Efficient production and application of ssDNA for CRISPR/Cas knockins in human primary T cells

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Abstract

The application of CRISPR/Cas9-based technology to engineer site-specific insertions of genes or sequences longer than 200 bp via homology-directed repair (HDR) is often very difficult. A primary challenge involves achieving efficient delivery of the DNA template used for HDR following Cas9-sgRNA-mediated cleavage at the genomic target site. Electroporation of ribonucleoprotein complexes (RNPs) is the preferred method for delivery of Cas9 and sgRNA *in vitro* due to the easy preparation of the reagents and their transient nature in the target cells, which limits the possibility of off-target effects. Two popular approaches that leverage this method to engineer knockins involve co-electroporation of the DNA donor template along with the Cas9-sgRNA RNPs, or adeno-associated virus (AAV)-mediated transduction of the donor as ssDNA following RNP electroporation. While the use of AAV often results in high editing efficiencies, it requires cloning into the appropriate vectors and producing viral particles prior to genome editing.

For the non-viral approach, co-electroporation of the Cas9-sgRNA RNP and donor sequence can be performed with either double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) as the HDR donor template. However, the application of ssDNA is associated with two important advantages: lower rates of random or off-target integration (Chen et al. 2011; Roth et al. 2018), and lower cytotoxicity (Roth et al. 2018), especially when working with hiPSCs or primary cells.

Here we present the Guide-it™ Long ssDNA Production System v2 which provides a simple and fast method for producing long ssDNA (up to 5,000 nt) for use as donor templates in gene editing applications. This system uses PCR followed by enzymatic degradation of one of the strands of the amplified product. The resulting ssDNA can be used in knockin experiments targeting endogenous genes in primary T cells. As a proof of concept, we specifically tagged two endogenous genes (*TUBA1A* and *SEC61B*) with a fluorescent protein and performed a knockin of murine αβ-TCR into the human endogenous TRAC locus (Schober et al. 2019).

References

Eyquem, J. *et al.* Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543,** 113–117 (2017).

Roth, T. L. *et al.* Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature* **559,** 405–409 (2018).

Schober, K. *et al.* Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. *Nat. Biomed. Eng.* **543,** 974–984 (2019).

1 A PCR-based method to create long, single-stranded DNA donors

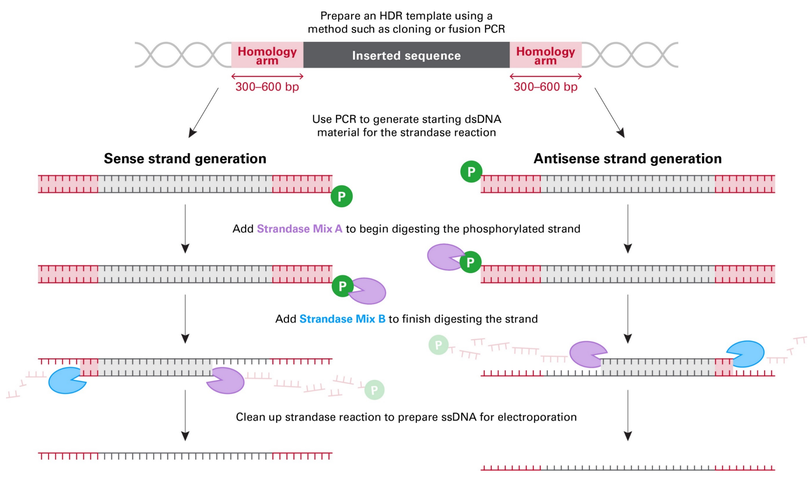

Figure 1. Preparation of long ssDNA donors using the Guide-it Long ssDNA Production System v2. First, the dsDNA template (insert sequence flanked by 5' and 3' homology arms) is prepared using cloning, fusion PCR, or other related methods. The template should contain arms homologous to the target gene flanking the sequence to be inserted. Next, either of two different dsDNA PCR products are generated from the template using appropriate phosphorylated (P) primers. These PCR products serve as substrates for the Strandase reaction: Strandase Mix A (purple enzyme) selectively digests the phosphorylated strand. Next, Strandase Mix B (blue enzyme) is added to finish the digestion and create ssDNA. Finally, the reaction is cleaned up to obtain ssDNA for use as an HDR template in gene knockin experiments. As depicted in the figure, we recommend creating ssDNA for both the sense and antisense strands and using each in separate knockin experiments.

Conclusions

- The Guide-it Long ssDNA Production System v2 enables production of long ssDNA fragments ranging from 0.5 to 5 kb in a simple and rapid manner
- The generated ssDNA can be used as template in HDR experiments together with RNP complexes to edit T cells (e.g., for tagging endogenous genes or replacing the T-cell receptor[s])

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2 Improved generation of ssDNA

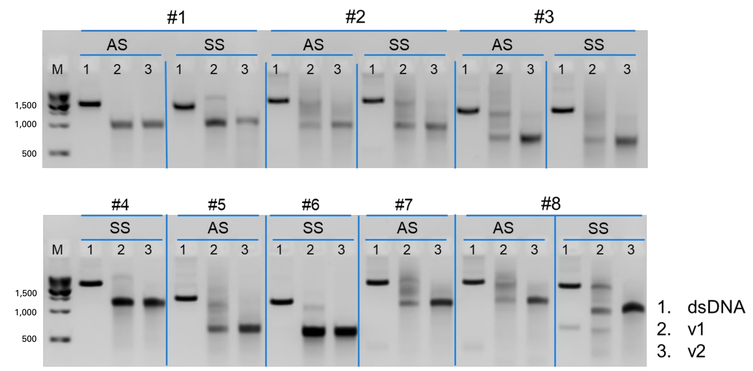

Figure 2. Improved generation of ssDNA with a new version of the Guide-it Long ssDNA Production Kit. Gel image showing the dsDNA (Lane 1) starting material and the sense (SS) and/or antisense (AS) ssDNA product for eight HDR templates generated using two respective versions of the Guide-it Long ssDNA Production System (v1 or v2). The templates consist of different inserts flanked by 5' and 3' homology arms targeting various genomic loci of interest. The results of the ssDNA production are analyzed via agarose gel electrophoresis using ethidium bromide as a staining agent. The ssDNA product runs at a smaller molecular weight than the corresponding dsDNA substrate. In all cases, the new version of the kit generates a cleaner band of ssDNA, suggesting more complete and uniform digestion.

3 Production of ssDNA ranging from 0.5 to 5 kb in length

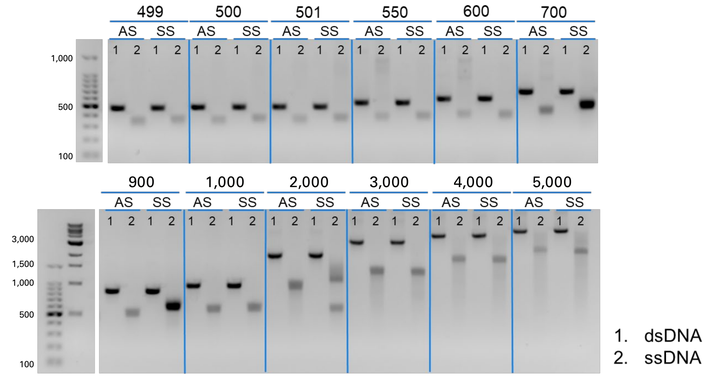

Figure 3. The Guide-it Long ssDNA Production System v2 enables successful production of ssDNA ranging from 500 bp to 5 kb in length. The gel images show the dsDNA starting material (Lane 1) and the ssDNA product (Lane 2) after cleanup of various sense (SS) and antisense (AS) HDR templates targeting the *CCR5* gene.

4 Tagging of endogenous genes (*TUBA1A* and *SEC61B*) with AcGFP1 in T cells

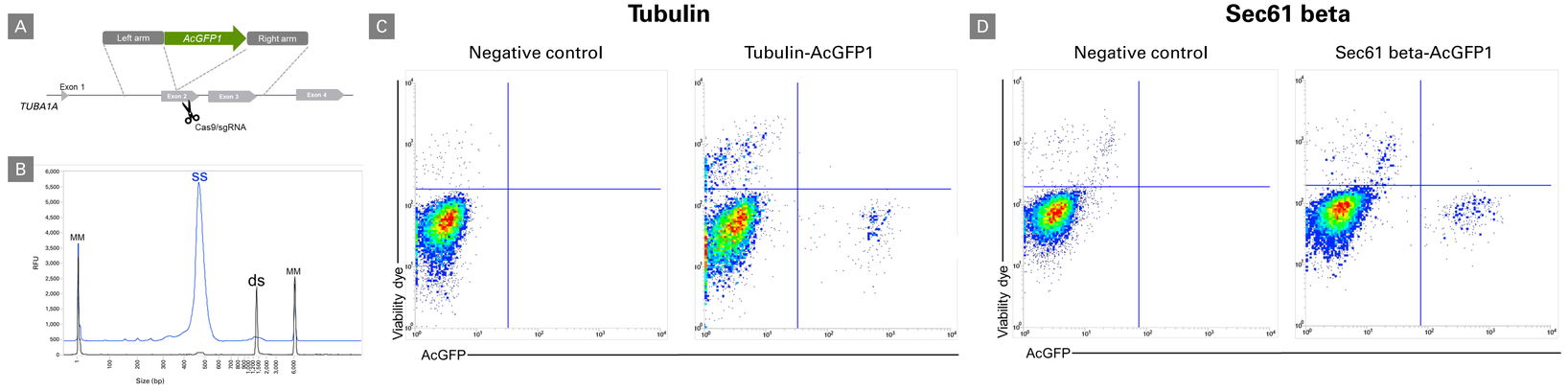

Figure 4. Tagging of endogenous genes with AcGFP1 in T cells using CRISPR/Cas9 gene editing. Panel A. Tubulin was tagged at its N-terminus with AcGFP1 using CRISPR/Cas9 together with a long ssDNA as an HDR donor template. The donor template had 350-bp homology arms to the insertion site and was 1.6 kb in length. **Panel B.** Analysis of the ssDNA product using the Agilent Fragment Analyzer system. Overlay of the starting fragment dsDNA (ds) and end-product ssDNA (ss) with two size markers (MM). The ssDNA product had a purity higher than 97%. **Panel C.** Flow cytometry plots of resulting T-cell populations following HDR-mediated knockin showing the percentage of AcGFP1-positive cells (approximately 2.45%), demonstrating a successful HDR. eBioscience Fixable Viability Dye eFluor 660 (Thermo Fisher Scientific) was used as a viability dye. **Panel D.** The same strategy was applied to tag the N-terminus of Sec61 beta, a protein that localizes in the endoplasmic reticulum. The percentage of AcGFP1-positive cells (equivalent to a successful HDR) was 2.1%.

5 Replacement of T-cell receptor α- and β-chains

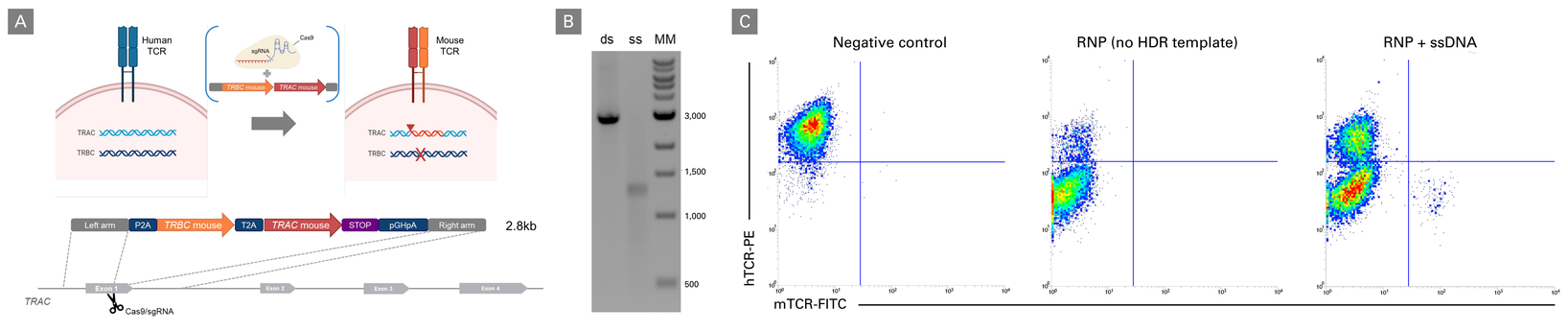

Figure 5. Knockin of murine T-cell receptor α- and β-chains in the endogenous human *TRAC* locus. Panel A. CRISPR/Cas9 RNP with sgRNAs targeting both *TRAC* and *TRBC* loci were electroporated with ssDNA encoding the murine T-cell receptor α- and β-chains. The HDR template was designed to have 350-bp homology arms targeting exon 1 of the *TRAC* locus with the following elements: P2A and T2A (self-cleaving peptide inserts); mouse *TRBC*; mouse *TRAC*; pGHpA, poly(A) tail. The total length of the ssDNA was 2.8 kb. **Panel B.** dsDNA (ds) and ssDNA (ss) templates used in the experiment were analyzed via agarose gel electrophoresis and show the high purity of the ssDNA HDR donor. **Panel C.** Flow cytometry analysis of T-cell populations ten days after editing using antibodies against mouse TCR-β (clone H57-597) and human TCR α/β (clone IP26). In nonedited cells (Negative control), cells were positive for the expression of human TCRs. When cells were electroporated with the CRISPR/Cas9 RNP with sgRNAs targeting both *TRAC* and *TRBC* loci in the absence of HDR template, the *TRAC* knockout was approximately 89%. When the RNPs were co-electroporated with ssDNA encoding murine T-cell receptor α- and β-chains (RNP + ssDNA), the knockin percentage was 1.6%.

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