Protocol for assembling annealed DNA oligonucleotides and a double-stranded DNA vector using NEBuilder HiFi DNA Assembly (NEB #E2621)

Materials Required but not Supplied

NEBuilder® HiFi DNA Assembly Bundle for Large Fragments
- Nuclease-free Water (NEB #B1500)

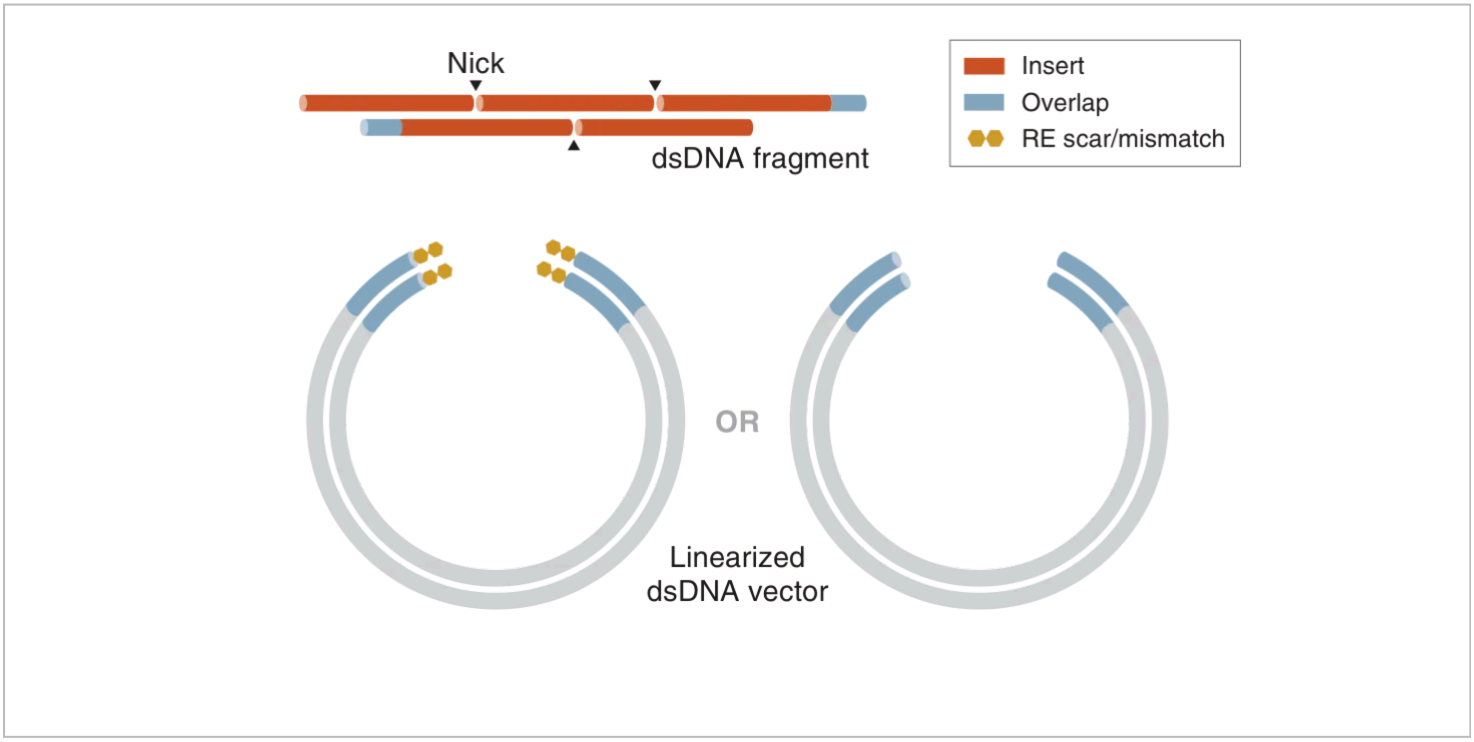
NEBuilder® HiFi DNA Assembly Master Mix
- Nuclease-free Water (NEB #B1500)

NEBuilder® HiFi DNA Assembly Cloning Kit
- Nuclease-free Water (NEB #B1500)

Overview

The following method allows you to anneal short overlapping single-stranded DNA oligos (generally 60 nucleotides (nt) each) to assemble a longer double-stranded DNA (dsDNA) fragment. The annealed nicked dsDNA fragment can then be combined and assembled with a linearized vector fragment. This annealed oligo protocol provides an alternative to short, synthesized dsDNA, such as gBlocks™.
This protocol is recommended for the assembly of the following types of DNA fragments:

- Annealed short DNA oligos forming a nicked dsDNA fragment
- dsDNA vector linearized by PCR or restriction digest



Annealed DNA Oligo Design

Short, annealed ssDNA oligos (60 nt each) should be designed with 30 nt overlaps with adjacent complementary oligos. When annealed, the overlapping oligos will form a nicked dsDNA fragment with no gaps, and ssDNA vector overlaps at each end. Please note that DNA oligos with 5' phosphates are not required.

DNA Quantities

This protocol uses a 1:50 (vector:insert) molar ratio with 0.02 picomoles of vector and 1 picomole of annealed oligos.

Protocol

1. Prepare oligos for annealing by adding 1 ul of each oligo (100 µM stock) to a final concentration of 0.2 µM (0.2 pmol/µl) using 1X NEBuffer r2.1*. This can be done by combining 1 µl of each 100 µM oligo stock in a single tube with an appropriate volume of buffer for a total volume of 500 µl.

Component	500 µl Volume	Final Conc. Or Amount
Overlapping Oligos (100 µM stock concentration)	1 µl of each oligo, for a total of X µl	0.2 µM (0.2 pmol/µl) of each oligo
1X NEBuffer r2.1*	Buffer volume = (500 µl - X µl)	1X

**Note: you can also use TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0) supplemented with 50 mM NaCl as an annealing buffer.*

2. Heat the oligo mixture solution at 100°C for 3 min and allow to cool at room temperature for 20 min. The annealed oligos are ready to assemble.

3. Set-up the following reaction on ice:

Component	20 µl Reaction	Final Conc. Or Amount*
Annealed Oligo Mixture (0.2 pmol/µl)	5 µl	1 pmol (1:50 vector: insert ratio)
Linearized Vector (0.02 pmol/µl)*	1 µl	0.02 pmol
Nuclease-free Water	4 µl	
NEBuilder HiFi DNA Assembly Master Mix (2X)	10µl	1X

* NEBioCalculator can help with DNA mass-to-molar quantity conversions for both ssDNA and dsDNA.

4. Incubate the reaction at 50°C in a thermocycler for 60 min. Transform 2 µl of assembled mix into 50 µl of NEB 5-alpha Competent *E. coli* (High Efficiency) (NEB #C2987) following the recommended protocol.

Notes:

- If you are working with large plasmids >10 kb in size we recommend NEB® 10-beta Competent *E. coli* (High Efficiency) (NEB #C3019H). If your plasmid or insert contain repetitive sequences, we recommend NEB® Stable Competent *E.*

coli (High Efficiency) (NEB #C3040H).

- For selection of transformed competent cells, we recommend LB plates with an appropriate antibiotic.
- For generating PCR Products, we recommend Q5® High-Fidelity DNA Polymerase (NEB #M0491) or related products, such as Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493) or Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)

Resources:

- NEBBuilder® Protocol Calculator
- Comparison of DNA Assembly Reaction Types
- NEBioCalculator
- High throughput cloning and automation solutions