Description

The Quick PCR™ **Plus** Assembly Kit is used as a molecular cloning tool to assemble long DNA fragment from multiple smaller fragments, or to insert DNA into a plasmid in a single reaction. The main kit component is a ready-to-use mix of enzymes in reaction buffer at a 2-fold concentration. This kit does not include *E. coli* cells; however, another version of the kit includes *E.coli* chemically competent cells for transformation (BPS Bioscience #78532).

Materials Required but Not Supplied

- DNA fragments from PCR (Inserts)
- Linearized plasmid (if using)
- Autoclaved/sterile or DNase-free distilled H_20

Storage Conditions

Store Quick PCR™ Assembly Mix at -80°C until first use and -20°C following first use. This kit will perform optimally for up to 12 months from date of receipt when the materials are stored as directed

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

1. Design of the PCR fragments

Design primers for the PCR with overhangs such that fragments will have 20 bp of homology (overlap) with one another or with the linearized plasmid.

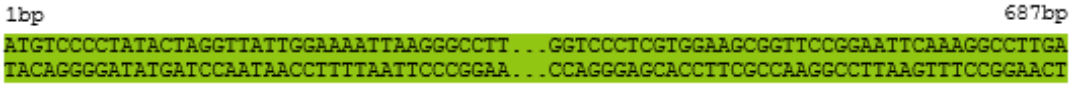
The standard overlap is 20 bp

- Ensure that at least 16 bp of homology exist between the plasmid and the overlapping DNA fragments (inserts)
- Regions of homology >50 bp between plasmid and overlapping DNA fragments (inserts) can lower the reaction efficiency.

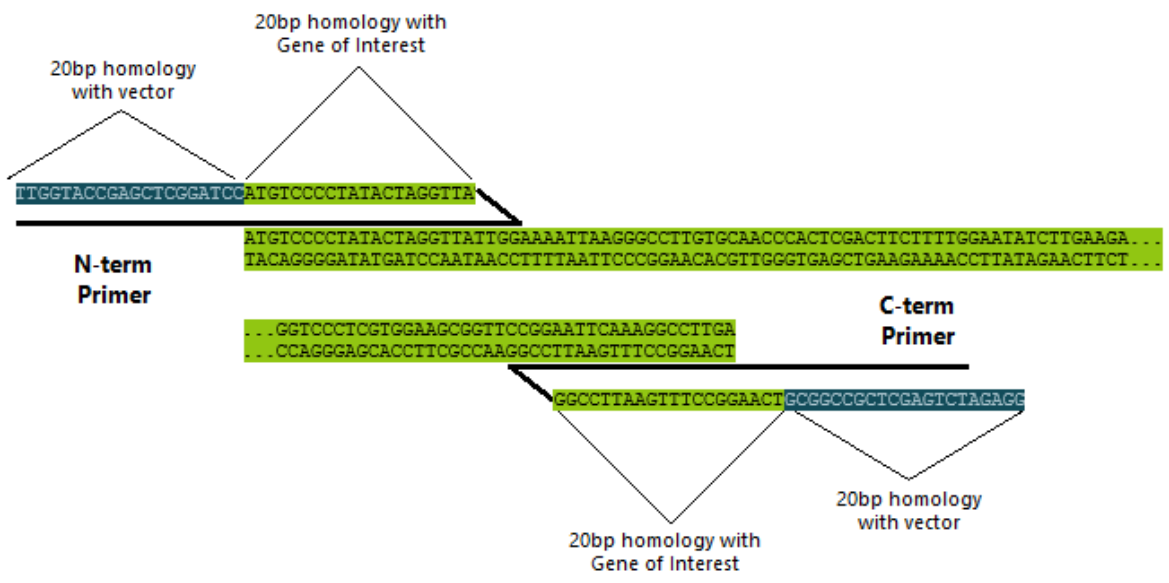
Primer Design #1: One insert and a linearized plasmid

Step 1: Linearize the plasmid (example: BamHI and NotI restriction digest)

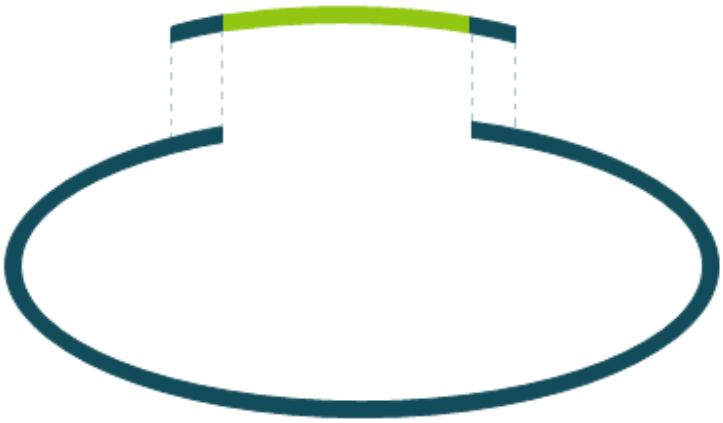
Step 2: Choose the DNA insert or the DNA fragments to assemble (Gene of Interest, open reading frame)



Step 3: Design the Primers (Gene of Interest with primers)



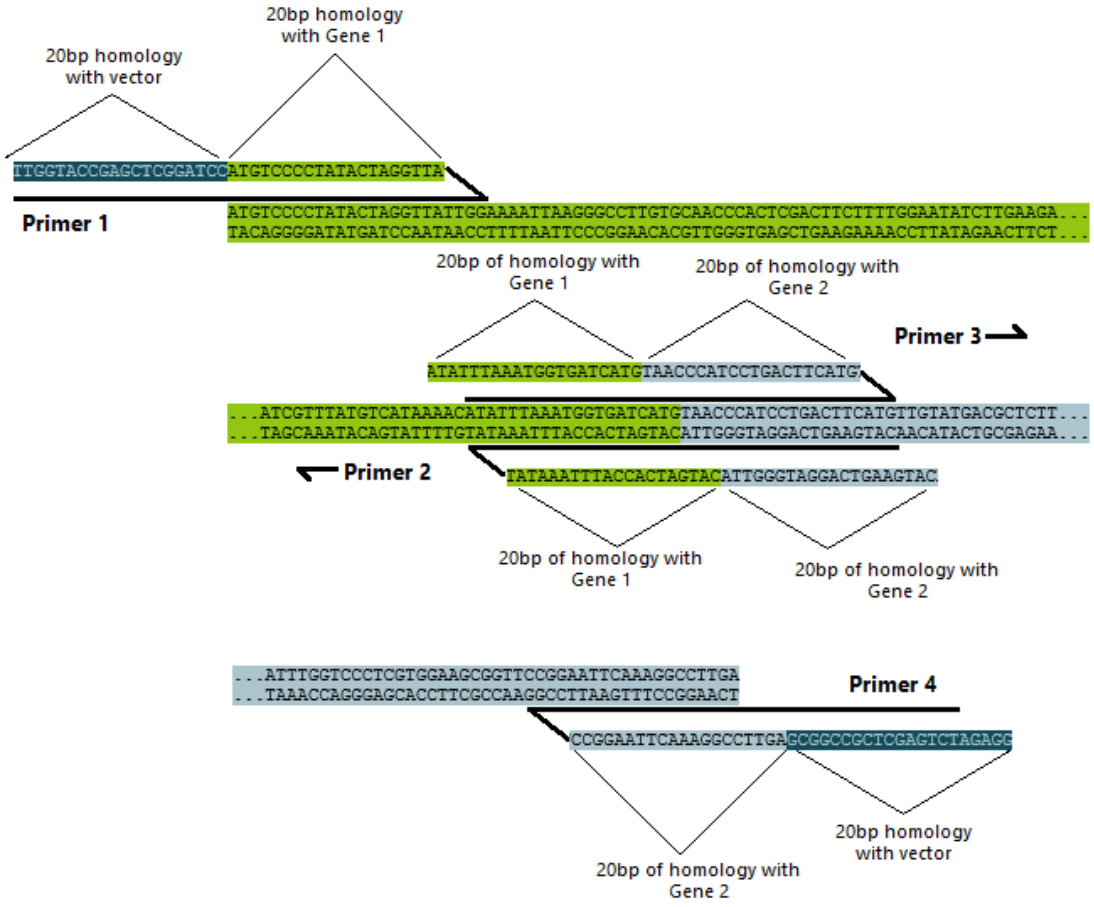
Schematic Figure for DNA insertion into the linearized plasmid:



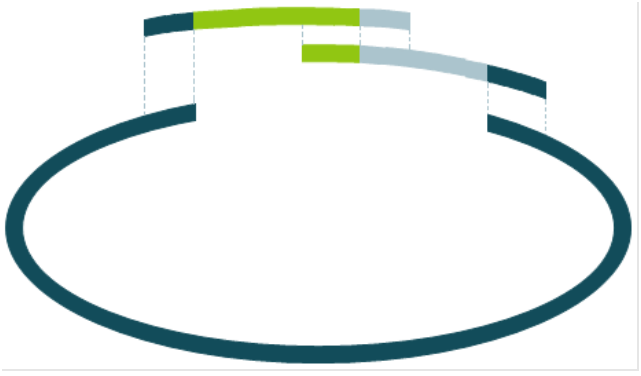
Primer Design: More Than One fragment to Insert in a linearized plasmid

Steps 1 and 2
See Above

Step 3: Design overlapping primers
(Desired assembly product with primers)



Schematic Figure for DNA insertion (2 fragments) into the linearized plasmid:



2. Protocol for DNA Assembly

A. Prepare the DNA fragment(s) of interest by PCR amplification followed by gel electrophoresis for size exclusion. Clean up the fragment(s) by gel extraction and column purification.

B. If using a plasmid, linearized by restriction digest followed by gel-extraction/clean up using your preferred column purification kit. It is highly recommended to use two different restriction enzymes to ensure complete linearization of the plasmid.
   a. A plasmid can alternatively be assembled using only linearized products
   b. A linear DNA product can be assembled from multiple DNA fragments for downstream application

C. Determine the amount of DNA, in molarity, needed for the assembly reaction depending on the number of fragments to assemble and on their sizes (in bp/kb).
   a. If inserting into a plasmid: Use ≥ 2X the molar ratio of insert to vector.
   b. Use equimolar amounts of DNA fragments if assembling a single DNA fragment from multiple linear DNA fragments.
   c. Note: See page 5 for calculating DNA molar quantities and reaction examples.

D. Assemble the reaction on ice using the 2X Assembly Mix and the DNA fragments. Use sterile dH_20 to bring the reaction up to desired volume. A standard reaction has a total volume of 20 µl.

E. Incubate the reaction at 50°C for 30 min if using 3 or fewer DNA fragments (including the plasmid) or 60 min if using ≥4 DNA fragments. Following the incubation place the tube on ice to cool down before transformation. The reaction can be stored at -20°C until transformation.

3. Quality Control of Quick PCR™ Assembly Kit

A 20 µl reaction was performed using 10 µl of the 2X Quick PCR™ Assembly Mix, two DNA fragments of 700 bp each, and a linearized pcDNA3.1 (+) (Thermo Fisher #V79020, AmpR) plasmid double-digested with EcoRI-HF and NotI-HF (New England Biolabs R0101 and R3189.) The total amount of insert DNA used was in a 2-molar excess of the linearized vector. A negative control was also performed in absence of the two DNA fragments.

Reactions were incubated at 50°C for 30 min in a thermocycler and cooled on ice for 5 min. 10 µl of the reaction was added to *E.coli* chemically competent cells. Cells and DNA were incubated on ice for 5 min, followed by a heat shock at 42°C for 40 sec. Transformed cells were incubated on ice for 2 min, after which 500 µl of room temperature antibiotic-free LB was added to the cells and incubated in a 37°C shaker for 15 min. Transformed cells were spun down at 6000 rpm for 2 min, 400 µl of supernatant (SUP) removed, and cells resuspended in the remaining SUP volume. 50 µl of resuspended cells were plated onto prewarmed selection plates and incubated overnight at 37°C.

6 individual colonies were picked from the agar plate, grown overnight in Ampicillin-containing LB, and the plasmid was purified by mini-prep. A double-restriction digest was performed on each individual plasmid in a 10 µl reaction: 500 ng of plasmid was incubated at 37°C with EcoRI-HF and NotI-HF for 30 min and analyzed on an agarose gel. The gel was visualized and screened for bands of expected sizes 1.4 kb (assembled DNA fragments) and 5.4 kb (linearized pcDNA3.1 (+) plasmid). On average, 5 out of 6 colonies contained the 1.4 kb insert.

4. Reaction Examples and Molarity Conversion

Calculation of pmol from DNA mass and length (bp)

$$\text{pmols} = \frac{(\text{mass in ng}) \times 1000}{(\text{bp} \times 650 \text{ daltons})}$$

Example 1: (Standard one-insert and linearized vector)

Component	Size (kb)	Conc. (ng/µl)	Volume (µl)	Amount (pmol)
Vector	6.5	200	1.5 µl	0.05
Insert 1	1.5	50	2 µl	0.1
2X Assembly Mix	-	-	10 µl	-
dH20	-	-	6.5 µl	-
Total Volume			20 µl	

Example 2: (Standard two-inserts and linearized vector)

Component	Size (kb)	Conc. (ng/µl)	Volume (µl)	Amount (pmol)
Vector	5.5	100	1.75 µl	0.05
Insert 1	1.5	50	2 µl	0.1
Insert 2	2	100	1.25 µl	0.1
2X Assembly Mix	-	-	10 µl	-
dH20	-	-	5 µl	-
Total Volume			20 µl	

5. Troubleshooting

Issue	Reason	Solution
No colonies on selection plates following reaction with Quick PCR™ **Plus** Assembly and Cloning Kit and transformation into DH5α or High-efficiency *E.coli* chemically competent cells	Incorrect DNA amounts/ratios used	• Check calculations of DNA inserts and vector used in the reaction • Use a higher molar excess of insert(s) to vector
	Insufficient regions of homology	• Ensure that at least 20 bp of homology exist between vector and overlapping DNA fragments (inserts)
	Reaction volume too high	• Reaction efficiency is lowered in higher volumes; DNA concentration can be improved by eluting into lower volumes following gel/PCR cleanup
	Reaction incubation duration too short	• Incubate ≥4 DNA fragment reactions for *at least* 60 min at 50°C • All reactions can be incubated for up to 1.5X the suggested duration to improve efficiency
	Too little reaction volume transformed	• Increase the amount of the reaction volume added to *E.coli* competent cells
	Recovery/selection required	• Bactericidal antibiotics (ex. Kanamycin, Tetracycline) require at least 30 min of recovery at 37°C in antibiotic-free Luria Broth following transformation • Difficult assembly reactions will benefit from performing a recovery step following transformation, in which *E. coli* cells are incubated for 30 minutes at 37°C in a shaker, preferably in a larger tube for aeration, after the heat shock.
Colonies do not contain the plasmid or contain incorrect plasmid	DNA contamination from PCR fragments	• Use gel-extraction to clean up PCR fragments to minimize secondary PCR products or other DNA contaminants such as PCR template • Perform a negative control reaction with DNA inserts
	Insufficient regions of homology	• Ensure that at least 16 bp of homology exist between vector and overlapping DNA fragments (inserts)
	Regions of homology are *too* long	• Regions of homology >50 bp between vector and overlapping DNA fragments (inserts) can lower the reaction efficiency. Increase the duration of

		the reaction, or redesign primers such that the regions of homology are within 16-30 bp long
Colonies do not contain the plasmid or contain incorrect plasmid *(continued)*	Improper/partial digestion of vector	• Ensure that the plasmid has been digested with the proper amount of enzyme and for the duration suggested by the manufacturer • Perform a negative control assembly reaction using linearized vector • Use high-efficiency restriction enzymes to digest the plasmid • Use two different restriction enzymes to linearize the plasmid

6. Related Products

Kit Size	Catalog Number (Assembly Mix Only)	Catalog Number (Includes *E.coli* competent cells)
10 reactions	#78531-1	#78532-1
50 reactions	#78531-2	#78532-2