A PCR based method to create long, single-stranded DNA donors for gene knockin applications

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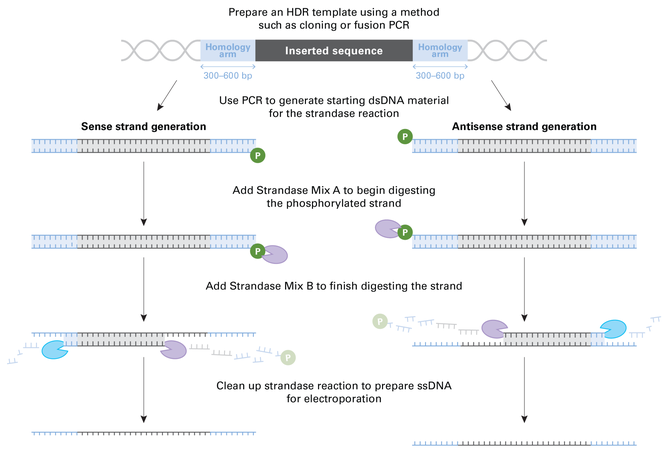
Abstract

CRISPR/Cas9-based gene editing has revolutionized the field of cell biology and has been quickly incorporated into the toolboxes of many laboratories. One of the most interesting applications of CRISPR/Cas9 is the precise introduction of a DNA sequence (donor) at the target site via the cellular homology-directed repair (HDR) mechanism activated after a DNA strand break.

The length of the introduced donor DNA varies depending on the nature of the desired modification: a site-specific SNP can be introduced by a short single-stranded DNA (ssDNA) oligo whereas other applications, such as tagging an endogenous gene with a fluorescent reporter for example, require a longer DNA donor. These longer DNA donors can either be double-stranded DNA (dsDNA) or ssDNA. However, dsDNA has the tendency to randomly integrate into the genome, and therefore is of very limited use for precise gene editing applications. On the other hand, ssDNA has a drastically reduced tendency to randomly integrate into the genome and mainly inserts into the site specifically targeted by the Cas9/sgRNA complex. In addition, ssDNA does not trigger a strong cytotoxic response after being delivered into cells, unlike dsDNA. Despite being the preferred choice for use as the DNA donor fragment for knockin applications, ssDNA can only be synthetically produced in an affordable and error-free manner up to a length of 200 base pairs. Above this length, ssDNA synthesis becomes very costly and error-prone.

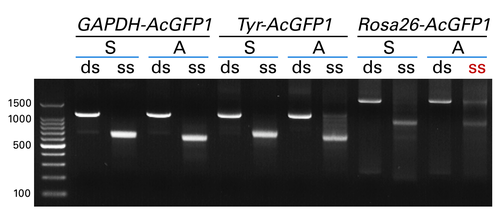
Here we report a simple method to produce long ssDNA of up to 5 kb based on a PCR reaction followed by enzymatic degradation of one of the strands. The ssDNA produced with this novel method can be electroporated in conjunction with an electroporation-ready recombinant Cas9 and *in vitro* transcribed sgRNA in knockin experiments targeting difficult to manipulate cells like hiPSCs.

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



This schematic describes the steps involved in preparing long ssDNA donors using Guide-it™ Long ssDNA Production System. 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 with the appropriate phosphorylated primers. These PCR products would be substrates for the Strandase reaction: 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 obtain the ssDNA template for use in gene knockin experiments. We recommend creating ssDNA for both the sense and antisense strands and using each in separate knockin experiments.

2 Production of ssDNA encoding different endogenous targets



Gel image showing the dsDNA starting material and the ssDNA product after cleanup for both sense (S) and anti-sense (A) orientations for three different HDR templates. The templates consist of the *AcGFP1*-encoding sequence, flanked by the 5′ and 3′ homology arms to the respective target sequences: *GAPDH*, tyrosine kinase, or the *Rosa26* locus. The results of the ssDNA production can be analyzed via agarose gel using ethidium bromide as staining agent. The ssDNA runs at a smaller molecular weight than the corresponding dsDNA. The anti-sense ssDNA product includes two bands for the *Rosa26* locus, suggesting an incomplete digestion, and is considered a failed synthesis. We have observed that this problem can be solved by shifting the primers for the initial PCR reaction as little as one nucleotide in either direction.

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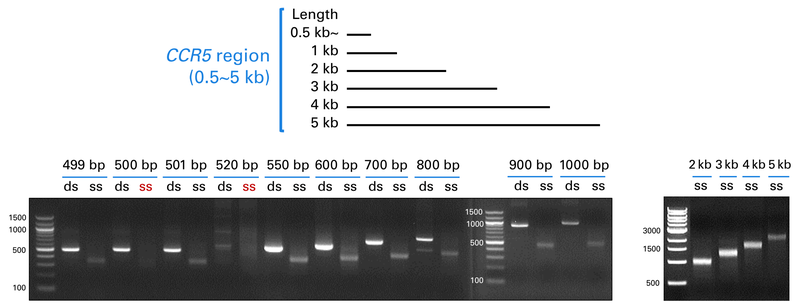
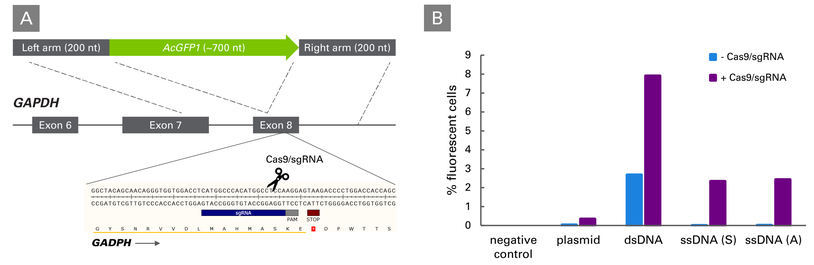
3 Production of ssDNA ranging from 0.5 to 5 kb in length



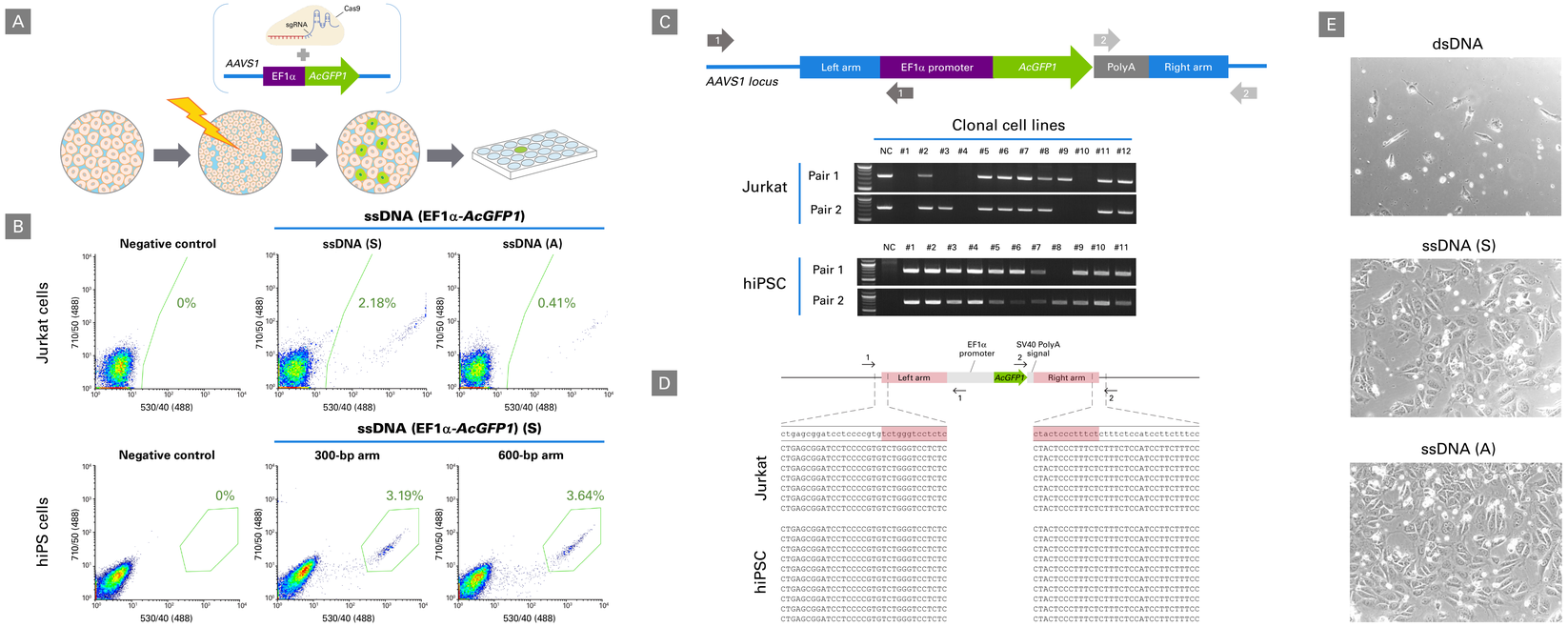
Guide-it Long ssDNA Production System allows the successful production of ssDNA of the *CCR5* locus ranging from 500 bp to 5 kb in length. The gel images show the dsDNA starting material and the ssDNA product after cleanup for the sense strand of the *CCR5* gene. In some cases the ssDNA could not be produced (marked in red). This problem could be solved by shifting the primer position used in the first PCR step.

4 Tagging GAPDH with AcGFP1 in HEK293 cells



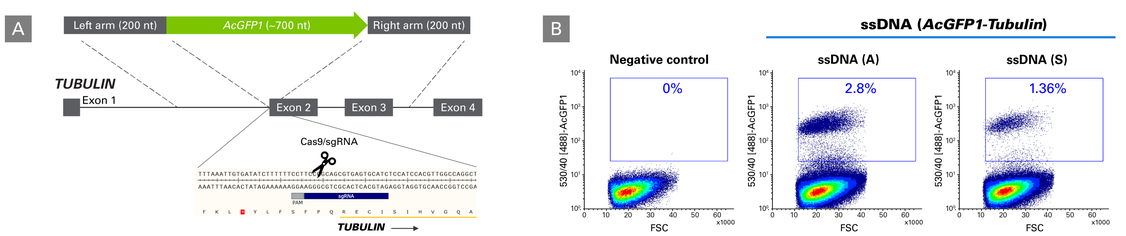
Tagging GAPDH with AcGFP1 in HEK293 cells. Panel A. This experiment demonstrates the use of long ssDNA to tag an endogenous protein with a green fluorescent protein (AcGFP1). An HDR template was designed in order to fuse AcGFP1 in-frame with GAPDH specifically at the C-terminus. **Panel B.** HEK239 cells were transfected with plasmids encoding Cas9 and sgRNA targeting the *GAPDH* locus together with the template for HDR experiment. Cells were grown for 3 days and FACS analysis was performed. A plasmid template showed very little integration as seen with a very low percentage of fluorescent cells, while the dsDNA demonstrated significant AcGFP1 expression even in the absence of RNP complex suggesting non-specific integration. Both the sense and the anti-sense long ssDNA repair templates underwent significant integration at the target site with very low background expression in the negative controls.

5 Precise and efficient insertion of a fluorescent protein gene at the *AAVS1* site using long ssDNA as donor template in different cell types



Insertion of a fluorescent protein encoding cassette into the *AAVS1* site using long ssDNA oligo as donor template in different cell types. Panel A. Workflow for targeted knockin of the fluorescent protein expression cassette encoding AcGFP1 under the control of the EF1α promoter into the *AAVS1* locus of Jurkat or hiPS cells. CRISPR/Cas9 ribonucleoprotein complex (RNP) together with the donor template, a long ssDNA encoding for AcGFP1 expression cassette with homology arms related to the *AAVS1* site, were delivered using electroporation. Fluorescent cells that resulted from a successful HDR were isolated by flow cytometry and expanded into edited clonal cell lines. **Panel B.** FACS plots of populations after the HDR. In the case of Jurkat cells, two independent experiments were performed with the sense (S) or antisense (A) ssDNA donor template. In the case of hiPSC cells, two different lengths of the homologous arms (300 or 600 base pairs) were tested achieving a slightly higher percentage of knockin when 600-bp homology arm was used. In both cell-types, the fluorescent population was isolated via flow cytometry and single cells were isolated by limiting dilution in the condition of higher HDR . **Panel B.** The expanded clonal cell lines were further characterized by their fluorescence (data not shown) and the correct insertion of the EF1α-*AcGFP1* in *AAVS1* locus. Using two pairs of primers, pair 1 (dark gray) and pair 2 (light gray), the insertion of the EF1α-*AcGFP1* construct in the *AAVS1* site was confirmed in the different clonal cell lines. One of the primers in each pair annealed outside the region spanned by the homology arm in the *AAVS1* locus to avoid false detection of residual repair template. **Panel D.** Sanger sequencing of the junctions in the different edited cell lines showed a seamless insertion of the long ssDNA donor template since no mutations could be detected and there was full alignment with the wild-type sequence. **Panel D.** As seen in the microscopy data, the dsDNA repair template induced significantly higher cellular toxicity in comparison with the ssDNA template in hiPSC cells when it was electroporated with RNP complex. The images were taken 5 days after electroporation.

6 Tagging of Tubulin with AcGFP1 in hiPSCs



Tagging of Tubulin with AcGFP1 in hiPS cells. Panel A. Tubulin was tagged at its N-terminus with AcGFP1 using CRISPR/Cas9 in hiPS cells using long ssDNA as donor template. The donor templates had 350-bp homology arms to the insertion site and were 1.5 kb in length. **Panel B.** FACS plots of the hiPS cell population after the HDR experiment showing the percentage of positive cells ranging from 1 to 3%. Single positive cells are being isolated for further characterization.

Conclusions

- The Guide-it Long ssDNA Production System enables production of long ssDNA fragments ranging from 0.5 to 5 kb with an easy protocol
- The produced ssDNA can be used as template in HDR experiments together with RNP complexes in different cell lines (Jurkat, hiPS cells, etc.)
- In comparison with dsDNA, ssDNA template has a lower random integration rate and is less toxic to cells when electroporated

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