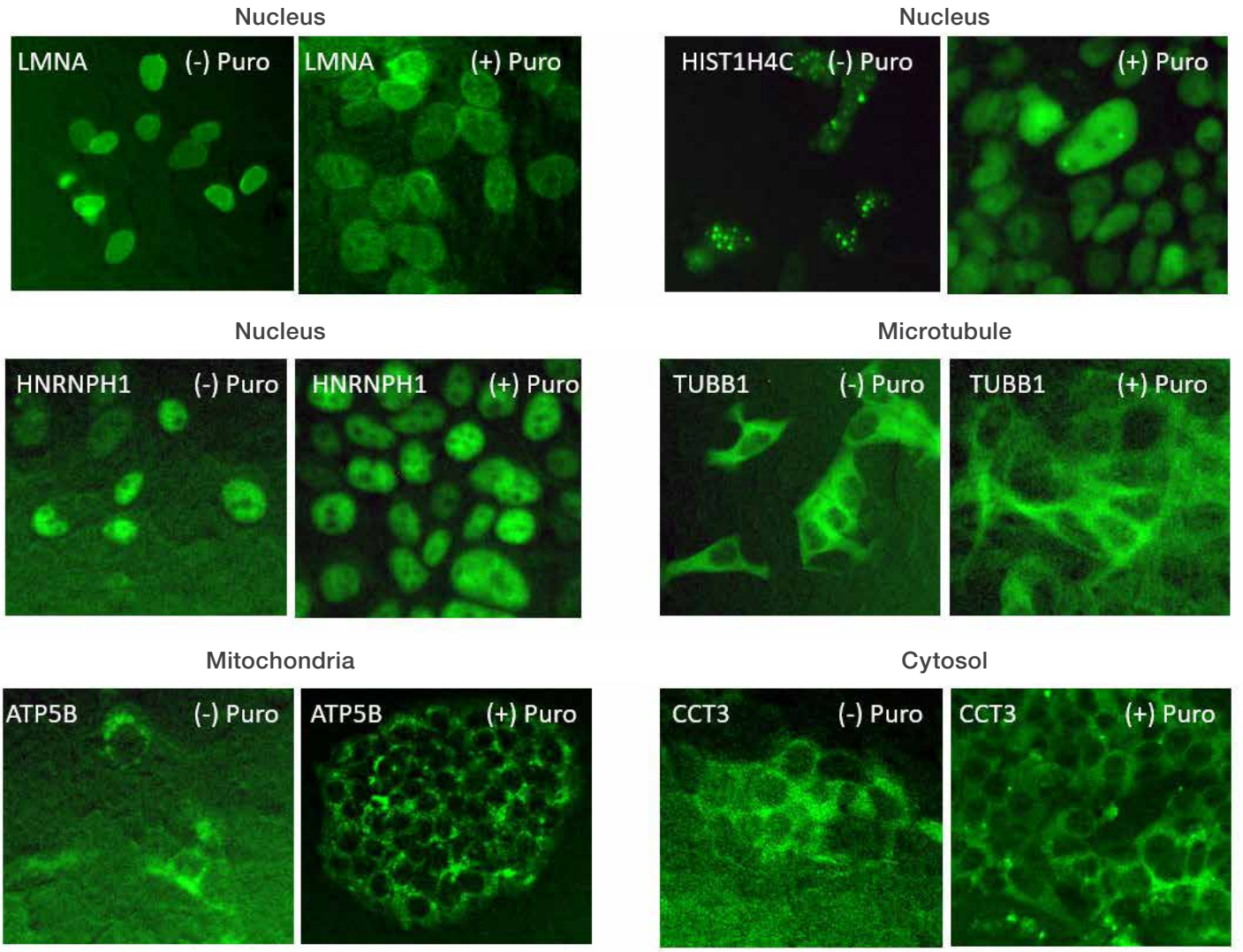

Figure 3. Visualization of the subcellular location of endogenous tagged proteins. Donor DNA targeting six different genes was prepared using the TrueTag Donor DNA Kit. Endogenous proteins localized at different subcellular compartments were tagged with GFP by co-delivering TrueCut Cas9 Protein v2, target-specific gRNA, and the appropriate donor DNA into 293FT cells. At 48 hr posttransfection, the cells were treated with puromycin for 5–7 days and then visualized using the EVOS Imaging System.

The tagging of genes in stem cells has broad applications in gene and stem cell therapy research. Donor DNA molecules prepared using the TrueTag Donor DNA Kit were used to tag two different genes in iPSCs. Results showed highly efficient tagging after puromycin selection (Figure 4). Tagging efficiency was further verified in HSCs both before and after cell sorting (Figure 5). Fusion protein expression was maintained after plating of cells for differentiation.

For GFP-tagging experiments in stem cells where the target protein may not be expressed until after differentiation, we utilized the Invitrogen™ TrueTag™ Donor DNA Kit, GFP Stem (Cat. No. A53812). The donor DNA template provided in this kit is specially designed to permit enrichment with a selection marker driven by its own promoter (Figure 6A), while the GFP tag is under control of the endogenous promoter. The utility of this DNA donor strategy was verified by tagging the *GFAP* gene in iPSCs that were then differentiated into astrocytes (Figure 6B). *GFAP* is only expressed in mature astrocytes, and accordingly the GFP expression was not detected until enrichment of mature astrocytes was carried out.

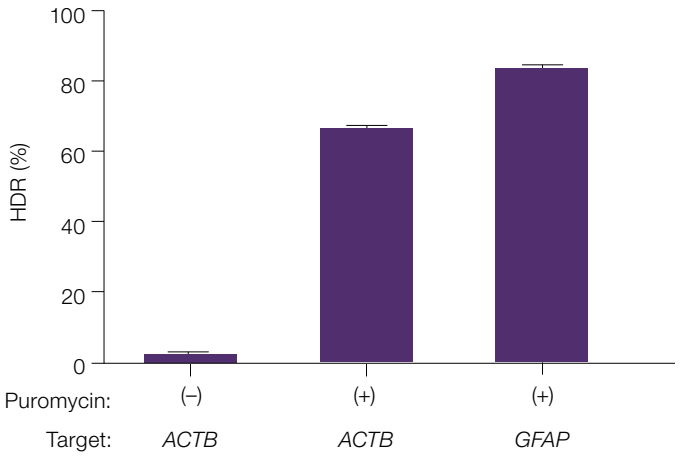

Figure 4. The GFP-tagging efficiency of *ACTB* and *GFAP* in iPSCs. Donor DNA targeting either the *ACTB* or *GFAP* locus was prepared using the TrueTag Donor DNA Kit. TrueCut Cas9 Protein v2, target-specific gRNA, and the appropriate donor DNA were co-delivered into iPSCs using electroporation. At 48 hr posttransfection, the cells were selected with puromycin for 7 days. The resulting colonies with the *ACTB* target were examined by flow cytometry for determination of HDR efficiency. The colonies with the *GFAP* target were randomly picked and expanded for determination of HDR efficiency by junction PCR and sequencing.

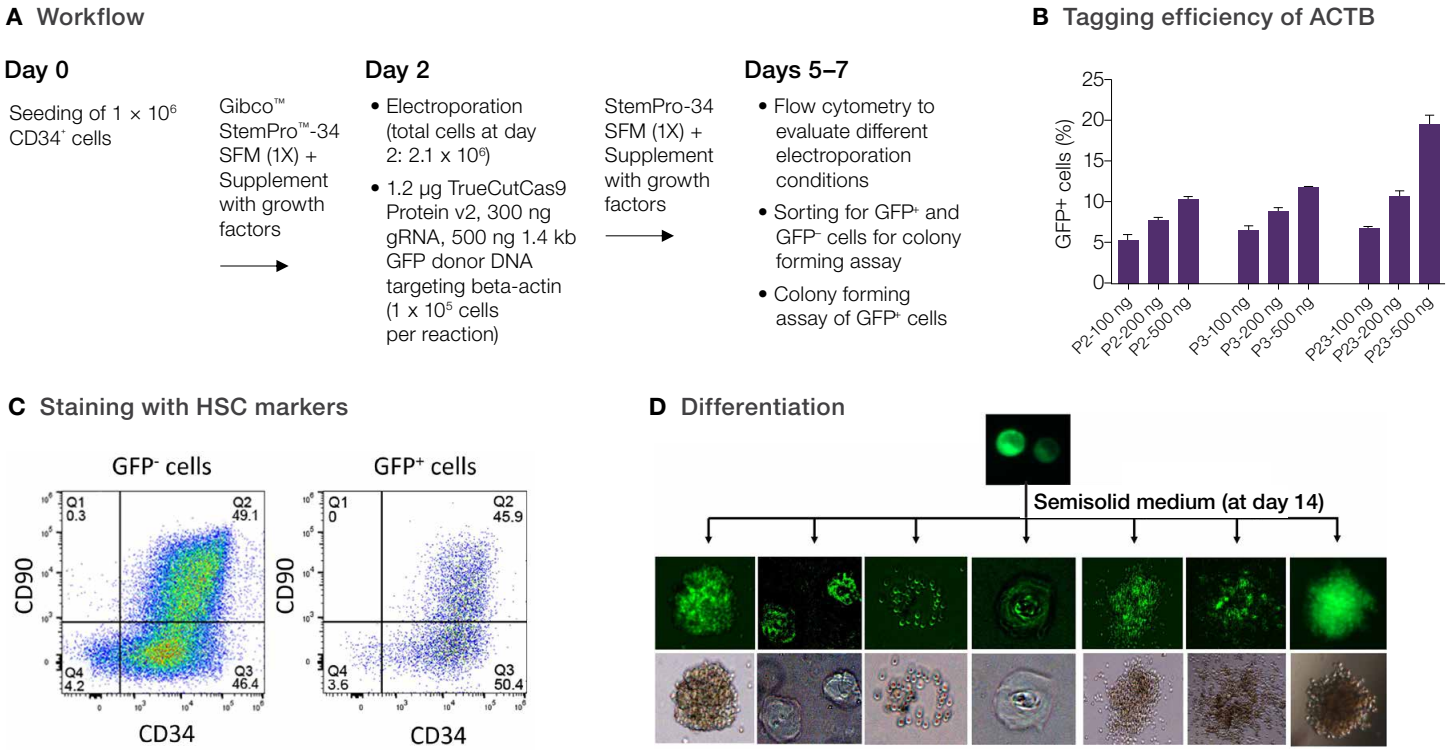

Figure 5. HSC genome editing and differentiation. (A) HSC editing workflow. **(B)** Tagging efficiency of *ACTB* with GFP using different electroporation conditions. **(C)** The transfected cells were stained with CD90 and CD34 antibodies and sorted. **(D)** The GFP-positive cells were plated onto semisolid medium for 14 days for differentiation, and colony formation was visualized by fluorescence microscopy.

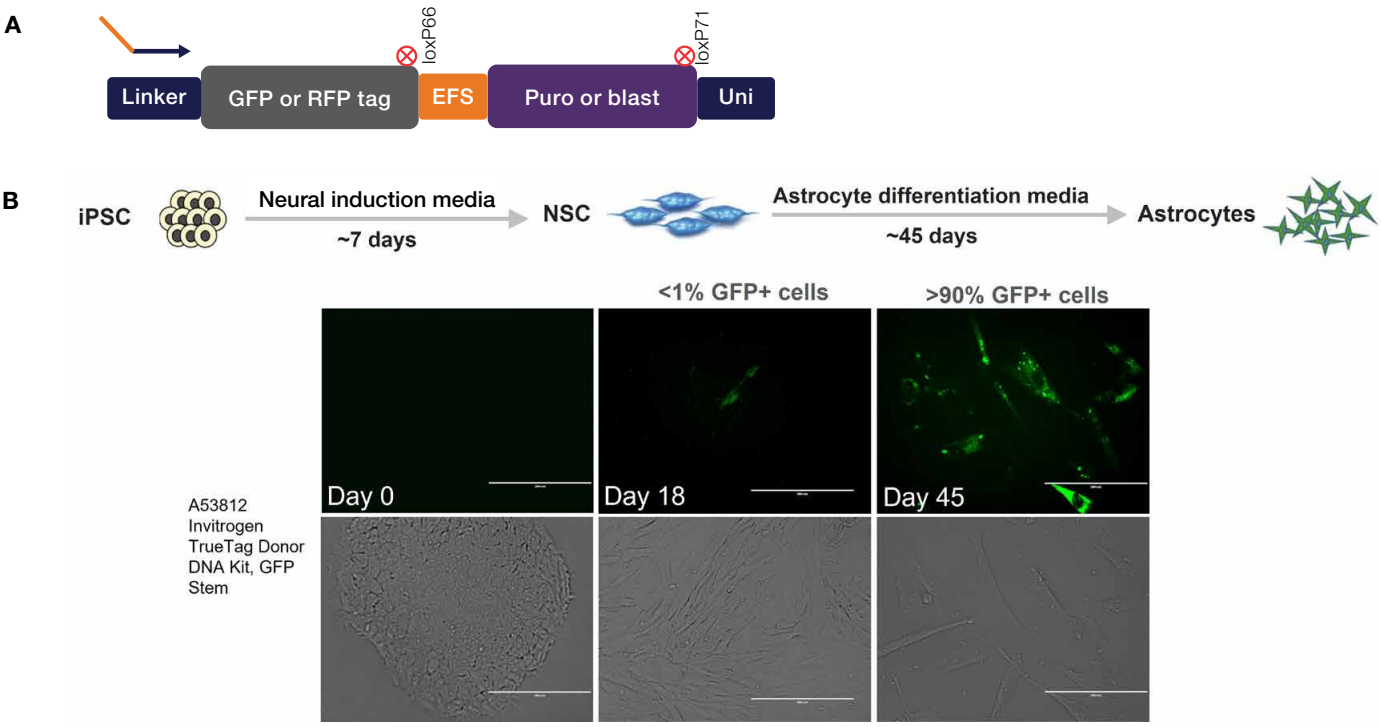

Figure 6. Expression of GFP-tagged *GFAP* after differentiation of iPSCs into astrocytes. (A) Donor DNA targeting the *GFAP* locus was prepared using the TrueTag Donor DNA Kit, GFP Stem. TrueCut Cas9 Protein v2, gRNA, and donor DNA were co-delivered into iPSCs using electroporation. At 48 hr posttransfection, the cells were selected with puromycin for 7 days, and the resulting colonies were randomly picked and expanded. **(B)** The stable cell lines with GFP-tagged *GFAP* were first induced to differentiate into neural stem cells (NSCs) for 7 days. The resulting NSCs were then differentiated into astrocytes with fluorescent images taken at days 18 and 45.

Conclusions

TrueTag Donor DNA Kits offer a simple method for donor DNA preparation without the need for preparation of donor plasmid. When combined with efficient genome editing tools like TrueCut Cas9 Protein v2, the tagging efficiency can reach nearly 100% with antibiotic selection. This method has been verified for different targets in different cell lines. TrueTag Donor DNA Kits provide a full line of options for gene tagging with fluorescent tags (GFP, RFP, BFP, and YFP) as well as epitope tags (Myc, HA, 6xHis, and DYKDDDDK).

References

1. Xiang X, Li C, Chen X et al. (2019) CRISPR/Cas9-mediated gene tagging: a step-by-step protocol. *Methods Mol Biol* 1961:255–269.
2. Liang X, Potter J, Kumar S et al. (2015) Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* 208:44–53.

Get started with the TrueDesign Genome Editor

This free online software was developed to streamline the design and ordering of necessary reagents for performing gene tagging with TrueTag Donor DNA Kits as well as other types of gene editing experiments for cell line engineering.

Start designing today at **thermofisher.com/truedesign**

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