



DNA MODIFYING ENZYMES

NEBuilder[®] HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit

Instruction Manual

NEB #E2621S/L/X,
NEB #E5520S

 NEW ENGLAND
BioLabs[®] Inc.



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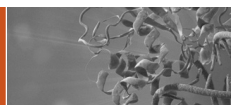


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Components:

NEBuilder HiFi DNA Assembly Master Mix:

Important Note: Upon arrival, store the kit components at –20°C.

NEBuilder HiFi DNA Assembly Master Mix

Positive Control:

2 overlapping dsDNA fragments for control assembly

NEBuilder HiFi DNA Assembly Cloning Kit:

Important Note: Upon arrival, store the kit components at –80°C. Before use, thaw and vortex the master mix thoroughly and keep on ice. After first use, store the HiFi DNA Assembly Master Mix, SOC Outgrowth Medium and positive controls at –20°C. Store the competent cells at –80°C.

NEBuilder HiFi DNA Assembly Master Mix

NEB 5-alpha Competent *E. coli* (High Efficiency)

SOC Outgrowth Medium

Positive Controls:

2 overlapping dsDNA fragments for control assembly

pUC19 Control DNA for NEB 5-alpha Competent *E. coli*

Required Materials Not Included:

DNA Polymerase (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).

LB (Luria-Bertani) plates with appropriate antibiotic.

Introduction:

NEBuilder HiFi DNA Assembly Master Mix was developed to improve the efficiency and accuracy of DNA assembly. This method allows for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. This method has been used to assemble either single-stranded oligonucleotides or different sizes of DNA fragments with varied overlaps (15–80 bp). 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. The reaction includes different enzymes that work together in the same buffer:

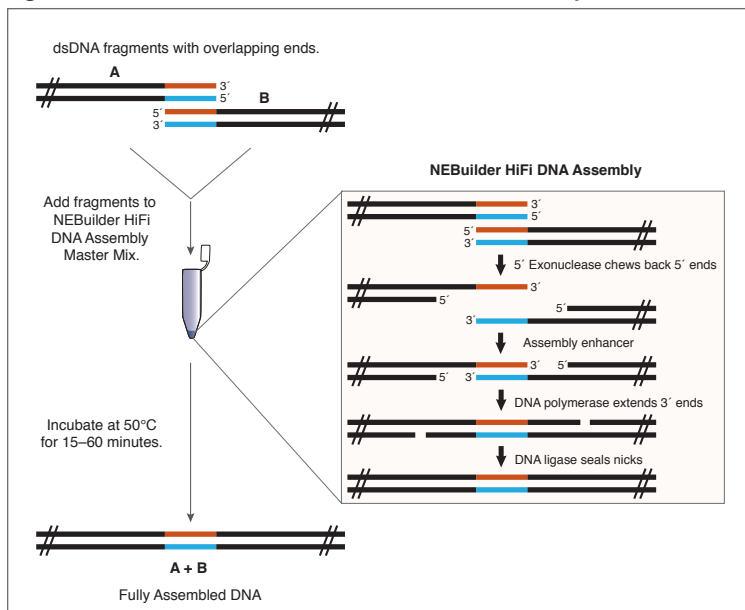
- The exonuclease creates single-stranded 3' overhangs that facilitate the annealing of fragments that share complementarity at one end (the overlap region)
- The assembly enhancer increases the efficiency of DNA assembly
- The polymerase fills in gaps within each annealed fragment
- The DNA ligase seals nicks in the assembled DNA

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation of *E. coli*.

Improved Features vs Gibson Assembly®:

- High-fidelity for accurate assembly
- Compatible with DNA fragments designed for cloning with GeneArt®, In-Fusion® and Gibson Assembly technology
- Increased efficiency of fragment assembly and transformation
- Ability to stitch two fragments with single-stranded oligonucleotides

Figure 1: Overview of the NEBuilder HiFi DNA Assembly Method



Specification:

10 μ l of 2X NEBuilder HiFi DNA Assembly Master Mix was incubated with 6 DNA fragments [4 fragments of 1,000 bp and one fragment of 1,152 bp with 80 bp overlap, and a vector of 3,373 bp (20 bp overlap), 0.05 pmol each] in a final volume of 20 μ l at 50°C for 60 minutes. NEB 5- α Competent *E. coli* (NEB #C2987) were transformed with 2 μ l of the assembled products according to the transformation protocol. Successfully assembled fragments result in *lacZ* gene in pACYC184 vector, and yield blue colonies on an IPTG/Xgal/Chloramphenicol plate when incubated overnight at 37°C after transformation. Greater than 100 blue colonies were observed when 1/10 of the outgrowth (500 μ l) was spread on a plate.

Overview of NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit Protocol:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps (see pages 4–10).
- Amplify fragments using a high-fidelity DNA polymerase.
- Prepare linearized vector by using a high-fidelity DNA polymerase or by restriction enzyme digestion.
- Determine concentration of fragments and linearized vector using agarose gel electrophoresis, a NanoDrop® instrument or other method.
- Add fragments and linearized vector to NEBuilder HiFi DNA Assembly Master Mix and incubate at 50°C for 15 minutes to 1 hour, depending on number of fragments being assembled.
- Transform into NEB 5-alpha Competent *E. coli* (provided with cloning kit or purchased from NEB) or use directly in other applications.

Design and PCR of Fragments for DNA Assembly:

Note: We highly recommend using our web tool, NEBuilder Assembly Tool, available at nebuilder.neb.com, to design PCR primers with overlapping sequences between the adjacent DNA fragments and for their assembly into a cloning vector.

NEBuilder Assembly Tool is the fastest and easiest approach to obtaining ready-to-use sequences for overlapping primers. However, it does not give details about the primer-design workflow. In some cases, it might be appropriate to further manually alter primer sequences in order to adapt them for the use in more complex assemblies, such as those that include site-specific mutagenesis. For this purpose, it is absolutely necessary to understand the general requirements and rules that apply to PCR primers used in conjunction with HiFi DNA Assembly Mix. The sections below offer step-by-step directions and recommendations for the manual design of primers for the assembly of two or more PCR fragments, as well as primer design for assembly of PCR fragments into a cloning vector prepared either by PCR or by restriction digestion.

Structure of the Overlapping Primers

PCR primers for use in HiFi DNA assembly must have two sequence components:

- an overlap sequence, required for the assembly of adjacent fragments;
- a gene-specific sequence, required for template priming during PCR.

The non-priming overlap sequence is added at the 5' end of the primer. This sequence is homologous to the 5'-terminal sequence of the adjacent fragment. The length of overlap sequence is dependent on the GC content of the sequences.

The priming gene-specific sequence is added at the 3' end of the primer after the overlap sequence. The priming sequence should meet the criteria required for template annealing during PCR.

The T_m of the 3' gene-specific sequence of the primer can be calculated using the T_m calculator found on the NEB website at tmcalculator.neb.com.

General Recommendations for Design of Overlapping Primers

To achieve efficient assembly of PCR fragments into a vector, we suggest using a 15–25 nt overlap with a T_m equal to or greater than 48°C (assuming A-T pair = 2°C and G-C pair = 4°C). To prevent errors in primer design it is highly recommended to first perform DNA fragment assembly *in silico* and create a final sequence file displaying both DNA strands (Fig. 2A, Step I). This virtual sequence may then be used as a template to design overlapping primers. Figure 2A shows the workflow for overlapping primer design by using an *in silico*-created DNA sequence file. First, mark the junctions between the adjacent fragments 1, 2 and 3 (Fig. 2A, step II). Next, at or near each junction choose 15–25 nucleotide sequences to serve as the overlap region between the two adjacent fragments (Fig. 2A, step III). For the best fit, in terms of length and T_m , the overlap sequence can be composed of nucleotides which belong to only one fragment (overlap shown in blue) or it can be split between the two adjacent fragments in any combination (overlap shown in orange). Mark the first 5' and the last 3' nucleotide of the overlap sequence on both DNA strands (boxed sequence). Finally, starting from the first 5' nucleotide, copy the entire overlap sequence in the 5' to 3' direction and, if necessary, continue to add nucleotides to the 3' end until the gene-specific priming sequence length is reached (Fig. 2A, step IV). The reverse overlapping primer is designed following the same steps as described above but copying the sequence from the complementary DNA strand in the 5' to 3' direction. Keep in mind that the two primers sharing the same overlap sequence are always used in separate PCR reactions, each in combination with the primer which primes the complementary sequence on the opposite end of the respective DNA fragment (Fig. 2A, step V).

Figure 2A: Primer design using an *in silico* created final DNA sequence file.

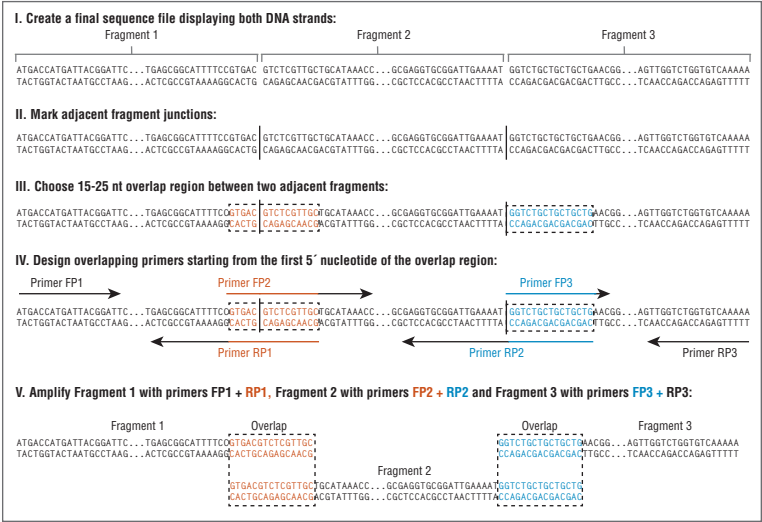
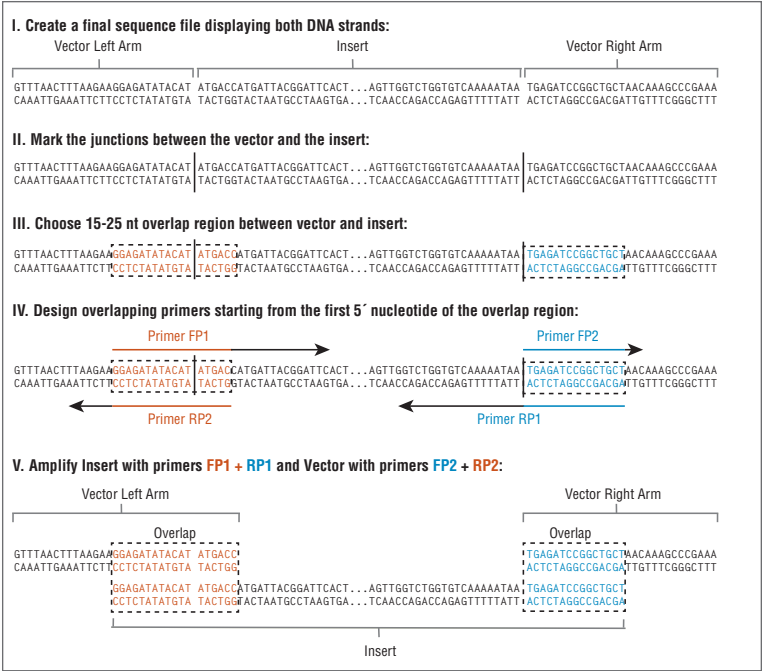


Figure 2B: Primer design for PCR-generated vector and insert using an *in silico* created final DNA sequence file.



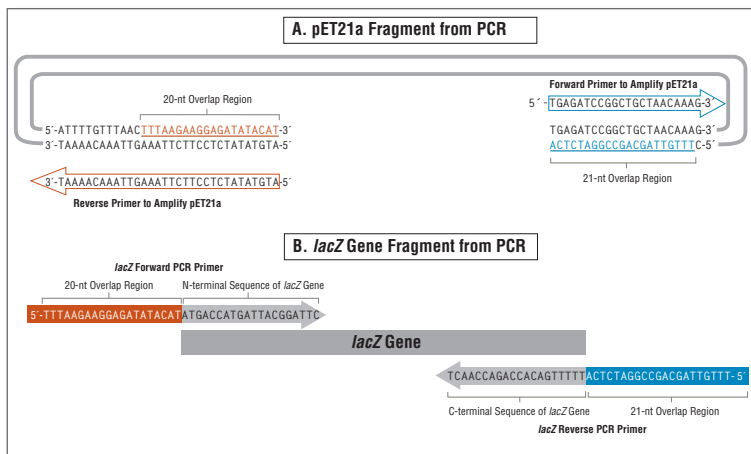
Primer Design for PCR-Generated Vector and Insert

For the purposes of primer design, the vector and the insert may be viewed as two PCR fragments that have to be assembled into a circular DNA molecule. This means that the primer design rules described above may also be applied for generation of the vector fragment and the insert fragment sharing overlapping ends. Use the *in silico*-created final sequence file as a template to design overlapping primers between the vector and the insert by accomplishing the same steps as described above, and as shown in Figure 2B.

If you intend to use a PCR-generated vector for one specific insertion, then the overlap sequence may be split between the vector and the insert in any combination to make shorter primers (Figure 2B, step III, overlap shown in orange). However, if the same PCR-generated vector will be used for assembly of various inserts, then the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert (Figure 2B, step III, overlap shown in blue). The latter case is also illustrated in Figure 3 for assembly of the *lacZ* gene into a pET21a vector. The pET21a forward primer (orange arrow) and the reverse primer (blue arrow) start at the position where the *lacZ* gene must be inserted. Both vector-specific primers completely match the vector sequence on the respective strands. This inverse PCR strategy yields a linear vector fragment. Generally, 10–100 pg of a vector is recommended as a template in the inverse PCR reaction.

To amplify the *lacZ* gene, both forward and reverse *lacZ*-specific priming sequences (gray) at their 5′ end are fused with the respective vector sequences to be used as overlap sequences in assembly with the vector. Within the *lacZ* Forward PCR primer, the overlap sequence (orange) is identical to the 20-nt terminal sequence on the top strand (orange) of the vector's left-arm (in the 5′→3′ direction). Within the *lacZ* Reverse PCR primer, the overlap sequence (blue) is identical to the 21-nt terminal sequence on the bottom strand (blue) of the vector's right-arm (in the 5′→3′ direction). The length of the overlap sequence is determined by the number of nucleotides needed to reach a $T_m \geq 48^\circ\text{C}$. If necessary, one may add additional nucleotides between the overlap sequence and the *lacZ*-specific sequence, for example, to introduce a unique restriction site.

Figure 3: Primer Design for Vector pET21a and *lacZ* Gene Assembly.



Primer Design for Assembly of Restriction Enzyme Digested Vector and PCR-Generated Insert.

Restriction enzyme-treated vectors can have 5' overhangs, 3' overhangs or blunt ends. When vector is linearized by restriction digestion, the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert. The overlap region of the forward primer for the gene of interest (orange) should line up with the 3' end of the overhang on the vector's left arm and extend back until the $T_m \geq 48^\circ\text{C}$ (Fig.4A, Left side shown in a, b and c). This primer also includes gene-specific sequence at the 3'-end (gray). Keep in mind that the restriction site, which was used to digest the vector, will be lost in the assembled product. However, additional nucleotides may be added between the overlap region and gene-specific sequence region of inserted fragment to restore the pre-existing restriction site, to introduce a new restriction site, or keep the translation of the fusion protein in frame. A similar principle is applied to the design of the reverse primer for the gene of interest (Fig. 4A, Right side).

One of the unique features of the NEBuilder HiFi DNA Assembly Master Mix is the ability to remove both 3' and 5' end flap sequences upon fragment assembly. As shown in Figure 4A, panel d, additional 3' and 5' end flap sequences after a particular restriction enzyme digestion can be removed depending on the design of the insert sequence. This allows fragments generated by restriction enzyme digestion to assemble while eliminating the remaining restriction site sequences on both the 5' and 3' ends in the fragment junction.

Figure 4B shows primer design for assembly of the *lacZ* gene and pMAL-c5X, digested with NcoI and SbfI. In this example, the forward primer of the gene has a "C" nucleotide (underlined) inserted between the 18-nt overlap and the N-terminal sequence of the *lacZ* gene to ensure the *lacZ* protein is in frame with the maltose binding protein.

Figure 4A: Assembly of Restriction Enzyme-Digested Vector and PCR-derived Insert

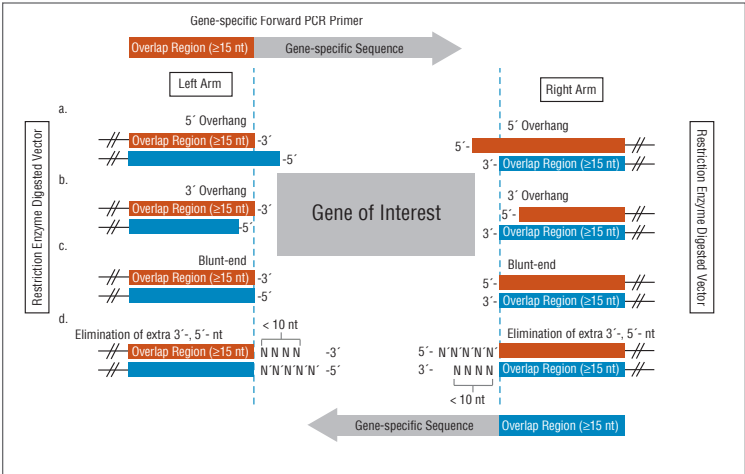
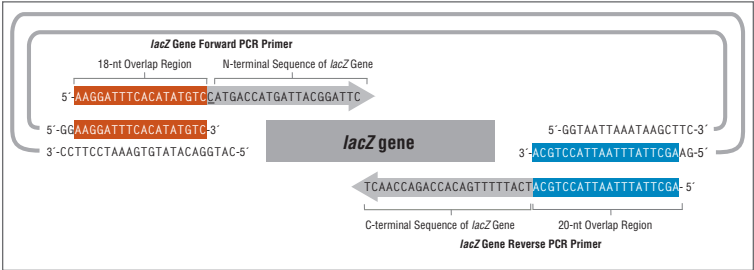


Figure 4B: Primer Design for *lacZ* Gene and NcoI/SbfI-cut pMAL-c5X Assembly



Useful Recommendations for Vector Digestion with Restriction Enzymes

In general, the cloning vector can be linearized by any restriction endonuclease or by any combination of two restriction endonucleases displaying unique site(s) at the desired locations within the vector sequence.

Note: Double digestion of vector DNA with two restriction endonucleases is the best approach to reduce the uncut vector background.

- Some restriction endonucleases cannot efficiently digest supercoiled DNA and thus may leave behind different amounts of uncut vector DNA. If not gel purified, the uncut vector is transformable, and will show up after transformation of the HiFi DNA assembly reaction, thereby, reducing the overall fraction of recombinant clones. The table “Cleavage of Supercoiled DNA” found at www.neb.com/nebecomm/tech_reference/restriction_enzymes/ may be used as a reference for choosing the most

suitable restriction endonucleases and the number of activity units required for complete digestion of plasmid vector.

- Restriction endonucleases might have a reduced activity on plasmid DNA purified using various plasmid purification kits. In such cases, extended incubation time or increased enzyme concentration may be necessary to digest plasmid vector to completion (or as nearly as possible to completion). When applicable, NEB highly recommends using High-Fidelity (HF[®]) restriction endonucleases to avoid star activity, which may occur when digesting DNA for extended periods of time with elevated amounts of standard restriction endonuclease.
- Purification of restriction endonuclease-digested vector is not necessary unless the same restriction site is present in insert DNA. In such cases, either heat inactivate the restriction endonuclease or purify the linearized vector, either by phenol-chloroform extraction/alcohol precipitation or by electrophoresis on an agarose gel before adding to NEBuilder HiFi DNA Assembly Master Mix reaction.

Useful Recommendations for PCR

NEB recommends using Q5 High-Fidelity DNA Polymerase (NEB #M0491) or related products (NEB #M0493 or NEB #M0494) to amplify fragments of interest prior to assembly. The use of this high-fidelity DNA polymerase yields PCR products with blunt ends, thereby reducing the error rates at the fragment junctions.

- When using circular plasmid DNA as a template, it is important to use a minimal amount of DNA (e.g., 0.1–0.5 ng of plasmid template per 50 µl PCR reaction) in order to reduce the template background after transformation. If higher amounts of plasmid template must be used in the PCR reaction or higher amounts of PCR product must be used in the HiFi DNA assembly reaction, it is recommended to digest the PCR product with DpnI restriction endonuclease in order to destroy plasmid template before setting up the NEBuilder HiFi DNA assembly reaction (for protocol, see below).
- Verify PCR product purity and yield by gel electrophoresis. If non-specific DNA fragments are obtained, you will need to purify the target fragment from the agarose gel to ensure the correct product assembly during the NEBuilder HiFi DNA assembly reaction.

- PCR product purification is not necessary as long as the product is > 90% pure. You can add unpurified PCR product directly from the PCR reaction into the assembly reaction, for up to 20% of the total reaction volume (i.e., PCR products should account for 4 µl, or less, in a 20 µl NEBuilder HiFi DNA assembly reaction). Larger volumes of unpurified PCR products could significantly inhibit both the assembly and the transformation. In such cases, it is recommended to column purify PCR products and, if necessary, to concentrate DNA by ethanol precipitation.

(Optional) DpnI Digestion Protocol

When higher amounts of plasmid template must be used in the PCR reaction, it is recommended to digest the PCR product with DpnI (NEB #R0176) in order to destroy the plasmid template before setting up the assembly reaction. DpnI cleaves only *E. coli* Dam methylase-methylated plasmid DNA, but does not cleave the PCR product, since it is not methylated.

DpnI Digestion Protocol:

1. In a 10 µl reaction, mix 5–8 µl of PCR product with 1 µl of 10X CutSmart™ Buffer and 1 µl (20 units) of DpnI.
2. Incubate at 37°C for 30 minutes.
3. Heat-inactivate DpnI by incubating at 80°C for 20 minutes.
4. Proceed with the NEBuilder HiFi DNA Assembly Protocol, as described below.

NEBuilder HiFi DNA Assembly Reaction 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, or using the tool, NEBcalculator (nebiocalculator.neb.com)

$$\text{pmols} = (\text{weight in ng}) \times 1,000 / (\text{base pairs} \times 650 \text{ daltons})$$

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.

HiFi DNA 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 μ l	0.2–0.5 pmols** X μ l	10 μ l
NEBuilder HiFi DNA Assembly Master Mix	10 μ l	10 μ l	10 μ l
Deionized H ₂ O	10–X μ l	10–X μ l	0
Total Volume	20 μ l††	20 μ l††	20 μ l

- * 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 NEBuilder HiFi DNA 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: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section on page 15).

3. Transform NEB 5-alpha Competent *E. coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 μ l of the assembled product, following the transformation protocol on page 12.

NEBuilder HiFi DNA Assembly Transformation Protocol

1. Thaw chemically-competent cells on ice.
2. Add 2 μ l of the chilled assembled product to the competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tubes to ice for 2 minutes.
6. Add 950 μ l of room-temperature SOC media to the tube.

7. Incubate 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 selection plates. **Use Amp plates for the positive control sample.**
10. Incubate overnight at 37°C.

Usage Notes:

To ensure the successful assembly and subsequent transformation of assembled DNAs, NEB recommends the following:

- **DNA:** PCR product purification is not necessary if the total volume of all PCR products is 20% or less of the assembly reaction volume. Higher volumes of PCR products may reduce the efficiency of high-fidelity DNA and transformation due to the elevated carryover amounts of PCR reaction buffer and unused primers present in the PCR product. Column purification of PCR products may increase the efficiency of both high-fidelity DNA assembly and transformation by 2–10 fold and is highly recommended when performing assemblies of three or more PCR fragments or assembling longer than 5 kb fragments. Purified DNA for assembly can be dissolved in ddH₂O (Milli-Q® water or equivalent is preferable), TE or other dilution buffers.
- **Insert:** When directly assembling fragments into a cloning vector, the concentration of assembly fragments should be at least 2 times higher than the concentration of vector. **For assembly of 4 or more fragments into a vector, we recommend using an equimolar ratio of fragments.**
- **Transformation:** NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987) provided with the NEBuilder HiFi DNA Assembly Cloning Kit are recommended for use for assembled products of less than 20 kb. 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 high-fidelity DNA assembled products.
- **Electroporation:** Electroporation can increase transformation efficiency by several logs. When using the NEBuilder HiFi DNA Assembly Master Mix, use 1 µl of the assembled product for electroporation.

Should you require the use of Electrocompetent cells, please use the following protocol:

Electrocompetent Cells Transformation Protocol:

1. Thaw electrocompetent cells on ice.
 2. Transfer 50 μ l of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mm gap.
 3. Add 1 μ l of the assembly product to electrocompetent cells.
 4. Mix gently by pipetting up and down.
 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 μ l 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 μ l of the cells onto the plates.
 10. Incubate overnight at 37°C.
- *Biology:* Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation or small colonies.

Frequently Asked Questions (FAQs):

What are the advantages of this method compared to traditional cloning methods?

NEBuilder HiFi DNA assembly allows insertion of one or more DNA fragments into virtually any position of the linearized vector and does not rely on the presence of restriction sites within a particular sequence. Therefore, the user has complete control over what is assembled and insertion of unwanted additional sequence, often used to facilitate the manipulation of multiple DNA sequences, can be avoided. Furthermore, the NEBuilder HiFi DNA assembly method is fast, relative to standard restriction enzyme-based cloning. Lastly, a greater number of DNA fragments can be joined in a single reaction with greater efficiency than conventional methods.

Are there any differences between NEBuilder HiFi DNA Assembly Master Mix and NEBuilder HiFi DNA Assembly Cloning Kit ?

The NEBuilder HiFi DNA Assembly Master Mix in both products is the same. The NEBuilder HiFi DNA Assembly Cloning Kit includes additional NEB 5-alpha chemically competent *E. coli*.

What is the difference between NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit and the current Gibson Assembly Master Mix/Cloning kit?

The NEBuilder HiFi DNA Assembly Master Mix utilizes a high-fidelity polymerase. While protocols for these kits are similar, the assembled products from NEBuilder HiFi DNA Assembly Master Mix and NEBuilder HiFi Cloning Kit will typically result in more colonies with higher accuracy. When large DNA (> 10 kb) or multiple fragments (4+) need to be assembled, increasing the overlap region to 30 bp improves the efficiency of assembly and transformation. There are also no licensing requirements with the NEBuilder products.

What is the largest single fragment that has been assembled with NEBuilder HiFi DNA Assembly Master Mix?

NEBuilder HiFi DNA Assembly Master Mix has been used to clone a 12 kb DNA fragment into a 7.4 kb plasmid in *E. coli*, totaling up to 19 kb in length. For assembled products greater than 15 kb, NEB recommends NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

How many fragments of DNA can be assembled in one reaction?

The number of DNA segments that can be assembled in one reaction is dependent on the length and sequence of the fragments. NEBuilder HiFi DNA Assembly Master Mix has been used to efficiently assemble up to eleven, 0.4 kb inserts into a vector at one time. However, we recommend the assembly of five or fewer inserts into a vector in one reaction, in order to produce a clone with the correct insert. A strategy involving sequential assembly can be used if all of the fragments cannot be assembled in a single reaction.

Is this method applicable to the assembly of repetitive sequences?

Yes. However, one must ensure that each DNA fragment includes a unique overlap so that the sequences may anneal and are properly arranged. The repetitive sequence can also be internalized in the first stage of a two-stage assembly strategy. If having repetitive sequences at the ends of each fragment is unavoidable, the correct DNA assembly may be produced, albeit at lower efficiency than other, unintended assemblies.

What are the shortest overlaps that can be used with this assembly method?

Productive assembly has been achieved for DNA fragments with as little as a 12 bp overlap, however, it depends on the GC content of the overlap. We recommend using at least 15 bp overlaps, or more, for dsDNA assembly with a $T_m \geq 48^\circ\text{C}$ (AT pair = 2°C and GC pair = 4°C). Increasing the length of overlap between fragments also reduces the number of fragments needed for assembly.

What are the longest overlaps that can be used with this method?

Both the quantity of 5' exonuclease in the NEBuilder HiFi DNA Assembly Master Mix and a 15 minute assembly reaction time have been optimized for the assembly of DNA molecules with ≤ 25 bp overlaps. If assembly reaction time is increased to 60 minutes, overlaps up to 40 bp may be used with the NEBuilder HiFi DNA Assembly Master Mix.

Can ≤ 200 bp dsDNA fragments be assembled by this method?

Yes. For optimal results, use these fragments in ≥ 5 -fold excess.

Can ssDNA oligonucleotides be assembled with dsDNA fragments?

Yes. However, the optimal concentration of each oligonucleotide should be determined. As a starting point, we recommend using 45 nM of each oligonucleotide that is less than or equal to twelve 60-base oligonucleotides containing 30-base overlaps.

Can longer or shorter incubation times be used?

Yes. For assembling 2–3 fragments, 15 minute incubation times are sufficient. For assembling 4+ fragments, 60 minute incubation times are recommended. 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.

Will the reaction work at other temperatures?

The reaction has been optimized at 50°C, but it has been shown to work at temperatures between 40°C and 50°C.

Is it necessary to purify PCR products?

Purification of PCR products is generally not necessary. You can use unpurified PCR products directly, as long as the total volume of PCR products in the reaction is 20% or less. If greater amounts of PCR products are used, a column cleanup kit is sufficient. It is advantageous to gel purify the target DNA fragment if the PCR product is contaminated by either non-specific amplification products, primer-dimers or large quantities of unused PCR primers.

Is it necessary to inactivate restriction enzymes after vector digestion?

Inactivation of restriction endonucleases is generally not necessary, but in some cases it might increase the transformation efficiency. If the insert and final assembled product also carry the restriction site that was used to linearize the vector it is necessary to heat inactivate the restriction enzyme before mixing the linearized vector with the insert in the assembly reaction. If a heat-resistant restriction enzyme was used to linearize the vector, then vector should be purified by phenol-chloroform extraction or extracted from agarose gel after electrophoresis, before coming into contact with the insert.

I would like to produce overlapping dsDNA fragments by PCR. Do I need to use PCR primers that have been purified by PAGE or HPLC?

No. Standard, desalted primers can be used.

I would like to assemble ssDNA oligonucleotides into dsDNA fragments. Do I need to use oligonucleotides that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

Can I use a 15-nt overlap that is entirely composed of His-tag repeats (i.e., CACCACCACCACCAC)?

No, you must flank the His-tag sequence on both sides with at least 2 nucleotides that are not part of the His-tag repeating sequence. You should avoid repeating sequences at the end of an overlap.

Can I PCR amplify the assembled product?

Yes. The assembled DNA molecule is covalently joined and can be PCR amplified. Additionally, if the final product is a closed circular DNA molecule, it can also be used as a template in rolling-circle amplification (RCA).

The control reaction is not resulting in any colonies. Why?

Our testing indicates that the choice of competent cells is critical. We recommend the use of high-efficiency chemically-competent cells such as NEB 5-alpha Competent *E. coli* (High Efficiency) (NEB #C2987).

What should I do if my assembly reaction yields no colonies, a small number of colonies, or clones with the incorrect insert size following transformation into *E. coli*?

- Assemble and transform the positive control provided with the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit (see page 11,12). Successful assembly of a positive control will demonstrate that the assembly mixture is functional and the transformation conditions are suitable.
- Analyze the reaction on an agarose gel. An efficient assembly reaction will show assembled products of the correct size and the disappearance of fragments.
- Check the primer design of the overlapping DNA fragments to ensure that there is sufficient overlap to facilitate assembly.
- Consider whether the cloned insert may be toxic to *E. coli* and a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently closed molecule, it may be alternatively amplified by PCR.

How can I reduce the number of vector-only background colonies?

To significantly reduce the background of unwanted vector-only colonies, the vector should be a PCR product, rather than a restriction fragment. If background continues to be a problem, the PCR amplified vector can be treated with DpnI to remove the template carry-over, if applicable, and extracted from an agarose gel following electrophoresis.

What type of competent cells are suitable for transformation of DNA constructs created using NEBuilder HiFi DNA Assembly Master Mix?

The resulting DNA constructs are compatible with most *E. coli* competent cells. NEB recommends using NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987). If the assembled products are larger than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020). If the assembled genes contain repetitive sequences, NEB Stable Competent *E. coli* (NEB #C3040) should be used.

Can I use electroporation instead of chemical transformation?

Yes, electroporation can be used in place of chemical transformation.

Are there any differences between the requirements for 2–3 fragment assemblies versus 4+?

The major differences between the two are the length of overlapping sequences between the adjacent fragments and the incubation time of the assembly reaction. The 15 minute assembly reaction protocol is recommended for assembly of 2–3 fragments that are flanked by 15–25 nt overlaps. The 1 hour assembly protocol is recommended for the assembly of 4+ fragments, flanked by 20–40 nt overlaps. (see the chart on page 12).

Can I use PCR product amplified from Taq DNA polymerase?

Yes. The additional A base at the 3' end of PCR product will be removed during DNA assembly if it becomes a mismatched residue once fragments anneal.

Troubleshooting

Positive Control Yields No Colonies Following Transformation into *E. coli*

- Be sure to perform a positive control assembly reaction with NEBuilder HiFi DNA Assembly Master Mix, as described on page 11.
- Use the competent cells provided with the cloning kit (NEB 5-alpha, Competent *E. coli*, NEB #C2987). The components of the NEBuilder HiFi DNA Assembly Master Mix may inhibit the functionality of competent cells from other companies.
- Perform the transformation procedure exactly as described on page 12.
- Competent cells may be thawed only once and cannot be repeatedly frozen and thawed without extreme loss in competency. Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears.
- Do not vortex competent cells. Mix cells and DNA by gently pipetting up and down. Check cell competency by transforming 100 pg of pUC19 plasmid provided with the kit. Expect $1\text{--}3 \times 10^9$ colonies formed/ μg DNA after overnight incubation on LB-ampicillin plates at 37°C.

NEBuilder HiFi DNA Assembly Master Mix/DNA Assembly Cloning Kit Reaction Yields No Colonies Following Transformation into *E. coli*.

- Assemble and transform the positive control provided with the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit. Successful assembly of a positive control will demonstrate that the NEBuilder HiFi DNA Assembly Master Mix is functional, and the transformation conditions are suitable.
- Check the primer design of the overlapping DNA fragments to ensure that there is sufficient and correct overlap to facilitate assembly.
- Avoid overlaps with highly palindromic sequences, as they may cause up to a 10-fold reduction in recombinant colonies. When assembling fragments into a multiple cloning site (MCS) of a cloning vector, it is strongly recommended to use restriction sites that are located at the edges of the MCS to avoid overlap regions with highly-palindromic sequences. Plate higher amounts of transformation reaction when using restriction sites that are located in the middle of the MCS of the cloning vector.
- Repeat the NEBuilder HiFi DNA Assembly Master Mix/Cloning Kit reaction using higher concentrations of fragments and/or vector. Make sure that the total volume of PCR-amplified products does not exceed 20% of the high-fidelity DNA assembly reaction. If necessary, purify PCR fragments and/or PCR-amplified vector before the assembly reaction.
- Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation.

- Test the success of the DNA assembly by performing PCR with primers that flank the assembled product.
- Consider whether the cloned insert may be toxic to *E. coli*, and whether a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently-closed molecule, it may be alternatively amplified by PCR.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields High Number of Clones with Incorrect Inserts

- Make sure that your PCR product is a single band of the correct size. If the PCR product is contaminated with non-specific bands, it is necessary to gel purify the PCR product to ensure cloning of the correct insert.
- Consider whether the cloned insert may be toxic to *E. coli* and whether a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently-closed molecule, it may be alternatively amplified by PCR.
- Consider using NEB Stable Competent *E. coli* (NEB #C3040) if inserts contain repetitive sequences.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields High Number of Small Colonies

- Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation efficiency or small colonies. Use a low-copy number vector (i.e., pACYC184) or a vector with tight control of protein expression. When assembling into the pUC19 vector, make sure that your gene is not in frame with *lacZ* alpha fragment.

NEBuilder HiFi DNA Assembly Master Mix Reaction Yields a High Number of Clones without the Insert

- PCR products may carry over large quantities of uncut plasmid template. To remove plasmid template, treat PCR products with DpnI restriction endonuclease before performing high-fidelity DNA assembly. Protocol for DpnI digestion can be found on page 11.
- Restriction enzyme-digested vector may carry over large quantities of uncut plasmid. Some restriction enzymes do not cut supercoiled plasmids to completion. The best way to reduce uncut vector background is to digest the vector with two different restriction endonucleases. If a single enzyme must be used, avoid restriction enzymes that leave four-base single-stranded overhangs rich in C/G (i.e., CCGG overhang). These overhangs may self-anneal to form the transformable form of the vector molecule. Also, increase units and/or incubation time and/or purify the linear vector from agarose gel.

Appendix A

NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987)

Store at -80°C

Genotype: *fhuA2 D(argF-lacZ)U169 phoA glnV44 f80D(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

CAUTION: This product contains DMSO, a hazardous material. Review the MSDS before handling.

Quality Control Assays

Transformation Efficiency:

100 pg of pUC19 plasmid DNA was used to transform NEB 5-alpha Competent *E. coli* (High Efficiency) following the protocol provided. 1–3 x 10⁹ colonies formed/μg after an overnight incubation on LB-ampicillin plates at 37°C.

Untransformed cells were also tested for resistance to phage φ80, a standard test for resistance to phage T1 and sensitivity to ampicillin, chloramphenicol, kanamycin, nitrofurantoin, spectinomycin, streptomycin and tetracycline. The cells were shown to be suitable for blue/white screening by α-complementation of the β-galactosidase gene using pUC19.

Transformation Protocol Variables:

- **Thawing:** Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will dramatically decrease the transformation efficiency.
- **Incubation of DNA with Cells on Ice:** For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes this step is shortened.
- **Heat Shock:** Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 30 seconds at 42°C is optimal.
- **Outgrowth:** Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes this step is shortened. SOC gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.
- **Plating:** Selection plates can be used warm or cold, wet or dry, without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.

Antibiotics for Plasmid Selection

Antibiotics	Working Concentration
Ampicillin	100 µg/ml
Carbenicillin	100 µg/ml
Chloramphenicol	33 µg/ml
Kanamycin	30 µg/ml
Streptomycin	25 µg/ml
Tetracycline	15 µg/ml

Ordering Information

PRODUCT	NEB #	SIZE
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 rxns
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X	10/50/250 rxns
COMPANION PRODUCTS		
Q5 High-Fidelity DNA Polymerase	M0491S/L	100/500 units
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
Q5 High-Fidelity 2X Master Mix	M0492S/L	100/500 rxns
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 rxns
NEB 5-alpha Competent <i>E. coli</i> (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB Stable Competent <i>E. coli</i> (High Efficiency)	C3040I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Competent <i>E. coli</i> (High Efficiency)	C3019I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Electrocompetent <i>E. coli</i>	C3020K	6 x 0.1 ml
SOC Outgrowth Medium	B9020S	4 x 25 ml



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