NEBuilder Assembly Tool v1.11.1

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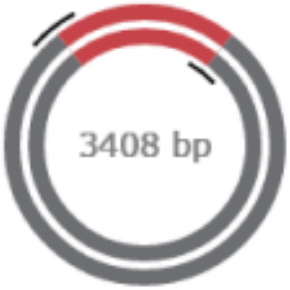
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	2-3 fragments (including vector)
Construct Length	less than 10 Kb
Min. Overlap	15 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

Vector Digestion

Vector backbone opened with EcoRI



Required Primers

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *	6-Frame
pUC19	aaacgacggccagtgTTTATACAGTTCATCCATTCCATG	sfGFP_plus	Fwd	58.1°C	**61.1°C**	view
pUC19	cgggtaccgagctcgAGGAGGACAGCTATGTCG	sfGFP_plus	Rev	61.0°C	**61.1°C**	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- Any element of a construction that includes the 5' overhang of a restriction site will be altered upon assembly. For example, the essential cysteine codon at the N-terminus of the intein segment of IMPACT vectors is present in the 5' overhang of the SapI site in those vectors. The bases removed in the assembly reaction can be added back by including them in the PCR primers for the corresponding insert.

Assembled Sequence

GGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAA
ACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC
TATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTT
GCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTT

CCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGG
TCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTG
CATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCA
AGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA
TACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAA
CTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGAT
CTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTAT
TGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA
AACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTAT
TATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGT
GATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGG
ATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCT
TAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCAC
AGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGG
AAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAG
GCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGTT
TATACAGTTCATCCATTCCATGGGTAATGCCCGCGGCGGTCACGAACTCCAGCAGCACCATATG
GTCACGTTTTTCGTTCGGATCTTTGCTCAGCACTGACTGGGTGCTTAAATAGTGATTATCCGGG
AGCAGCACCGGGCCGTCGCCGATCGGTGTGTTCTGCTGGTAATGGTCGGCCAGTTGTACACTCC
CGTCCTCCACGTTGTGCCGGATTTTGAAATTCGCCTTGATACCGTTTTTCTGTTTGTCGGCGGT
GATATAAACGTTATGACTGTTAAAGTTGTATTCTAATTTGTGACCGAGAATGTTACCATCTTCC
TTGAAATCAATACCCTTCAGTTCAATGCGATTAACCAGGGTATCACCCTCGAATTTCACTTCTG
CCCGGGTTTTGTAGGTCCCGTCATCTTTAAAGCTAATCGTACGTTCCTGCACATAGCCCTCGGG
CATGGCGCTTTTGAAGAAGTCATGGCGTTTCATGTGATCTGGATAACGCGAAAAACACTGAACC
CCGTACGTCAGTGTGGTGACCAAGGTCGGCCAAGGGACCGGCAGCTTGCCGGTAGTGCAAATGA
ATTTAAGGGTAAGTTTGCCATTTGTGGCATCCCCTTCACCCTCACCACGGACGCTAAATTTATG
GCCGTTCACGTCCCCATCCAGTTCTACCAGAATCGGAACCACACCGGTAAACAGTTCTTCTCCT
TTCGACATAGCTGTCCTCCTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCAT
GCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACT
CACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT
TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGC
TCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT
AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAA
AAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACG
AGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCA
GGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCG
CTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTG
GCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA
AGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCC
AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGA
TCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAG
ATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAA
AGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG
CGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACG
GGAGGGCTTACCATCT

HiFi DNA Assembly® Protocol

Optimal Quantities

NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–0.5 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly*	4-6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols* X µl	10 µl
Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

* Optimized cloning efficiency is 50–100 ng of vectors with 2 fold excess of inserts. Use 5 times more inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
** To achieve optimal assembly efficiency, it is recommended to design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).
† Control reagents are provided for 5 experiments.
‡ If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional Assembly Master Mix.

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

   Note: Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the assembly reaction overnight.

3. Transform NEB 5-alpha Competent E. *coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 µl of the assembled product, following the appropriate transformation protocol.

Transformation Protocols

Transformation with chemically competent cells.

1. Thaw chemically competent cells on ice.

2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).

3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacture, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O.

Add 2 µl of the diluted assembled product to competent cells.

4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds.* Do not mix.
6. Transfer tubes on ice for 2 minutes.
7. Add 950 µl of room temperature SOC media* to tubes.
8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Spread 100 µl of the cells onto the plates with appropriate antibiotics. Use Amp plates for positive control sample.
11. Incubate plates overnight at 37°C.

* Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically, transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of Gibson Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the Gibson Assembly reaction.

Transformation with electrocompetent cells.

1. Thaw electrocompetent cells on ice.
2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mM gap.
3. Dilute assembled products 3-fold with H_2O prior electroporation. This can be achieved by mixing 5 µl of assembled products with 10 µl of H_2O. Add 1 µl of the diluted assembly product to electrocompetent cells.
4. Mix gently by pipetting up.
5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
6. Add 950 µl of room temperature SOC media to the cuvette immediately after electroporation.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.