SOP-2XX: Gibson Assembly Cloning - General Protocol

Version 1.0

Date Created: [Insert Date]

Purpose: General protocol for cloning DNA fragments into plasmid vectors using Gibson assembly. Enables seamless joining of 2-6 DNA fragments with homologous overlapping ends.

Applications: Gene cloning, synthetic construct assembly, vector modification, library construction, multi-fragment assembly

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1. OVERVIEW & WORKFLOW

1A. Purpose

Gibson assembly enables seamless, sequence-independent cloning of multiple DNA fragments with overlapping homology arms (15-40 bp). This protocol uses NEBuilder HiFi DNA Assembly Master Mix, which combines exonuclease, polymerase, and ligase activities for high-efficiency assembly.

1B. General Timeline

Day	Tasks
Day 1	Inoculate overnight culture for plasmid miniprep
Day 2	Miniprep + restriction digest (1-2 hours); Optional: Set up overnight digest
Day 3	Gel purify linearized vector; PCR amplify insert fragments; Gel purify PCR products
Day 4	Gibson assembly; Transform competent cells; Plate on selective media
Day 5	Screen colonies by colony PCR or miniprep + restriction digest
Day 6	Submit verified clones for Sanger sequencing; Prepare glycerol stocks

Total time: 6 days from start to validated clone

1C. Key Design Considerations

- **Homology arms:** 15-40 bp overlaps between adjacent fragments (20-25 bp recommended)
- **Overlap Tm:** >48°C for efficient assembly
- **Fragment sizes:** 200 bp - 10 kb per fragment; total assembly <15 kb recommended
- **Number of fragments:** 2-6 fragments per assembly (2-3 optimal)
- **End structure:** Use blunt or 5' overhangs; avoid 3' overhangs

2. MATERIALS & EQUIPMENT

2A. Reagents

For Plasmid Preparation:

- Destination vector plasmid (appropriate strain)
- LB broth with appropriate antibiotic
- QIAprep Spin Miniprep Kit (or equivalent)

For Restriction Digest:

- Restriction enzyme(s) for vector linearization (e.g., EcoRI, BamHI, BsaI)
- Appropriate restriction buffer (10×)
- Nuclease-free water

For PCR Amplification:

- Q5 High-Fidelity DNA Polymerase (NEB #M0491)
- 5× Q5 Reaction Buffer
- 10 mM dNTP mix
- Forward and reverse primers with 15-40 bp homology arms (10 µM stocks)
- Template DNA (plasmid, genomic DNA, or synthetic gene)
- Nuclease-free water

For Gel Purification:

- Agarose
- TAE or TBE buffer
- DNA gel stain (SYBR Safe or GelRed)
- QIAquick Gel Extraction Kit (or equivalent)

For Gibson Assembly:

- **NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)**
- Nuclease-free water

For Transformation:

- Chemically competent E. coli (DH5α, TOP10, or strain-specific)
- SOC medium
- LB agar plates with appropriate antibiotic

2B. Equipment

- Shaker incubator (37°C)
- Microcentrifuge (16,000×g capable)
- Centrifuge for 50 mL tubes (2,000×g capable)
- Thermocycler
- Gel electrophoresis system with power supply
- UV or blue light transilluminator
- NanoDrop, Qubit, or plate reader (DNA quantification)
- Heat blocks (37°C, 50°C, 65°C)
- Micropipettes (P2, P10, P20, P200, P1000)

3. PLASMID MINIPREP (HIGH-YIELD PROTOCOL)

Purpose: Generate high-quality, high-concentration plasmid DNA for restriction digest and PCR template.

3A. Overnight Culture

1. Inoculate 50 mL LB + appropriate antibiotic with single colony or glycerol stock
2. Incubate at 37°C with shaking (200-250 rpm) for 16-18 hours

Note: *For smaller plasmids (<5 kb) or lower-copy vectors, use full 50 mL culture*

3B. Cell Harvest

1. Transfer culture to two 50 mL conical tubes (25 mL per tube)
2. Centrifuge at 2,000×g for 10 minutes at 4°C
3. Discard supernatant completely; remove residual LB with pipette

3C. Alkaline Lysis

Resuspension:

- Add 400 µL Buffer P1 (with RNase A) to each pellet
- Resuspend thoroughly by vortexing or pipetting
- Transfer both resuspensions to single 1.5 mL tube (800 µL total)

Lysis:

- Add 400 µL Buffer P2 (lysis buffer)
- Invert 4-6 times until solution becomes clear/viscous
- Incubate 3-5 minutes at room temperature

DO NOT vortex or exceed 5 minutes

Neutralization:

- Add 400 µL cold Buffer N3 or P3 (neutralization buffer, from 4°C)
- Invert immediately until white precipitate forms
- Centrifuge at 16,000×g for 6 minutes

3D. Column Binding & Washing

1. Transfer supernatant to QIAprep spin column (avoid white pellet)
2. Stand 5 minutes at room temperature (allows DNA binding)
3. Centrifuge at 16,000×g for 30 seconds; discard flow-through
4. Add 500 µL Wash Buffer (PE or equivalent)
5. Centrifuge at 16,000×g for 30 seconds; discard flow-through
6. Repeat wash 2 more times (3 washes total for high purity)
7. Dry spin at 16,000×g for 2 minutes (remove residual ethanol)

3E. Elution

1. Transfer column to clean 1.5 mL tube
2. Add 30-40 µL Elution Buffer (EB) or nuclease-free water (pre-warmed to 65°C)
3. Stand 5 minutes at room temperature
4. Centrifuge at 16,000×g for 2 minutes
5. Measure concentration on NanoDrop or Qubit

Expected yield: 500-1,500 ng/µL

Quality: A260/A280 = 1.8-2.0

6. Store at -20°C or proceed immediately to restriction digest

4. VECTOR LINEARIZATION (RESTRICTION DIGEST)

Purpose: Linearize circular plasmid vector at specific restriction site(s) to create compatible ends for Gibson assembly.

4A. Restriction Digest Setup

Component	1× Reaction	5× Scale	Notes
Plasmid DNA	___ µL (1 µg)	___ µL (5 µg)	From miniprep
Restriction Buffer (10×)	5 µL	25 µL	Match enzyme
Restriction Enzyme	___ µL	___ µL	10-20 U per µg
Nuclease-free H_2O	___ µL	___ µL	To final volume
Total Volume	**50 µL**	**250 µL**	-

4B. Digestion Procedure

1. Mix gently by pipetting up and down 5-10 times
2. Incubate at appropriate temperature (usually 37°C) for 1-2 hours
   • For overnight digest: 16 hours at 37°C
3. Heat-inactivate (if enzyme allows): 65-80°C for 20 minutes
4. Cool on ice before gel loading

4C. Verification & Gel Purification

1. Run 5 µL digest on 1% agarose gel to confirm linearization
2. Expected: Single band at predicted size (linear vector)
3. Load remaining digest onto preparative gel (0.8-1.2% agarose)
4. Excise linearized vector band under UV or blue light
5. Gel extract using QIAquick Gel Extraction Kit:
   • Weigh gel slice, add 3 volumes Buffer QG
   • Dissolve at 50°C for 10 minutes
   • Bind to column, wash, elute in 30-40 µL
6. Quantify DNA (expect 50-200 ng/µL)
7. Store at -20°C or use immediately for Gibson assembly

5. PCR AMPLIFICATION OF INSERT FRAGMENTS

Purpose: Amplify insert DNA with primers containing 15-40 bp homology arms for Gibson assembly.

5A. Primer Design Guidelines

- **Homology arms:** 15-40 bp overlaps (20-25 bp optimal)
- **Overlap Tm:** >48°C (use NEB Tm Calculator)
- **Primer structure:** [Homology arm]—[Gene-specific sequence]
- **Avoid:** Secondary structures, primer dimers, long repeats

5B. PCR Master Mix Preparation (21.5 µL per reaction)

Prepare on ice. Always make 1-2 extra reactions for overage.

Component	1× (µL)	___×	Total (µL)
5× Q5 Reaction Buffer	5.00	×	_____
10 mM dNTPs	0.50	×	_____
Q5 High-Fidelity Polymerase	0.25	×	_____
Nuclease-free H_2O	15.75	×	_____
Total Master Mix	**21.5 µL**	**×**	**_____ µL**

Example calculation for 5 reactions:

For 5 reactions + 1 overage = 6×

• 5× Q5 Buffer: 5.00 µL × 6 = 30.00 µL

• 10 mM dNTPs: 0.50 µL × 6 = 3.00 µL

• Q5 Polymerase: 0.25 µL × 6 = 1.50 µL

• Water: 15.75 µL × 6 = 94.50 µL

• Total: 129.00 µL

5C. Primer/Template Mixtures (3.5 µL per reaction)

Prepare individual tubes on ice for each fragment.

Reagent	1× (µL)	_____	_____	_____
Template DNA	1-2	___ µL	___ µL	___ µL
Forward Primer (10 µM)	~1.25	___ µL Primer F: _____	___ µL Primer F: _____	___ µL Primer F: _____
Reverse Primer (10 µM)	~1.25	___ µL Primer R: _____	___ µL Primer R: _____	___ µL Primer R: _____
Total	**3.5 µL**			

Notes:

- Adjust template volume based on concentration (aim for 10-100 ng total)
- Adjust primer volumes based on concentration (final 0.5 µM in 25 µL reaction)
- Add water as needed to bring total to 3.5 µL

5D. PCR Reaction Assembly

1. Prepare Master Mix on ice (Section 5B)
2. Prepare Primer/Template mixtures in individual PCR tubes on ice (Section 5C)

3. Add 21.5 µL Master Mix to each Primer/Template tube

4. Mix by pipetting gently

5. Final volume per reaction: 25 µL

IMPORTANT: Only 25 µL goes into each PCR tube for thermocycling.

5E. PCR Cycling Conditions

Step	Temperature	Time
Initial Denaturation	_____°C	_____ sec
Cycling (_____ cycles):		
Denaturation	_____°C	_____ sec
Annealing	_____°C	_____ sec
Extension	_____°C	_____ sec/kb
Final Extension	_____°C	_____ min
Hold	4°C	∞

Typical Q5 conditions:

• Initial: 98°C / 30 sec

• Cycling (30-35×): 98°C / 10 sec → 50-72°C / 20-30 sec → 72°C / 20-30 sec/kb

• Final: 72°C / 2 min

Note: *Annealing temperature depends on primer Tm. For primers with long homology arms, use 2-step PCR or touchdown PCR.*

5F. PCR Verification

1. Run 5 µL PCR product on 1% agarose gel
2. Verify single band at expected size
3. If clean and strong, proceed to gel extraction
4. If multiple bands or smearing, optimize PCR or gel-purify

6. GEL EXTRACTION & PURIFICATION

Purpose: Purify PCR products and digested vector from agarose gel to remove primers, enzymes, and non-specific products.

6A. Gel Electrophoresis

1. Prepare 1-1.5% agarose gel in TAE or TBE buffer
   - Use 0.8% for large fragments (>5 kb)
   - Use 1.5-2% for small fragments (<1 kb)
2. Add DNA gel stain (SYBR Safe or GelRed) to molten agarose
3. Load remaining PCR product (~20 µL) with loading dye
4. Run at 80-100 V until fragments are well-separated (30-60 min)

6B. Band Excision

1. Visualize DNA under UV or blue light transilluminator
2. Excise target band with clean scalpel
3. Minimize UV exposure time (<1 minute)
4. Transfer gel slice to pre-weighed 1.5 mL tube

6C. Gel Extraction (QIAquick Protocol)

1. Weigh gel slice; add 3 volumes Buffer QG per 100 mg gel
   - Example: 200 mg gel slice = 600 µL Buffer QG
2. Incubate at 50°C for 10 minutes, vortexing every 2-3 minutes
3. Check that gel is completely dissolved (yellow color)
4. Add 1 gel volume of isopropanol, mix
   - Example: 200 mg gel = 200 µL isopropanol
5. Apply to QIAquick column, centrifuge 16,000×g for 1 minute
6. Discard flow-through
7. Add 750 µL Buffer PE, centrifuge 16,000×g for 1 minute
8. Discard flow-through; dry spin 16,000×g for 1 minute
9. Elute DNA:
   - Add 20-30 µL Buffer EB or nuclease-free water to column center
   - Stand 2 minutes at room temperature
   - Centrifuge 16,000×g for 1 minute
10. Quantify DNA (expect 10-100 ng/µL)
11. Store at -20°C or use immediately for Gibson assembly

7. GIBSON ASSEMBLY REACTION

Purpose: Assemble purified DNA fragments with overlapping homology arms into circular plasmid construct.

7A. Calculate DNA Amounts

Use NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation):

1. Enter vector size and concentration
2. Enter insert sizes and concentrations
3. Calculator provides volumes for equimolar ratios

General guidelines:

- **Vector amount:** 50-100 ng per 20 µL reaction
- **Insert ratio:** 2-3× molar excess over vector
- **Total DNA:** 0.02-0.5 pmol recommended
- **Multiple inserts:** Use equimolar amounts of each insert

7B. Gibson Assembly Setup

Component	Amount	Notes
Linearized vector	___ µL (50-100 ng)	Gel-purified
Insert fragment(s)	___ µL (2-3× molar)	Gel-purified PCR
NEBuilder HiFi (2×)	10 µL	Master Mix
Nuclease-free H_2O	___ µL	To 20 µL
Total	**20 µL**	-

7C. Assembly Reaction

1. Set up reactions on ice in PCR tubes or strip tubes
2. Mix gently by pipetting up and down 5-10 times
3. Incubate in thermocycler or heat block:
   - 50°C for 15 minutes (2-3 fragments)
   - 50°C for 60 minutes (4-6 fragments or troubleshooting)
4. Place on ice immediately after incubation
5. Use 2-5 µL for transformation or store at -20°C

IMPORTANT: Do NOT heat-inactivate. Gibson assembly product is ready for transformation.

8. BACTERIAL TRANSFORMATION

Purpose: Transform assembled plasmid into competent E. coli for propagation and screening.

8A. Heat Shock Transformation Protocol

1. Thaw competent cells on ice (10-15 minutes)
2. Add 2-5 µL Gibson assembly mix to 50 µL competent cells
3. Mix gently by flicking tube (do NOT pipette)
4. Incubate on ice for 20-30 minutes
5. Heat shock at 42°C for 30-45 seconds (water bath or heat block)
6. Return to ice for 2 minutes
7. Add 450 µL pre-warmed SOC medium (37°C)
8. Recover at 37°C with shaking (200-250 rpm) for 1 hour

8B. Plating

1. Prepare LB agar plates with appropriate antibiotic (pre-warmed to 37°C)
2. Plate 100 µL and 400 µL on separate plates
3. For low-efficiency assemblies: pellet remaining cells, resuspend in 50 µL, plate
4. Incubate plates inverted at 37°C for 16-18 hours

Expected: 10-100 colonies per plate (good assembly)
Low colonies (<10): Check DNA quality, molar ratios, or extend assembly time
Very high colonies (>500): Possible undigested vector; verify linearization

9. COLONY SCREENING & VERIFICATION

9A. Colony Picking

1. Pick 4-8 well-isolated colonies per construct
2. Inoculate 5 mL LB + antibiotic in culture tubes
3. Grow overnight at 37°C with shaking (200-250 rpm)

9B. Screening Methods

Option 1: Colony PCR (faster, lower cost)

- Use vector and insert-specific primers
- Pick colony, resuspend in 20 µL water, use 1 µL for PCR
- Run products on gel; select positive clones for miniprep

Option 2: Miniprep + Restriction Digest (higher confidence)

- Miniprep all overnight cultures
- Digest with diagnostic restriction enzymes
- Run on gel; verify expected banding pattern

9C. Sequencing Validation

1. Select 2-3 clones with correct screening results
2. Submit miniprep DNA for Sanger sequencing
3. Design primers to sequence:
   - All junction regions (vector-insert overlaps)
   - Complete insert sequence
   - Promoter and terminator regions
4. Verify no mutations, correct sequence at junctions

9D. Glycerol Stock Preparation

1. Mix 850 µL overnight culture + 150 µL 50% glycerol in cryovial
2. Vortex to mix thoroughly
3. Label with strain name, plasmid name, date, and initials
4. Freeze at -80°C for long-term storage

10. QUALITY CONTROL CHECKPOINTS

Step	Expected Result	QC Criteria
Miniprep	500-1,500 ng/µL	A260/A280 = 1.8-2.0
Linearization	Single band, expected size	No circular vector visible
PCR products	Clean bands, no smearing	Correct sizes, bright bands
Gel purification	10-100 ng/µL	No residual gel buffer
Transformation	10-100 colonies	Well-isolated colonies
Sequencing	100% sequence match	No mutations at junctions

11. TROUBLESHOOTING

Problem	Possible Cause	Solution
No PCR product	Poor primer design; Low template; Wrong annealing temp	Run gradient PCR; Increase template; Check primers
Multiple PCR bands	Non-specific amplification; Low annealing temp	Increase annealing temp; Use touchdown PCR; Gel purify
No transformants	Poor assembly; Bad competent cells; Wrong antibiotic	Test cells with control plasmid; Check molar ratios; Extend assembly time
High background	Uncut vector; Self-ligation	Gel-purify linearized vector; Increase digest time
Wrong clones	PCR errors; Poor assembly efficiency	Screen more colonies; Use high-fidelity polymerase; Verify primers
Mutations in insert	PCR polymerase errors; Template contamination	Always use high-fidelity polymerase; Verify template quality; Screen multiple clones

12. SAFETY NOTES

Chemical Hazards

- **Ethidium bromide / DNA stains:** Potential mutagens; wear gloves, dispose as hazardous waste
- **UV light:** Use protective equipment; minimize skin exposure
- **Isopropanol/Ethanol:** Flammable; use in ventilated area
- **Acrylamide (if making gels):** Neurotoxin; use PPE, avoid powder inhalation

Biological Hazards

- **E. coli work:** BSL-1 minimum; follow institutional biosafety guidelines
- **Waste disposal:** Autoclave all bacterial cultures and contaminated materials
- **PPE required:** Lab coat, gloves, safety glasses at all times

VERSION HISTORY

v1.0 ([Insert Date]):

- Initial generalized Gibson assembly protocol
- Comprehensive workflow from miniprep to validated clone
- High-yield plasmid preparation method
- Master mix preparation system for multiple PCR reactions
- Fillable tables for restriction digest, PCR master mix, and cycling conditions
- Detailed troubleshooting section
- QC checkpoints and safety protocols

END OF SOP-2XX