Highly specific gene knockins of long sequences using CRISPR/Cas9 and a single-stranded DNA donor template

Guide-it Long ssDNA Production System and Guide-it Long ssDNA Strandase Kit

- Efficient production of long single-stranded DNA (ssDNA) donor templates for gene editing
- Knockin of an AcGFP1 fluorescent tag to *GAPDH* and *AAVS1* sites in mammalian cells
- Dramatically reduced tendency of ssDNA templates to randomly integrate into the genome
- Reduced toxicity of ssDNA as compared to double-stranded DNA (dsDNA) when electroporated into target cells

Introduction

CRISPR/Cas9-based gene editing is an effective technique to obtain knockout mutations with high efficiency, but knocking-in longer genes or sequences (>200 bp) via homology directed repair (HDR) is difficult to complete successfully. Primary challenges of knockin experiments involve producing and delivering the donor template (also known as the repair template) along with the Cas9-sgRNA ribonucleoprotein complex (Cas9 RNP). Two methods have gained popularity: electroporation of a dsDNA donor template along with the Cas9 RNP, or separate delivery of Cas9 RNP and an ssDNA donor template via adeno-associated virus (AAV). While the use of AAV often results in high efficiency and specificity, it requires cloning into the appropriate vectors and producing virus particles prior to gene editing. As with AAV, our Guide-it Long ssDNA Production System provides the benefits of using ssDNA as a donor template but does not require virus production, and the ssDNA can be co-electroporated with the Cas9 RNP.

Benefits of using ssDNA templates for knockin experiments

Compared to dsDNA donor templates such as plasmids and PCR fragments, ssDNA templates have a dramatically reduced tendency to randomly integrate into the genome, as ssDNA templates typically insert only into the chosen target site (Chen et al. 2011; Figure 3). Also, ssDNA is far less toxic to cells than dsDNA when delivered as a donor template via electroporation (Figure 5).

The Guide-it long ssDNA Production System and Guide-it Long ssDNA Strandase Kit (referred to as the Long ssDNA kits hereafter) provide a simple method for producing long ssDNA (up to 5 kb) to use as donor templates in gene editing experiments. Here, we will discuss the production workflow and show data demonstrating the use of ssDNA as a donor template in HDR experiments.

Production of long ssDNA using strandase mixes

The Long ssDNA kits use an efficient and simple *in vitro* protocol for the production of long ssDNA strands. The method involves generating a double-stranded donor template using PCR where only one of the strands is phosphorylated at its 5' end, then selectively digesting the phosphorylated strand using the enzymes supplied in the kit (Strandase A and B). We recommend creating ssDNA for both the sense and antisense strands and using each in separate knockin experiments. The following schematic demonstrates the workflow involved.

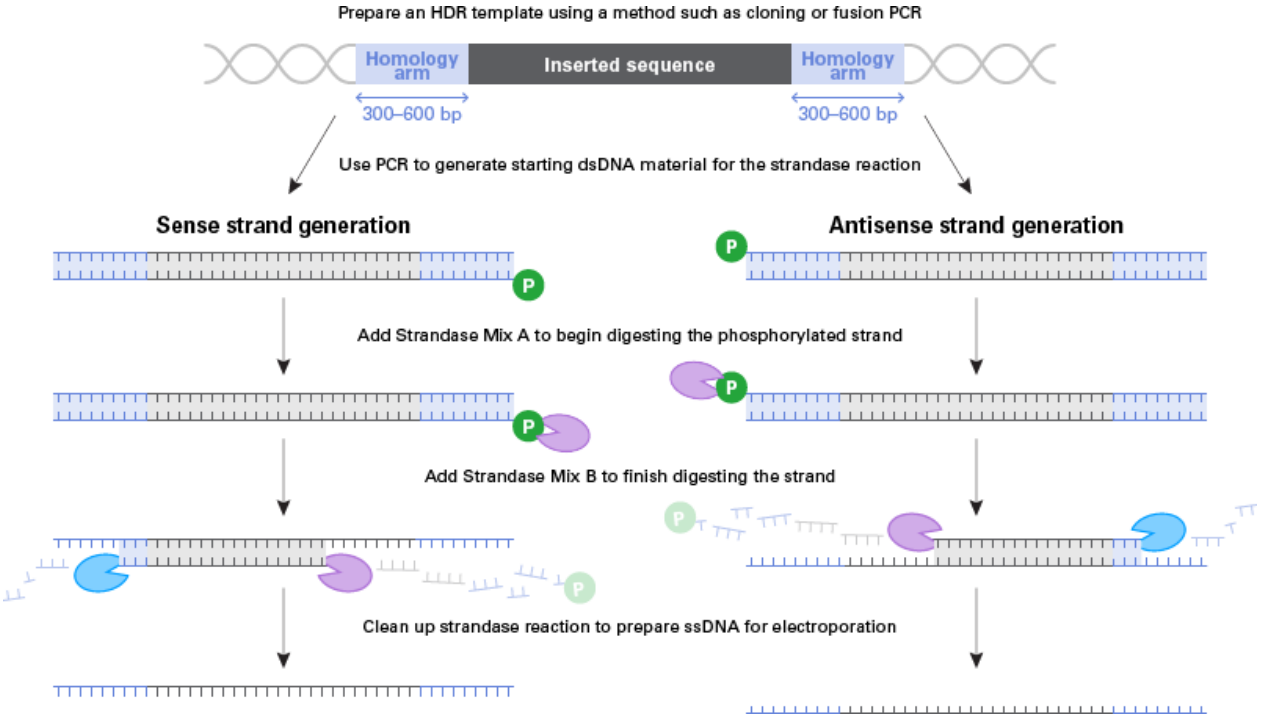

Figure 1. Schematic describing the steps involved in the preparation of long ssDNA donors for use in HDR experiments. First, the dsDNA template (insert sequence flanked by 5'- and 3'-homology arms) is prepared using cloning, fusion PCR, or other related methods. The template should contain arms homologous to the target gene flanking the sequence to be inserted. Next, two different dsDNA PCR products are generated as substrates with the appropriate phosphorylated primers for the strandase reaction. Strandase Mix A is added to begin digesting either the sense or the antisense strand. Strandase Mix A selectively digests the phosphorylated strand. Next, Strandase Mix B is added to finish the digestion and create ssDNA. Finally, the reaction is cleaned up to prepare the ssDNA template for use in your gene knockin experiment. We recommend creating ssDNA for both the sense and antisense strand and using each in separate knockin experiments.

Results

Production of ssDNA templates

Sense and antisense ssDNA donor templates were produced for each of the following experiments using the workflow described above since it is impossible to predict if the sense or the antisense DNA would be more efficient for knockin. Each template was created with 300- to 600-bp homology arms to the target site. To produce 2–4 µg of ssDNA, 10 µg of dsDNA PCR product was typically used. Production of long ssDNA was confirmed on an agarose gel with the ssDNA typically running faster than the dsDNA PCR substrate (Figure 2, Panel A). It is possible to make ssDNA templates ranging from 500–5,000 bp using this kit (Figure 2, Panel B).

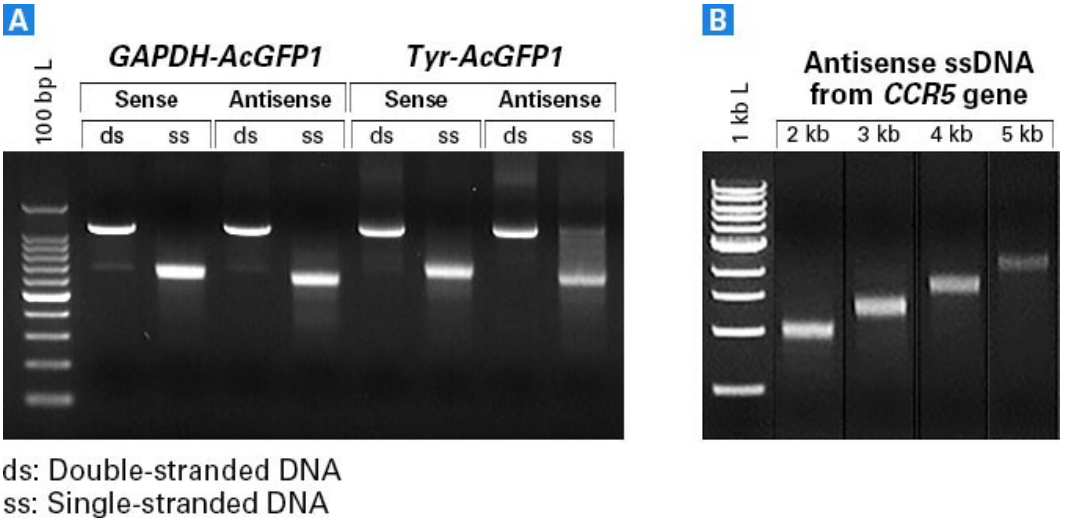

Figure 2. Production of ssDNA from a dsDNA template starting material. Long ssDNA was produced for tagging GADPH and Tyr genes with AcGFP1. The ssDNA has a smaller molecular weight than the corresponding dsDNA (Panel A). Panel B shows the successful production of ssDNA ranging from 2–5 kb for the CCR5 locus.

Gene knockin with long ssDNA

Long ssDNA produced with these kits can be used in combination with a nuclease such as Cas9 for gene knockin experiments. We produced several different ssDNA templates using the Guide-it Long ssDNA Strandase Kit and demonstrated gene knockin in HEK293 cells and human induced pluripotent stem cells, hiPSC. The details of the experiments are presented below. When using electroporation, the sgRNA was produced with the Guide-it sgRNA *In Vitro* Transcription Kit. We combined the sgRNA with Guide-it Recombinant Cas9 (Electroporation Ready) to form a ribonucleoprotein complex (Cas9 RNP). The Cas9 RNPs were delivered via electroporation into cells along with the ssDNA donor templates.

dsDNA integrates at sites other than the target

We designed a donor template to tag the endogenous gene *GAPDH* with a green fluorescent protein, AcGFP1 (Figure 3, Panel A), and compared results using either a dsDNA PCR fragment or ssDNA as the donor template. The donor template was transfected into the cells along with a plasmid expressing both Cas9 nuclease and an sgRNA against *GAPDH*. The data analyzed three days post-transfection shows that the cells transfected using the dsDNA donor template demonstrated a high level of GFP expression even in the absence of CRISPR/Cas9. This is a strong indication that dsDNA can integrate with high efficiency at sites other than the target site. Such Cas9-independent, random integration was not observed when using ssDNA templates (Figure 3, Panel B). Avoiding random integration is a critical requirement for successful gene editing experiments.

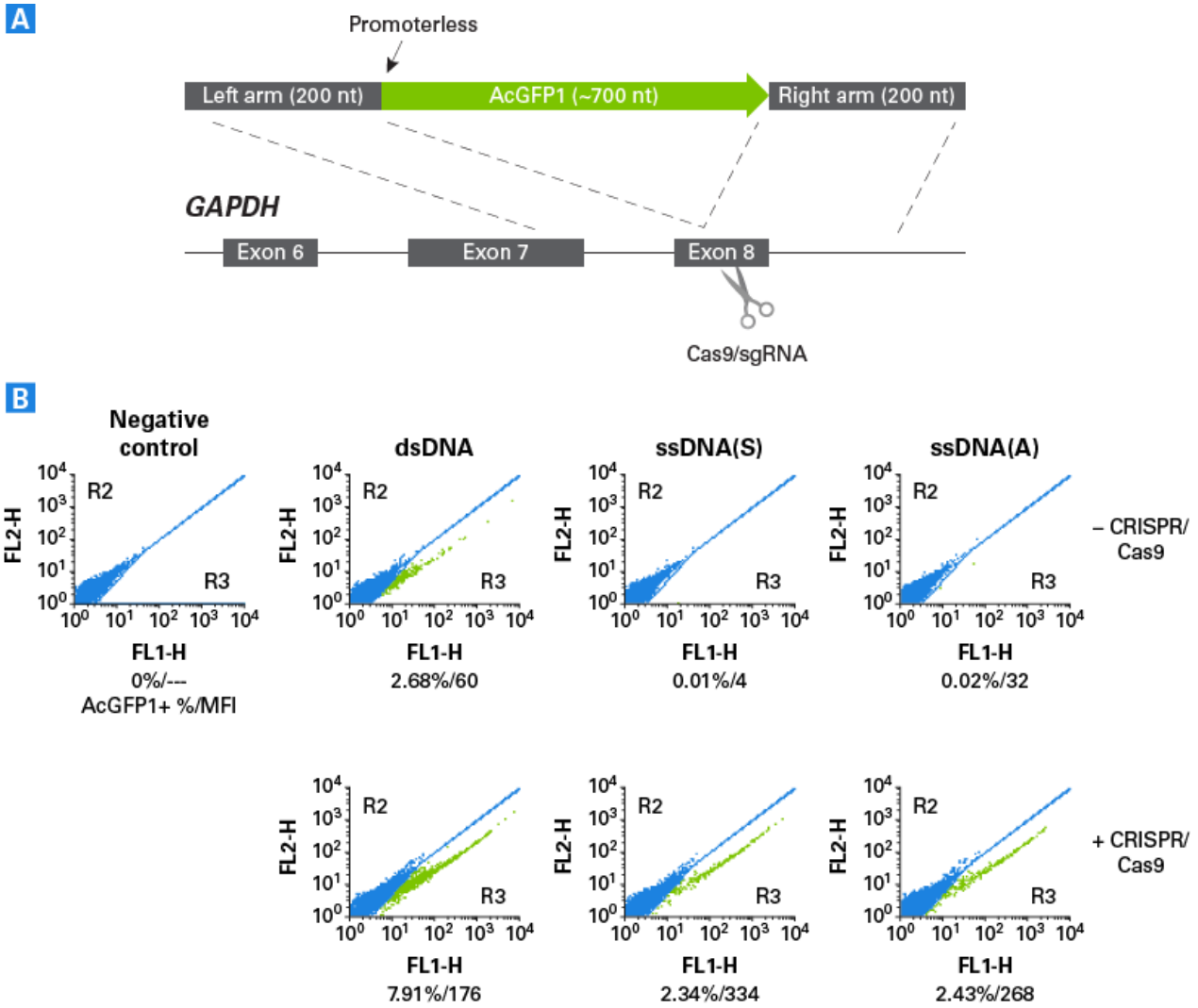

Figure 3. Knockin of *AcGFP1* at the C-terminus of *GAPDH* in HEK293 cells. This experiment demonstrates the use of long ssDNA to tag an endogenous protein with a fluorophore. We designed a donor template with homology arms to the end of exon 8 of *GAPDH* such that *AcGFP1* would fuse in-frame with *GAPDH* (Panel A). As shown by the flow cytometry data (Panel B), the use of linear dsDNA as a donor template results in a large number of green fluorescent cells even in the absence of CRISPR/Cas9. However, when using the sense or the antisense long ssDNA donor templates, integration only occurred when the templates were delivered together with Cas9 and sgRNA. If the ssDNA donor template was delivered into the cells in the absence of CRISPR/Cas9, almost no background could be detected.

ssDNA integrates seamlessly at the target site

In a separate experiment, we tagged the *AAVS1* site with *AcGFP1* under a constitutive promoter and tested it in human induced pluripotent stem cells (hiPSC). In this experiment, the donor template and the Cas9 RNPs were delivered via electroporation. After sorting and single-cell cloning of the GFP-positive cells, the genomic regions of the *AAVS1* site bordering the homology arms were amplified by PCR and sequenced to test for any undesired mutations near the site of integration (Figure 4). One of the primers in each pair was designed to anneal outside the region spanned by the homology arm in the *AAVS1* locus to avoid false detection of residual donor templates. All clones tested showed the expected fusion at the *AAVS1* target site, and none of the ten clones tested displayed any mutations in the region flanking the insertion site.

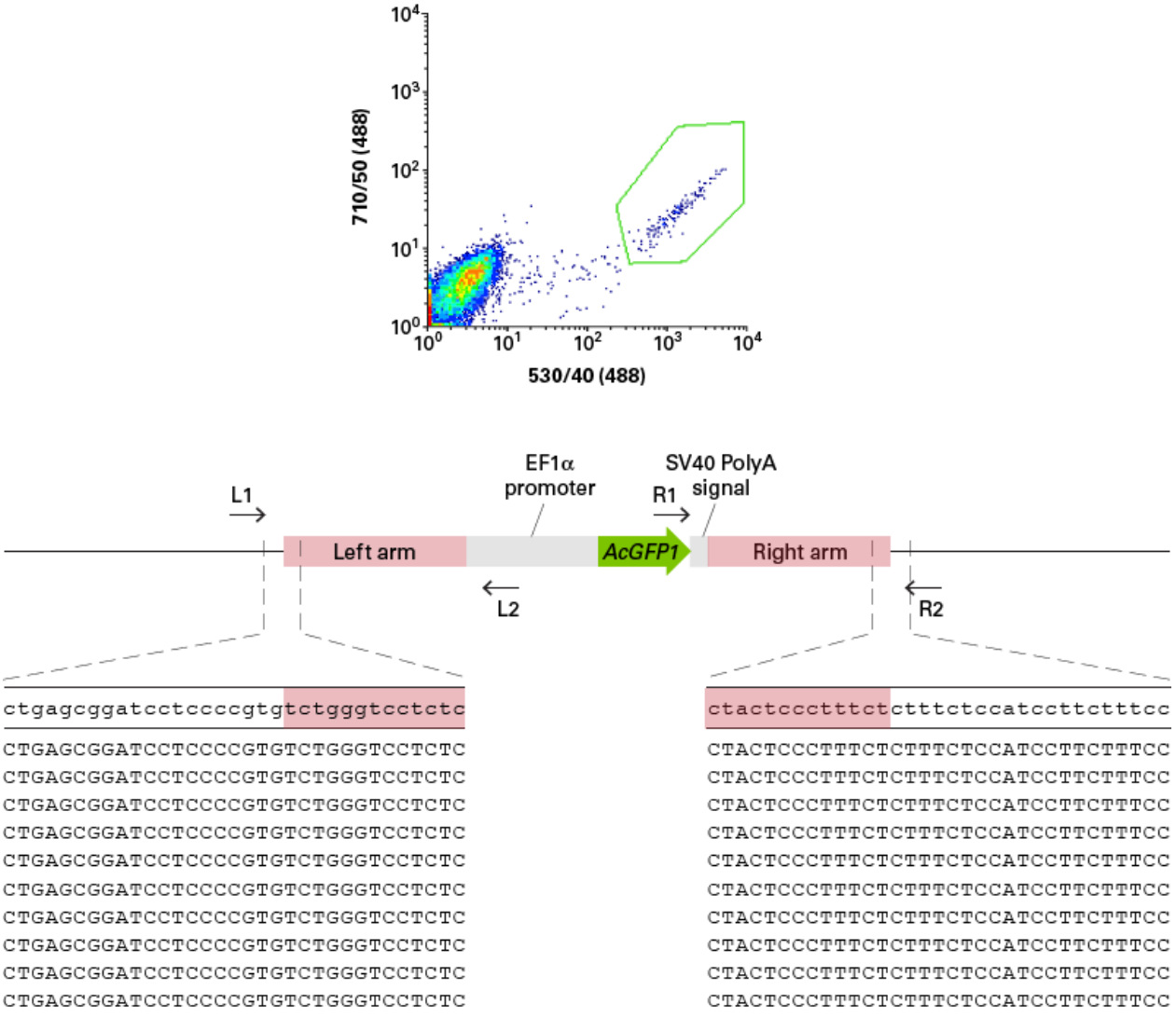

Figure 4. Screening for insertion-site mutagenesis in Cellartis human iPS cell line 18 (ChiPSC18). The ssDNA donor template containing *AcGFP1* driven by the EF1-alpha promoter had 600-bp homology arms with the target site in *AAVS1*. After isolating GFP-positive clones, the predicted insertion sites were sequenced to determine the insertion accuracy. We found that both the left and the right insertion sites had seamless integration in every clone tested.

ssDNA is significantly less toxic than dsDNA when delivered to target cells by electroporation

Finally, we tested dsDNA and ssDNA donor templates for cytotoxic effects on the cells after electroporation. dsDNA induced significant cellular toxicity irrespective of the presence or absence of Cas9 RNPs five days postdelivery (Figure 5).

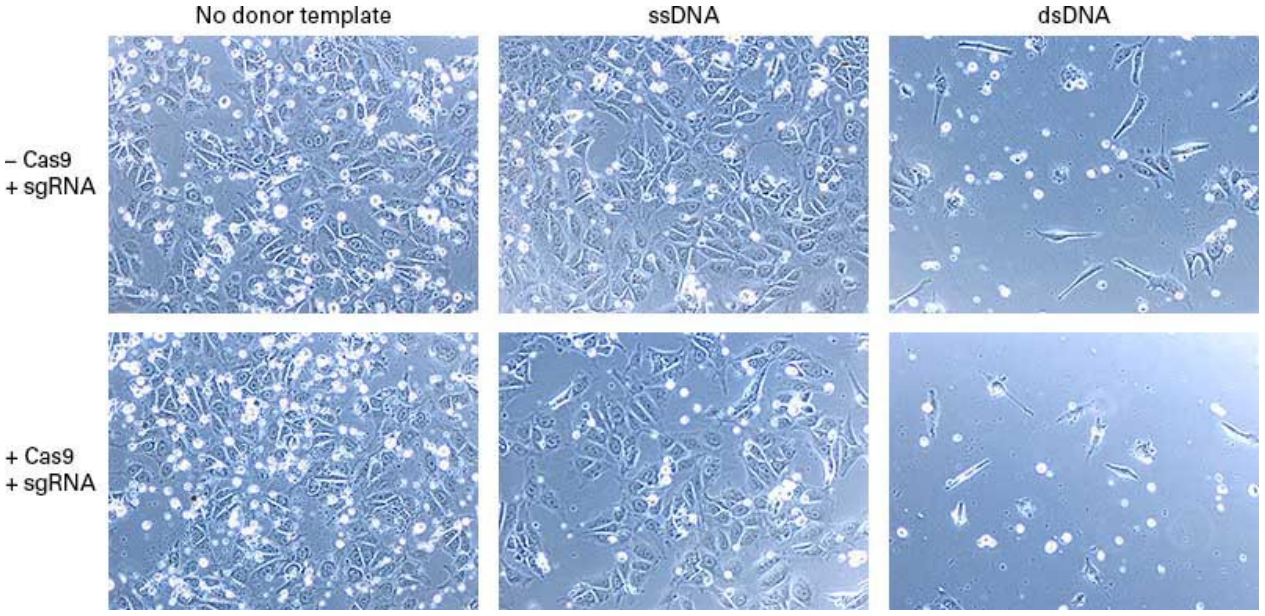

Figure 5. Comparison of cellular toxicity induced by dsDNA and ssDNA in ChiPSC18 cells during knockin experiments. Cells were electroporated with or without Cas9 and with either sense or antisense ssDNA or dsDNA donor templates designed to tag tubulin with AcGFP1. The donor templates were created with 350-bp homology arms to the insertion site and were 1.5 kb in length. As seen in the images, the dsDNA donor template caused significant toxicity when combined with or without Cas9.

Conclusions

Using ssDNA donor templates for CRISPR-based knockin applications enables more reliable editing with less nonspecific integration and lower cytotoxicity compared to double-stranded donor templates. However, the use of ssDNA donor templates has been limited to applications requiring >200-bp fragments due to the cost and difficulty associated with producing longer ssDNA templates. The Long ssDNA kits enable the production of ssDNA up to 5 kb in length in an error-free and cost-effective manner.

The ssDNA produced using these kits showed significantly less non-specific integration and toxicity in comparison to dsDNA when introduced using Cas9 RNPs into two mammalian cell lines. Furthermore, ssDNA showed no undesired mutations at the site of integration.

Methods

For electroporation experiments, sgRNA was produced using the Guide-it sgRNA *In Vitro* Transcription Kit and combined with Guide-it Recombinant Cas9 (Electroporation-ready) to produce Cas9 RNPs. The ssDNA was produced according to the Guide-it Long ssDNA Production System Protocol-At-A-Glance. Cellartis hiPSC cells, ChiPSC 18 were electroporated using a Neon electroporation system according to the guidelines provided for each cell line. For each sample, 5 x 10^5 cells were electroporated with 1 µg of ssDNA or dsDNA in either the presence or absence of 2.25 µg of Cas9 nuclease combined with 0.45 µg of sgRNA. Cytotoxicity was assessed 48 hr after electroporation. Sequencing of genomic areas of interest was performed after establishing clonal lines.

The transfected cells were subjected to FACS analysis three or five days post-transfection for HEK293. Cytotoxicity analysis was performed five days post-transfection.

For the experiments with HEK293 cells, we used the Guide-it CRISPR/Cas9 System, a plasmid expressing Cas9 and the sgRNA. We transfected in the Cas9-sgRNA plasmid along with the donor template using Xfect Transfection Reagent. Again, long ssDNA was produced according to the user manual, and 1 µg of ssDNA or dsDNA was transfected along with the Cas9 expression plasmid.

References

Chen *et al.,* High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat. Meth.* **8,** 753–755 (2011).

Related Products

Cat. #	Product	Size	License	Quantity	Details
632644	Guide-it™ Long ssDNA Production System	25 Rxns		*	

The Guide-it Long ssDNA Production System enables production of single-stranded DNA (ssDNA) oligos up to 5 kb in length for use as repair templates in knockin experiments involving CRISPR/Cas9 or other genome editing tools. Included with the system are sufficient reagents for production of 50 different ssDNA oligos (25 pairs of sense and antisense ssDNAs), and the NucleoSpin Gel and PCR Clean-up kit for purification of ssDNA prior to target-cell delivery.

Documents	Components	You May Also Like	Image Data

632645	Guide-it™ Long ssDNA Strandase Kit	25 Rxns		*	

Add to Cart

Takara Bio USA, Inc.
United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999