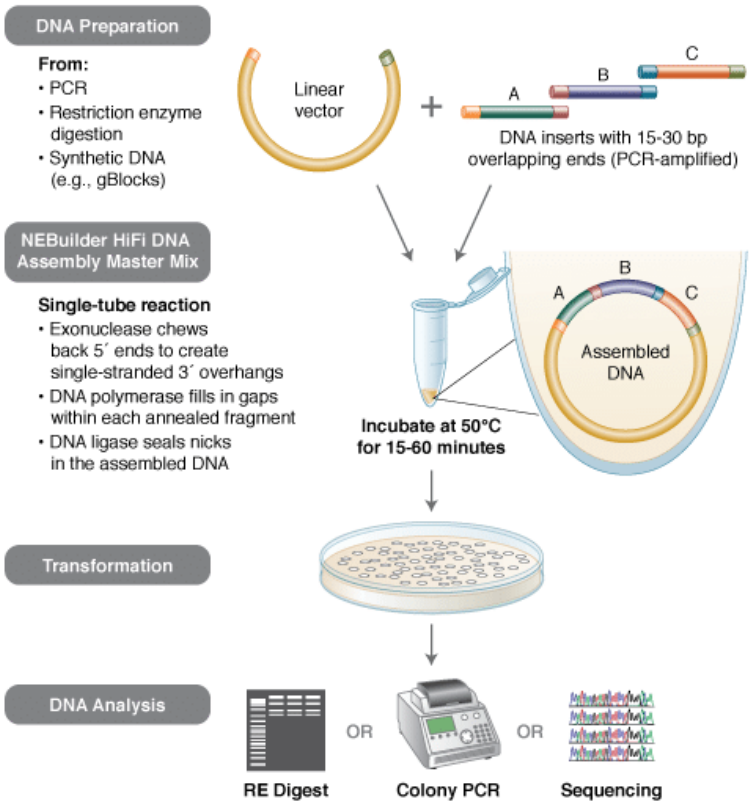
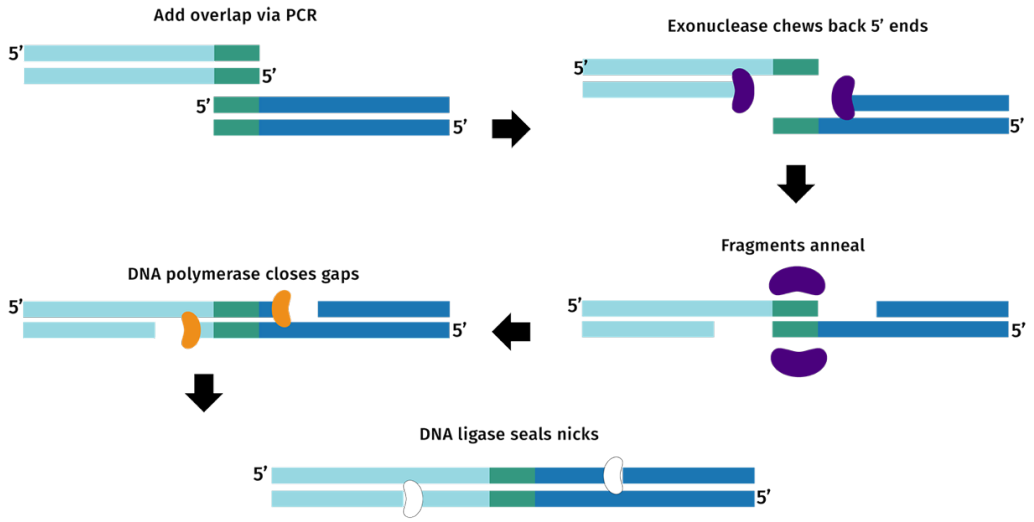
NEBuilder Protocol

Background:



Gibson Overlap Assembly is a well-known approach used for cloning together multiple linear DNA fragments without the need for restriction enzymes. Instead, Gibson Overlap relies on shared regions of homology, typically added to each fragment via PCR. These fragments are incubated together with an enzyme master mix containing an exonuclease that chews back the 5' ends of the fragments to generate long overhangs that allow homologous regions to anneal, a polymerase to repair any gaps in the bound sequences, and a DNA ligase to seal the ends of adjacent fragments. All of these components have been designed to operate at the same temperature (50°C), and the entire reaction takes an hour or less to complete. Samples are then ready for immediate transformation into competent cells. The Gibson assembly process can be used to assemble up to 6 fragments in a single step, resulting in scar-free assembly that does not require the presence of specific restriction sites. Furthermore, the long overlapping regions of complimentary sequence between overlapping fragments also better ensures correct assembly of the fragments compared to the smaller overlapping sequences created with restriction enzymes. The NEB kit for Gibson overlap PCR (NEBuilder) recommends using fragments with 15-40 bp of complimentary sequence (20 works best) and a primer melting temperature > 48°C. Additionally, fragments should be larger than 200 bp to avoid the exonuclease chewing through the entire fragment before the annealing and polymerization can occur. Another factor to consider is secondary folding of single-stranded DNA (hairpin or a stem loop) that can directly compete with the annealing and priming of neighboring assembly fragments. Despite these limitations, Gibson assembly is commonly used in synthetic biology.



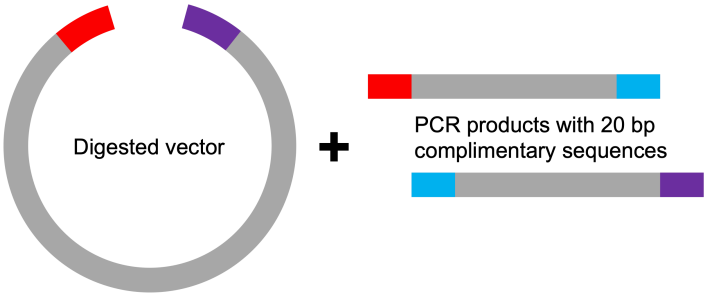
Reagents:

- Primers
- Restriction digestion enzymes
- Gel electrophoresis materials
- Agarose purification kit
- NEBuilder HiFi DNA Assembly Master Mix
- Competent *E. coli* cells (see the **Preparation of chemically competent E. coli protocol**)

Note: This protocol uses a more conservative amount of reagents to extend the use of the kit; however, the results are just as effective. The Master Mix should be divided into 30 µL aliquots stored in 200 µL tubes at -20°C (10 rxns ea).

Designing primers:

- Design primers with complimentary overhangs of 20 bp for the fragments you wish to assemble.



PCR amplification of inserts:

- Using the primers containing complimentary overhangs, perform PCR following the protocol for your polymerase of choice (see the **Q5 polymerase chain reaction protocol**).
    - Note: because Gibson overlap PCR relies heavily on complimentary regions, it is imperative to use a polymerase that does not add 3' A overhangs. Most "Taq" polymerases are therefore incompatible.
- Following PCR, perform gel electrophoresis on your products and purify from gel using an agarose gel DNA purification kit of your choice (see the **Agarose gel electrophoresis protocol** and that **DNA purification from agarose gel protocol**)

Restriction digestion of the backbone:

- Linearize the vector for insertion of the amplified fragments via endonuclease restriction digestion with the appropriate enzymes (See the manufacture instructions for your restriction enzymes).
    - Note: Proper restriction digestion should always be confirmed via Agarose gel electrophoresis.
- Perform gel electrophoresis on your restriction digestion product and purify the cut vector from gel (see the **Agarose gel electrophoresis protocol** and that **DNA purification from agarose gel protocol**)
    - Note: Gibson overlap PCR can requires concentrations of cut vector that are larger than can be recovered from gel purification, particularly if your cut vector is larger than 5 kb. If you find that your gel purification recovery of cut vector DNA is particularly low, it is possible to proceed directly from the restriction digestion step to Gibson overlap PCR without purification; however, this may lead to the presence of some undigested plasmids in your transformation, resulting in transformants of cells with un-modified plasmids. If you choose to take this approach, it is recommended that your run your restriction digestion for 1 hour (rather than the recommended 15 min), and to heat inactivate the restriction enzymes following the manufacturer information. The concentration of the resulting cut plasmid mixture can be calculated by dividing the concentration of DNA used in the restriction digestion divided by the total volume of the reaction (for NEB, this is ~20 ng/µL of cut vector).
    - Note: Depending on the size of your vector, sometimes more than 200 ng is needed, which is often incompatible with the concentrations retrieved from a normal restriction digestion. If this is the case, the amount of DNA added to the restriction digestion can be increased, so long as the amount of enzyme used is also increased in a 1:1 ratio as well. (e.g., 2 µg DNA requires 2 µL of enzyme).
    - Note: If your vector is smaller than 4 kb, you can instead design primers for PCR amplification of the backbone as a linear DNA fragment, skipping the need for restriction digestion altogether.

Gibson overlap PCR:

- The concentrations for each insert in the assembly depends on the number of fragments being assembled.

Recommended Amount of Fragments Used for Assembly		
	2–3 Fragment Assembly*	4–6 Fragment Assembly**
Recommended DNA Molar 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
NEBuilder HiFi DNA Assembly Master Mix	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl
Total Volume	20 µl††	20 µl††

* It is recommended to use 50 – 100 ng of vector with 2-fold excess of each insert (5-fold for insert(s) less than 200 bp). Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
** For 4+ fragment assembly, design 20-30 bp overlap regions for each fragment with equimolarity of all fragments (suggested: 0.05 pmol each).

- Once the number of inserts has been determined, the concentration needed can be calculated using the following equation.

ng = (pmols * bp * 650) / 1,000

  - Note: The following table has been set up to automatically calculate the concentration needed for you. Simply enter the size of your fragment in the size columne, and the nanodrop reading for your PCR/RD product in the Nano Read column, to determine the amount needed for the assembly:

double click the table to bring up the excel file with automatic formulas:

Fragment	Size (bp)	pmol Need	Nano Read (ng)	Con Need (µg)	Use (µL)
Cut Vector		0.025		0	#DIV/0!
Insert 1		0.05		0	#DIV/0!
Insert…		0.05		0	#DIV/0!

Note: If the sum of your vector and fragments don't reach 10 µL, add Milli-Q up to 10 µL.

- Once the 10 µL mixture has been prepared, use a pipette to mix the suspension well and to transfer 3 µL of the mixture to a 200 µL tube.
- Add 3 mL of NEBuilder Master Mix to each tube.
- Quick spin the tubes and the incubate samples in a thermocycler at 50°C for 15 minutes for a 2 - 3 fragment assembly, or 60 minutes when 4-6 fragments are being assembled.
  - Note: increasing the incubate step to 60 min also increases the yield for 2-3 fragment assembly.
- Following incubation, store the samples on ice until transformation.
  - Note: Samples can be stored at -20°C for short term if needed.
- Transform competent strains of *E. coli* following the **Heat shock transformation of bacteria protocol**.