Gibson Assembly Protocol (combined)

Introduction

Protocol for PCR & Gibson adapted from the Gibson Assembly Cloning Guide 2nd edition
Protocol for template removal adapted from NEBcloner (link)
Protocol for PCR Purification adapted from Qiagen QIAquick® PCR Purification Kit
Protocol for MiniPrep adapted from Qiagen's QIAprep® Spin Miniprep Kit

Materials

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Procedure

PCR Amplfication of DNA Fragments

1. Prepare PCR reaction (see table)

PCR Components

	A	B
1	Component	Volume
2	Insert or vector DNA (100 pg/µL - 1 ng/µL in TE)	0.5 µL
3	10 µM Forward Primer	2.5 µL
4	10 µM Reverse Primer	2.5 µL
5	10 mM dNTPs	1 µL
6	5X Phusion HF Buffer	10 µL
7	Phusion DNA Polymerase (2 U/µL)	0.5 µL
8	Nuclease-free Water	33 µL
9	**Total**	**50 µL**

2. Run reaction in a thermocycler

Table1

	A	B	C	D
1	Step	Temperature	Duration	Number of Cycles
2	Initial denaturation	98 °C	30 seconds	1 cycle
3	Amplification	98 °C	10 seconds	25-30 cycles
4		Primer Tm	20 seconds	
5		72 °C	30 seconds/kb	
6	Final extension	72 °C	5 minutes	1 cycle
7	Hold	4 °C	-	1 cycle

Template Removal

3. Set up the reaction as follows:

DpnI digestion components

	A	B
1	Component	50 µl Reaction
2	DNA	1 µg
3	10X CutSmart Buffer	5 µl (1X)
4	DpnI	1.0 µl (or 10 units)
5	Nuclease-free Water	to 50 µl

4. Incubate at 37°C for 5–15 minutes (DpnI is Time-Saver qualified.)

5. **Optional:** Heat inactivate at 80 °C for 20 min if not doing a DNA purification step

PCR Purification

6. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow

7. Place a QIAquick column in a provided 2 ml collection tube

8. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back in the same tube.

9. To wash, add 750 µl Buffer PE to the QIAquick column centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back into the same tube.

10. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

11. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

12. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min and then centrifuge.

Mid-way analysis

13. Add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

14. Run the gel and evaluate the results

15. NanoDrop the purified DNA to determine the concentration

Gibson Assembly using a HiFi 1-Step Kit

16. Thaw Gibson Assembly HiFi 1-step Master Mix on ice

17. Combine insert and vector DNA to a total volume of:

    5 µL for the **2X** kit or
    7.5 µL for the **HC 4X** kit

18. Vortex the master mix

19. On ice, combine

    5 µL DNA and **5 µL Master Mix** for the **2X kit** or
    7.5 µL DNA and **2.5 µL Master Mix** for the **HC 4X** kit
    Mix well andbriefly centrifuge

20. Incubate at **50** °C for **1 hour**

21. Store reactions at -20 °C or use in 1-2 uL per reaction for transformation

Transformation

22. Thaw chemically-competent cells on ice

23. Add 2 uL of the chilled assembly product to the competent cells. Mix gently by pipetting up or down or by flicking the tube 4-5 times. Do NOT vortex

24. Place the mixture on ice for 30 minutes. Do not mix

25. Heat shock at 42°C for 30 seconds. Do not mix

26. Transfer tubes to ice for 2 minutes

27. Add 950 uL of room temperature SOC media to the tubes
28. Incubate the tube for 37°C for 60 minutes. shake vigerously (250 rpm) or rotate
29. Warm selection plates to 37°C
30. Spread 100uL of the cells onto the selection plates.

    Note: Use Amp plates for the positive control
31. Incubate overnight at 37°C.

Day 2:

32. Plate counting:

    Count CFU
    Check control plates
33. Innoculate LB Cam O/N culture

Day 3:

Miniprep

34. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
35. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
36. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
37. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
38. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
39. Apply 800 µl supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 30–60 s and discard the flow-through
40. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. Centrifuge for 30–60 s and discard the flow-through
41. Wash the QIAprep 2.0 spin column by adding 0.75 ml Buffer PE. Centrifuge for 30–60 s and discard the flow-through. Transfer the QIAprep 2.0 spin column to the collection tube.
42. Centrifuge for 1 min to remove residual wash buffer.

43. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.

44. If the extracted DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.