I. Introduction

Using single-stranded DNA (ssDNA) rather than double-stranded DNA (dsDNA) as a donor template for homology-directed repair (HDR) in CRISPR/Cas9 knockin experiments has several important advantages. ssDNA does not trigger a strong cytotoxic response upon delivery to target cells, and unlike dsDNA, is much less likely to randomly integrate into the genome (Roth et al 2018). 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 v2** (Cat. No. 632666) is designed to produce long ssDNA oligos (from 500 nt up to 5,000 nt) 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 dsDNA templates (PCR products) ranging in size from 500 bp to 5 kb. The following schematic shows the general steps in the protocol.

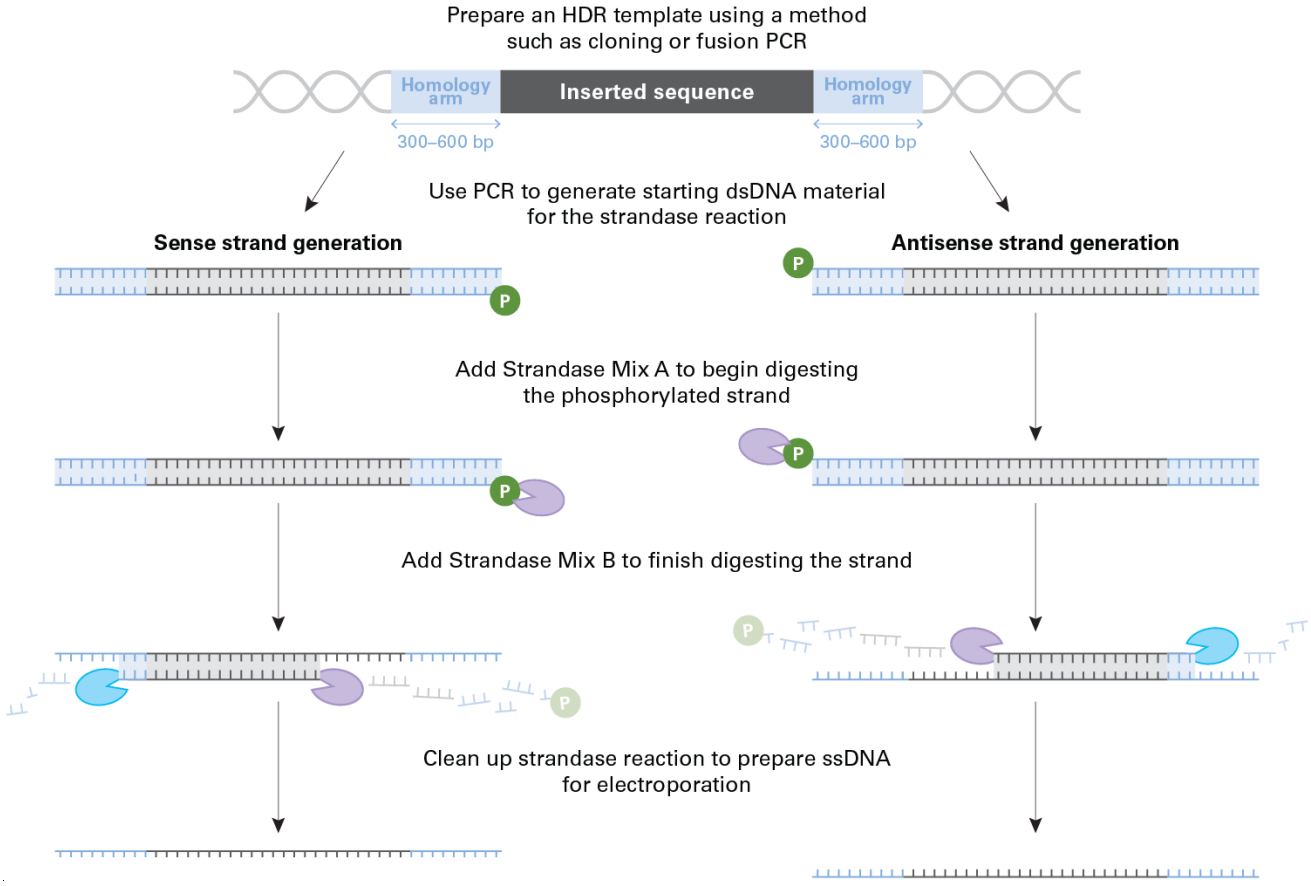

Figure 1. General steps involved in preparing long ssDNA donors for use in knockin experiments. Prepare the dsDNA template using In-Fusion® Cloning, fusion PCR, or another preferred method. For proper recombination, the template should contain arms 300 to 600 bp in length that are homologous to the genome sequence flanking the targeted site of integration. Perform 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. Note that if the forward primer is phosphorylated, the generated ssDNA will be the antisense strand; whereas if the reverse primer is phosphorylated, the final product will be the sense strand.

II. List of Components

The following components are included in the complete Guide-it Long ssDNA Production System v2:

- Guide-it Long ssDNA Strandase Kit v2
  - 4 x 625 µl PrimeSTAR® Max Premix (2X)
  - 250 µl Strandase Mix A
  - 250 µl Strandase A/B Buffer (10X)
  - 50 µl Strandase Mix B
  - 500 µl Digestion Enhancer (5X)
  - 5 x 1 ml RNase Free Water
  - 50 µl 2-kb Control Template (2 ng/µl)
  - 50 µl Control Primer Mix (16 µM)
- 2 x NucleoSpin Gel and PCR Clean-Up
- Buffer NTC

III. 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 tubes or plates
- 1.5-ml Eppendorf DNA LoBind Microcentrifuge Tubes (Catalog No. 022431021)
- Ice-cold 80% ethanol

IV. 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) using one of the following methods:

- **Cloning into a vector.** We recommend using In-Fusion HD Cloning Plus (Cat. No. 638909).
- **Performing an overlapping PCR with three fragments.** 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. In this manner, we have successfully created donor templates 1–2 kb in size.

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 due to the activity of Strandase Mix B. Therefore, we recommend preparing the template with homology arms that are 50–100 nucleotides longer than would be considered optimal given the length of the insert. This will ensure that the ssDNA is produced with sufficient homology to the target site for HDR.

V. Protocol: Preparation of dsDNA Substrate by PCR

Generate a PCR product using a forward and reverse primer against your donor template (Section IV). Note that one of the primers (forward or reverse) must contain a 5' phosphorylation in order to generate the antisense or sense ssDNA, respectively. Since it is impossible to determine which combination of primers will result in ssDNA of the highest yield and quality, we strongly recommend that you obtain four primers to generate both sense and antisense strands in parallel, 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: Since the phosphorylation of the primers is key for the generation of ssDNA, we strongly encourage ordering phosphorylated primers directly from your preferred oligo provider and avoiding any in-house phosphorylation method.

1. Set up two 100-µl PCRs as shown below:

PCR 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 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*
4°C	hold

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

NOTES: The PCR cycling conditions have been optimized for this application. No further modifications are needed.

PCR A (using a phosphorylated reverse primer) will provide the template to create ssDNA corresponding to the sense strand. PCR B (using a phosphorylated forward primer) will result in an antisense strand (Section I, Figure 1).

2. Analyze 5 µl of each PCR on an agarose gel to check for the correct amplification of dsDNA.

NOTE: Obtaining a clear, single band of dsDNA is essential for preparation of high-quality ssDNA.

3. Column-purify the dsDNA substrate using the provided NucleoSpin Gel and PCR Clean-Up kit.

a. **Adjust DNA binding conditions**. Mix one volume of sample with two volumes of Buffer NTI [i.e., each PCR (100 µl) is mixed with 200 µl of Buffer NTI].
b. **Bind DNA**. Place a NucleoSpin Gel and PCR Clean-up Column into a Collection Tube (2 ml) and load the sample. Centrifuge for 30 sec at 11,000*g*. Discard flowthrough and place the column back into the collection tube.
c. Wash silica membrane. Add 600 µl Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column. Centrifuge for 30 sec at 11,000*g*. Discard flowthrough and place the column back into the collection tube.
d. **Repeat wash step.**
e. **Dry silica membrane**. Centrifuge for 2 min at 11,000*g* to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flowthrough while removing it from the centrifuge and the collection tube.

NOTE: Strandase enzymes are very sensitive to residual ethanol from wash steps; make sure that membranes are dry before the elution step.

f. **Elute DNA**. Place the NucleoSpin Gel and PCR Clean-up Column into a new 1.5-ml microcentrifuge tube (not provided). To get a very concentrated sample, add 15–20 µl of Buffer NE to each column and incubate at room temperature (18–25°C) for 1 min. Centrifuge for 2 min at 11,000*g*.
g. (Optional) We recommend repeating the elution step with fresh Buffer NE (15–20 µl) in order to maximize the overall recovery yield.

NOTE: Recovery of larger DNA fragments (>1,000 bp) can be increased by preheating the elution buffer to 70°C and incubating the elution buffer on the column at 70°C for 5 min before centrifugation. This process can also be repeated in Step g.

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 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 spectrophotometer (Thermo Fisher Scientific) using the dsDNA setting, or an equivalent method. Typically, 7–10 µg of PCR product is generated from a single 100-µl PCR, and 10 µg of dsDNA will typically yield 2.5–4 µg of ssDNA (Section VI). If you require more than this, scale up your PCRs accordingly.

NOTES: If you are scaling up the PCR, you can purify 1.5 PCRs (150 µl) per purification column. Higher volumes are not advisable since the maximum binding capacity is 15 µg.

5. Save some of the dsDNA (approx. 100–150 ng) for comparison to the ssDNA on an agarose gel (Section VIII) and store–20°C.
6. Proceed to Section VI with the remainder of the dsDNA or store at –20° until ready to do so.

VI. Protocol: Preparation of ssDNA

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

1. Set up the Strandase A reaction as follows. While keeping the Strandase A enzyme on ice, pipette up and down several times in order to achieve a homogenous resuspension before adding the correct volume of enzyme to the reaction. Please follow the order of reagent addition as described below and prepare the reaction at room temperature.

   IMPORTANT: Briefly vortex (for 3–5 sec) and spin down the reaction before and after adding Strandase Mix A.

X µl	RNase-Free Water
5 µl	Strandase Buffer (10X)
10 µl	Digestion Enhancer (5X)
10–15 µg	dsDNA substrate (Section V; PCR A or B)
5 µl	Strandase Mix A
50 µl	**Total volume**

2. Incubate the Strandase A 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. Please make sure that the reaction and the tube (from Step 2) have cooled down to 4°C before adding Strandase Mix B. While keeping the Strandase B enzyme on ice, pipette up and down several times in order to achieve a homogenous resuspension before adding the correct volume of enzyme to the reaction. Prepare the reaction at room temperature.

   IMPORTANT: Briefly vortex (for 3–5 sec) and spin down the reaction after adding Strandase Mix B.

50 µl	Strandase A reaction mixture (entire reaction mixture from Step 2 above)
1 µl	Strandase Mix B
51 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.

NOTE: Reaction can be scaled up by increasing the number of PCRs performed (Section V).

VII. Protocol: Purify ssDNA

Column-purify the ssDNA to remove free nucleotides using the NucleoSpin Gel and PCR Clean-Up kit together with Buffer NTC (binding buffer specific for ssDNA), both of which are included in the Guide-it Long ssDNA Production System v2.

1. **Adjust DNA binding conditions**. Adjust the volume of the reaction mixture to 100 µl with water. Mix one volume of sample (100 µl) with two volumes (200 µl) of Buffer NTC.
2. **Bind DNA**. Place a NucleoSpin Gel and PCR Clean-up Column into a Collection Tube (2 ml) and load the sample. Centrifuge for 30 sec at 11,000*g*. Discard flowthrough and place the column back into the collection tube.
3. **Wash silica membrane**. Add 600 µl Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column. Centrifuge for 30 sec at 11,000*g*. Discard flowthrough and place the column back into the collection tube.
4. **Repeat the wash step**.
5. **Dry silica membrane**. Centrifuge for 2 min at 11,000*g* to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flowthrough while removing it from the centrifuge and the collection tube. Make sure that membranes are dry before the elution step.
6. **Elute DNA**. Place the NucleoSpin Gel and PCR Clean-up Column into a new low-binding DNA collection tube (not provided). Add 25 µl Buffer NE and incubate at room temperature (18–25°C) for 1 min. Centrifuge for 2 min at 11,000*g*.
7. Repeat the elution step with fresh 25 µl Buffer NE.

NOTE: Recovery of larger DNA fragments (>1,000 bp) can be increased by preheating the elution buffer to 70°C and incubating the elution buffer on the column at 70°C for 5 min before centrifugation.

VIII. Protocol: Check for ssDNA

1. Run 3 µl of each of the ssDNA samples on a 1–2% agarose gel, including 100–150 ng of the dsDNA substrate (Section V, Step 5) 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 below. If you do not need to scale up, proceed to Step 2.

   NOTES: Ethidium bromide is less effective in staining ssDNA than dsDNA. Therefore, we recommend that you don't estimate the amount of DNA by the strength of the signal.

   The ssDNA band will run lower than the respective dsDNA. However, due to possible secondary structure, it might not run at half of the dsDNA's size.

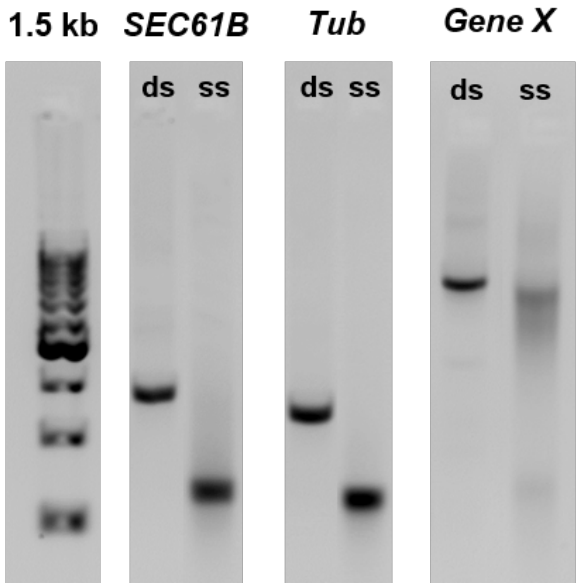

Figure 2. Gel image showing the dsDNA (ds) starting material and the ssDNA (ss) product for three different HDR templates. The first two templates consist of the AcGFP1-encoding sequence, flanked by the 5' and 3' homology arms to the respective target sequences: tubulin (*Tub*; sense strand) or *SEC61B* (antisense strand). In the third target (Gene X; undisclosed), the antisense ssDNA product runs at the same size as the dsDNA but shows a smear instead of a clean band, suggesting an incomplete digestion. As such, it is considered a failed synthesis. In the majority of cases, we have observed that this problem can be solved by shifting the PCR A or PCR B primers by at least one nucleotide in either direction.

- If you do not see a clear ssDNA band from either reaction, we recommend designing alternative primers and repeating Sections V, VI, and VII.

NOTE: In our experience, using a different primer set to amplify the dsDNA template (e.g., shifting the position of either the forward or reverse primer by at least one nucleotide) can greatly affect the efficiency of ssDNA production using this method.

2. Measure the DNA concentration by NanoDrop spectrophotometer (Thermo Fisher Scientific) using the 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.

IX. Protocol: Concentrate ssDNA Prior to Electroporation

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

1. Collect the ssDNA following column purification (Section VII).
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 VIII, Step 2), 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 spectrophotometer (Thermo Fisher Scientific) using the ssDNA setting, or an equivalent method.

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

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

X. References

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

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