I. Introduction

Using single-stranded DNA (ssDNA) rather than double-stranded DNA (dsRNA) as a donor for knockin CRISPR/Cas9 experiments has several important advantages. ssDNA does not trigger a strong cytotoxic response from the cells upon delivery, and unlike dsDNA, is much less likely to randomly integrate into the genome. For applications involving longer ssDNAs, such as tagging an endogenous gene with a fluorescent reporter, it is often a challenge to produce error-free long ssDNA strands (over 200 bases) in a cost-effective manner. The **Guide-it Long ssDNA Production System** and Strandase Kit are designed to produce long ssDNA oligos (up to 5 kb) for use as a donor template in knockin experiments involving CRISPR/Cas9 or other gene editing tools.

This protocol will enable the user to produce ssDNA donors for knockin applications from a 500 bp–5 kb dsDNA template (PCR product). The following schematic shows the general steps in the protocol.

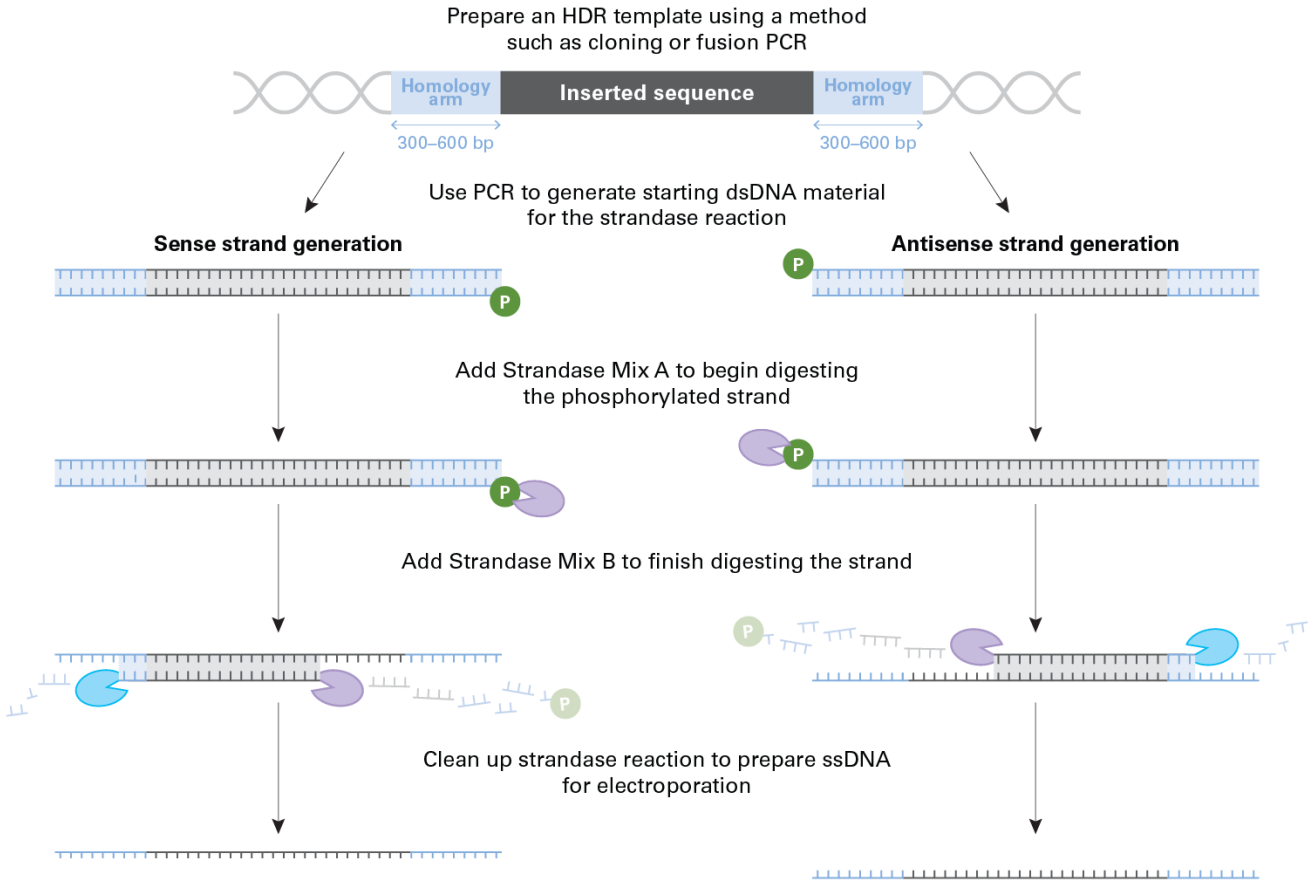

Figure 1. Steps involved in preparing long ssDNA donors for use in homology-directed repair experiments. Prepare the dsDNA template using cloning, fusion PCR, or another preferred method. For proper recombination, the template should contain arms 300 to 600 bp in length that are homologous to the genome sequence flanking the targeted site of integration. Perform a PCR with the appropriate phosphorylated primers to generate two different dsDNA PCR products prior to performing the Strandase reactions. Add Strandase Mix A to begin digesting either the phosphorylated sense or antisense strand. Next, add Strandase Mix B to finish the digestion. Finally, clean up the reaction to prepare the ssDNA donor for use in your gene knockin experiment.

II. General Considerations

A. Storage

Store all components at –20°C upon receipt.

B. Additional Materials Required

The following materials are required but not supplied:

- Target-sequence-specific PCR primers
- 200-µl PCR reaction tubes or plates

III. Protocol: Create donor (PCR) template

Create a donor template specific to your gene of interest with 300–600 bp homology arms to the target integration site (Figure 1, above) by cloning into a vector. We recommend using In-Fusion® HD Cloning Plus (Cat. No. 638909). Alternatively, you may be able to create a DNA template without cloning by performing an overlapping PCR with three fragments (e.g., combine ~20 pmol of each fragment corresponding to the left arm, the target, and the right arm with 15–20 bp of overlap at each junction). We have successfully created 1–2 kb donor templates by this method.

NOTE: The method for generating ssDNA employed by this kit may yield ssDNA that is slightly shorter than would be expected from the PCR product template. Therefore, we recommend preparing the template with homology arms that are 50–100 nucleotides longer than you would otherwise choose. This will ensure that the ssDNA is produced with sufficient homology to the target site for homology-dependent recombination (HDR).

IV. Protocol: Preparation of dsDNA substrate by PCR

Generate a PCR product using a forward and reverse primer against your donor template (Section III) (one of the primers must contain a 5' phosphorylation). Since it is impossible to determine which combination of primers will result in the highest yield and quality ssDNA, we strongly recommend that you obtain four primers, as follows:

Primer 1: Standard forward primer (F Primer)
Primer 2: 5'-Phosphorylated reverse primer (5'-P R Primer)
Primer 3: 5'-Phosphorylated forward primer (5'-P F Primer)
Primer 4: Standard reverse primer (R Primer)

NOTE: In our experience, shifting the position of either the forward or reverse primer by a single nucleotide can greatly affect the efficiency to effectively generate ssDNA using this method. See image under the Images and Data tab on the product page.

1. Set up three 100-µl PCR reactions as shown below:

PCR Reaction A:

50 µl	PrimeSTAR® Max Premix (2X)
20–40 ng	Template DNA
2 µl	Primer 1 (40 µM)
2 µl	Primer 2 (40 µM)
X µl	RNase Free Water
100 µl	Total volume

PCR Reaction B:

50 µl	PrimeSTAR Max Premix (2X)
20–40 ng	Template DNA
2 µl	Primer 3 (40 µM)
2 µl	Primer 4 (40 µM)
X µl	RNase Free Water
100 µl	Total volume

Positive Control Reaction:

50 µl	PrimeSTAR® Max Premix (2X)
5 µl	2-kb Control Template (2 ng/µl)
5 µl	Control Primer Mix (16 µM)
40 µl	RNase Free Water
100 µl	Total volume

Cycling conditions:

30–40 cycles:

98°C 10 sec
55°C 5 sec
72°C 5 sec/kb*

***The positive control template is 2 kb in length, so the Positive Control Reaction would require a 10-sec extension time.**

NOTE: PCR Reaction A will provide the template to create ssDNA corresponding to the sense strand. PCR Reaction B will result in an antisense-strand prep (Section I, Figure 1).

2. Analyze 5 µl of each PCR reaction on an agarose gel.

NOTE: Obtaining a clear single band of dsDNA is very important for preparation of high-quality ssDNA (Figure 2, below).

3. Column purify the dsDNA substrate. We recommend following the PCR cleanup protocol for our NucleoSpin Gel and PCR Clean-Up Kits (Cat. No. 740609.50 or 740609.250) (included in the Guide-it Long ssDNA Production System) or an equivalent method.

IMPORTANT: Strandase A stalls at lesions in dsDNA, and for this reason we strongly advise against purifying the dsDNA substrate in a manner that involves exposure to UV radiation (e.g., agarose gel purification). Instead, we recommend optimizing each PCR reaction to yield a single band, such that each dsDNA PCR product can be purified directly from solution without exposure to UV radiation. UV radiation has been shown to induce lesions in dsDNA, and in our hands, UV exposure combined with gel purification of a dsDNA substrate has interfered with production of ssDNA using this kit.

4. Measure the DNA concentration by NanoDrop (dsDNA setting) or an equivalent method. Typically, 7–10 µg of PCR product is generated from a single 100 µl-PCR reaction, and 10 µg of dsDNA will typically yield 2–4 µg of ssDNA (Section V). If you require more than this, scale up your PCR reactions accordingly.
5. Save some of the dsDNA for comparison to the ssDNA on an agarose gel (Section VI)

V. Protocol: Preparation of ssDNA

To generate ssDNA, each PCR product (Positive Control Reaction, PCR Reaction A and PCR Reaction B) is independently subjected to two short, consecutive Strandase reactions (Strandase A reaction and Strandase B reaction).

1. Set up Strandase A reaction as follows:

5–15 µg	dsDNA substrate (Section IV)
5 µl	Strandase A Buffer (10X)
5 µl	Strandase Mix A
X µl	RNase Free Water
50 µl	Total volume

2. Incubate the reaction as follows:

37°C	5 min/kb*
80°C	5 min
4°C	until next step

3. Set up the Strandase B reaction as follows:

50 µl	Strandase A reaction mixture (entire reaction mixture from steps 1–2 above)
50 µl	Strandase B Buffer (2X)
1 µl	Strandase Mix B
101 µl	Total volume

4. Incubate the reaction as follows:

37°C	5 min/kb*
80°C	5 min
4°C	until next step

*****For DNA fragments ≤1 kb, incubate at 37°C for 5 min, do not incubate for more than 15 min even for DNA fragments ≥3 kb. If your reaction produces smearing with 5 min/kb incubation at 37°C. Reduce the 37°C reaction time to 3 min/kb.

NOTE: Reaction can be scaled-up by increasing the number of PCR reactions (Section IV).

VI. Protocol: Check for ssDNA

1. Run 10 µl of each of the samples on a 1–2% agarose gel, including 100–200 ng of the dsDNA substrate, in a separate lane, as a control. Keep the rest of the samples at 4°C until the next step.
   - If a single band of ssDNA is confirmed from at least one of the reactions, consider scaling-up. For an example of how the bands should appear, see Figure 2. If you do not need to scale up, proceed to Step 2.

     NOTE: Ethidium bromide is less effective in staining ssDNA compared to dsDNA. Therefore, we recommend that you don't estimate the amount of DNA by eye.

   - If you do not see a clear ssDNA band from either reaction:
     a) Design alternative primers and repeat Sections IV, V, and VI.
     In our experience, shifting the position of either the forward or reverse primer by a single nucleotide can greatly affect the efficiency to effectively generate ssDNA using this method.
     b) Reduce amount of dsDNA substrate to 5 µg and repeat Sections V and VI.
     Lowering the template amount could eliminate residual dsDNA substrate.

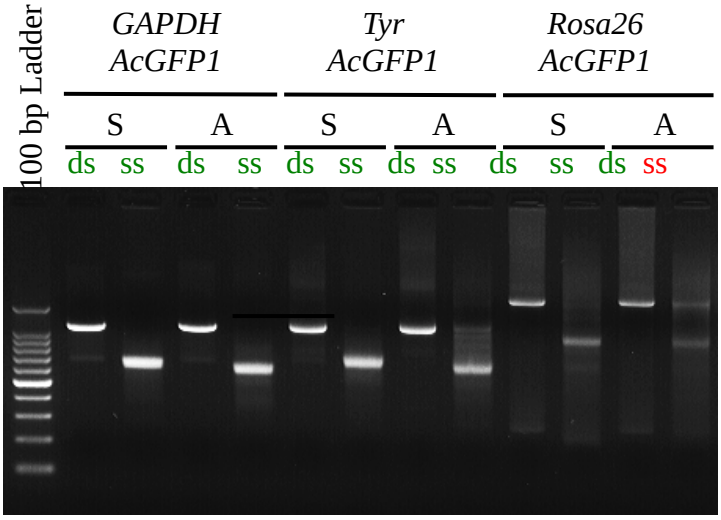

Figure 2. Gel image showing the dsDNA starting material and the ssDNA product after cleanup for both sense (S) and antisense (A) orientations for three different HDR templates. The templates consist of the *AcGFP1*-encoding sequence, flanked by the 5' and 3' homology arms to the respective target sequences: *GAPDH*, tyrosine kinase, or the *Rosa26* locus. The antisense 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 PCR reaction A or B (see above) as little as one nucleotide in either direction.

2. Column-purify the ssDNA to remove free nucleotides. We recommend following the PCR cleanup protocol for our NucleoSpin Gel and PCR Clean-Up Kits (Cat. No. 740609.50 or 740609.250) (included in the Guide-it Long ssDNA Production System) or an equivalent method.
3. Measure the DNA concentration by NanoDrop (ssDNA setting) or an equivalent method. We typically observe a concentration of 30–200 ng/µl.

NOTE: The concentration corresponding to 1 OD_{260} Unit for ssDNA is 33 µg/ml.

VII. Protocol: Concentrate ssDNA prior to electroporation

For electroporation, a high concentration of donor DNA is typically required (e.g., 1 µg/µl).

1. Collect the ssDNA following column purification (Section VI).
2. To the ssDNA, add 1/10th the volume of 3 M sodium acetate, pH 5.2, and an equal volume of isopropanol, and vortex well.
3. Incubate for 15 min on dry ice, then centrifuge at 12,000–16,000*g* for 10 min at 4°C.
4. Remove the supernatant carefully. Rinse the pellet with ice-cold 80% ethanol and centrifuge at 12,000–16,000*g* for 10 min at 4°C.
5. Air dry the pellet.
6. From the estimated amount of ssDNA (Section VI, Step 3), calculate the volume of water required to obtain a final concentration of 1 µg/µl or the desired concentration for your application. Resuspend in the appropriate amount of RNase Free Water to obtain your desired ssDNA concentration.
7. Measure the ssDNA concentration by NanoDrop or an equivalent method.

NOTE: The concentration corresponding to 1 OD_{260} Unit for ssDNA is 33 µg/ml.

8. For protocols on using the ssDNA template for gene editing experiments using electroporation with Cas9-sgRNA complexes, see the Guide-it Recombinant Cas9 (Electroporation Ready) User Manual.

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