

Bridging dsDNA with a ssDNA Oligo and NEBuilder[®] HiFi DNA Assembly to create an sgRNA-Cas9 Expression Vector

Peichung Hsieh, Ph.D., New England Biolabs, Inc

Introduction

NEBuilder[®] HiFi DNA Assembly Master Mix was developed to improve the efficiency and accuracy of DNA assembly over other DNA assembly products currently available. The method allows for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. Thus far, it has been adopted by the synthetic biology community as well as those interested in one-step cloning of multiple fragments due to its ease of use, flexibility and simple master mix format.

CRISPR/Cas9-based gene editing is quickly growing in popularity in the field of genome editing. Due to the size of most commonly used Cas9-containing plasmids, construction of an sgRNA or sgRNA library into a Cas9/sgRNA expression vector can be cumbersome. NEB has developed a protocol to solve this problem, using single-stranded DNA oligonucleotides, a restriction enzyme digested vector and the NEBuilder HiFi DNA Assembly Master Mix.

Protocol

Note: This protocol demonstrates the design of an sgRNA targeting a ~30 kb gene from *H. sapiens*.

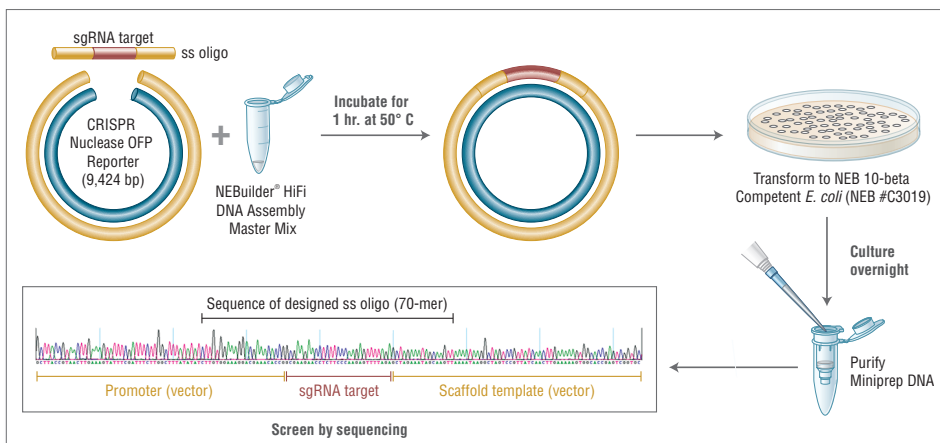
- Scan for a PAM sequence (NGG, in green) in the desired target sequence.
Example: 5'CGAAGAACCCTCTCCCAAGANGG3'
We suggest using the sgRNA design tool available at: <https://chopchop.rc.fas.harvard.edu>.
- Design a 70-base, single-stranded DNA oligonucleotide, containing a 21 nt target sequence (in red), flanked by a partial U6 promoter sequence (in blue) and scaffold RNA sequence (in purple),
Example:
5'ATCTTGTGGAAGGACGAAACACCCGGAAGAACCCTCTCCCAAGAGTTTTAGAGCTAGAAATAGCAAGTT3'
to construct a random library.
Example: 5'ATCTTGTGGAAGGACGAAACACCCG^{N₁₉₋₂₁}GTTTTAGAGCTAGAAATAGCAAGTT3'
- Prepare the ssDNA oligo in 1X NEBuffer 2 to a final concentration of 0.2 μM.
- Assemble a 10 μl reaction mix with 5 μl of ssDNA oligo (0.2 μM), 30 ng of restriction enzyme-linearized vector and ddH₂O.
- Add 10 μl of NEBuilder HiFi DNA Assembly Master Mix to the reaction mix, and incubate the assembly reaction for 1 hour at 50°C.
- Transform NEB 10-beta Competent *E. coli* with 2 μl of the assembled product, following the protocol supplied with the cells.
- Spread 100 μl of outgrowth on a plate with ampicillin antibiotic, and incubate overnight at 37°C.
- Pick 10 colonies to grow, and purify the plasmid DNA for sequencing.

* Researchers can find suitable vectors from Addgene, a non-profit organization.

We recommend Addgene plasmid #42230, pX330-U6-Chimeric_BB-CBh-hSpCas9 (for details, see <https://www.addgene.org/42230/>), although any plasmid containing an sgRNA scaffold under the control of a U6 promoter should be adequate.

 **FIGURE 1:**
sgRNA cloning workflow

Design an ssDNA oligo containing the target sequence (19-21 bases) of sgRNA flanked by 25 bases of sequence at both ends. Mix the single-stranded oligo, linearized vector DNA and NEBuilder HiFi DNA Assembly Master Mix together, incubate for 1 hour at 50°C and transform into *E. coli*.



Materials

- NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)
- Single-stranded oligonucleotides (100 μM)
- NEBuffer 2 (NEB #B7002)
- Plasmid containing an sgRNA scaffold under the control of a U6 promoter*
- NEB 10-beta Competent *E. coli* (NEB #C3019)
- LB plates with ampicillin

Results

Ten colonies were isolated, and plasmid DNA was purified using standard miniprep columns. Insertion of the target DNA sequence was confirmed by DNA sequencing. Of the 10 clones sequenced, 9 contained the target sequence in the correct orientation.

Summary

This Application Note demonstrates the convenience of inserting an sgRNA sequence into a 9.5 kb vector for targeted DNA assembly. Unlike traditional cloning methods, in which two oligos must be synthesized and re-annealed, this new protocol offers a simple way to design an oligo and assemble it with the desired vector. The NEBuilder HiFi DNA Assembly Master Mix represents a substantial improvement over traditional methods, specifically in time savings, ease-of-use and cost.

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