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F1-X™ Next-Gen Gibson Assembly® User Guide

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Preface

Over the past two decades, molecular biology has been transformed. DNA sequencing has become faster and more accessible, and the cost of synthetic DNA continues to decline. As these technologies advance, the ability to assemble DNA efficiently, accurately, and at scale must evolve in parallel.

At Racer Biosciences, our mission is to create next-generation tools that accelerate innovation in DNA assembly and preparation. The F1-X™ Next-Gen Gibson Assembly® System embodies that mission, bringing better performance, precision, and capabilities to a trusted workflow.

Over a decade ago, the invention of the Gibson Assembly method revolutionized cloning by enabling seamless, one-step assembly of multiple DNA fragments without the need for restriction enzymes or ligation scars. Since its introduction, the method has been cited in over 40,000 publications worldwide.

Building on the simplicity and legacy of the Gibson Assembly method, F1-X offers:

- ✓ **Fast and simple assembly:** complete reactions in a 1-Step process in as little as 15 minutes
- ✓ **Efficient multi-fragment assembly:** high-fidelity and effective assembly excels with complex builds
- ✓ **Visual positive control:** measure cloning efficiency quickly and easily
- ✓ **Small volume and automation friendly:** with a standard reaction volume of 10 uL and assembly verified down to 2.5 uL, F1-X is optimized for high throughput and low waste workflows

Whether you are building expression vectors, genetic circuits, or entire pathways, F1-X system delivers robust results and accelerates your innovation. This manual guides you through preparation, assembly, and transformation using the F1-X system.

At Racer Bio, we prioritize our customer experience and quality above all else.
For help and product feedback, contact help@racerbio.com
For purchase questions, contact sales@racerbio.com

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Limited Use Label License

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Limited Warranty

The F1-X™ system, and components and products thereof, is warranted to perform according to specifications stated on the certificate of analysis. No other warranty is made, whether express or implied, including any warranty of merchantability or fitness for a particular purpose. This warranty limits Telesis Bio Inc.'s and its licensors' liability to only the price of the system. Neither Telesis Bio Inc. nor its licensors shall have any responsibility or liability for any special, incidental, indirect, or consequential loss or damage whatsoever.

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Trademark Information

F1-X™ is a trademark of Racer Biosciences. Gibson Assembly® is a registered trademark of Telesis Bio Inc., used under license. This guide references third-party brands to identify products that are compatible with, or commonly used alongside, the F1-X™ Next-Gen Gibson Assembly® System. All such names are the property of their respective owners, and their use herein does not imply endorsement or affiliation.

Patents

F1-X™ system technology is protected under US patent numbers 7,776,532, 8,435,736, 8,968,999, and additional patents pending.

Regulatory Statement

For research use only. Not for use in diagnostic procedures.

Kit Information

F1-X™ Next-Gen Gibson Assembly® Kit Information

Each F1-X™ Next-Gen Gibson Assembly® Kit includes F1-X™ Master Mix (2×) and F1-X™ Positive Control (2×). Refer to Appendix A for more information about using the positive control.

F1-X™ Next-Gen Gibson Assembly® Kit	F1XGA-20R	F1XGA-100R	F1XGA-200R
No. of 10 µL reactions per kit	20	100	200
F1-X™ Master Mix (2×)	F1XMM-20R 100 µL	F1XMM-100R 500 µL	F1XMM-200R 1000 µL
F1-X™ Positive Control (2×)	F1X-CTRL 15 µL		
Storage	Store at −20°C; avoid repeated freeze-thaw; stable 12 months from manufacture		

Compatibility and Features

Parameter	**Range**
Reaction volume	2.5 – 20 µL with 10 µL standard reaction volume
Fragment size	100 bp – 32 kb per fragment
Fragment number	2-12 fragments per reaction
Assembly size	Up to 100 kb total
Homologous overlap length	• 20-40 bp (for 2-3 fragment assemblies) • 40+ bp (for 4+ fragment assemblies)
Reaction conditions	• 2-3 fragments: 50°C for 15 minutes • 4-12 fragments: 50°C for 60 minutes
Temperature	50°C (tolerance: 50-56°C)
Compatibility	• Mismatches in overlaps • Crude PCR products (up to 20% v/v)

Principles of Gibson Assembly® Technology

The Gibson Assembly® method enables seamless, 1-Step joining of multiple DNA fragments without restriction enzymes or ligation scars. The F1-X™ system builds upon this original chemistry with enhanced speed, fidelity, and flexibility. The method relies on user-defined overlapping ends designed into fragments, allowing homologous overlaps to guide assembly. DNA fragments with homologous overlaps are combined with the Gibson Assembly® Master Mix and incubated at a constant temperature (50-56°C). Within this isothermal environment, three coordinated enzymatic activities work in concert (Figure 1).

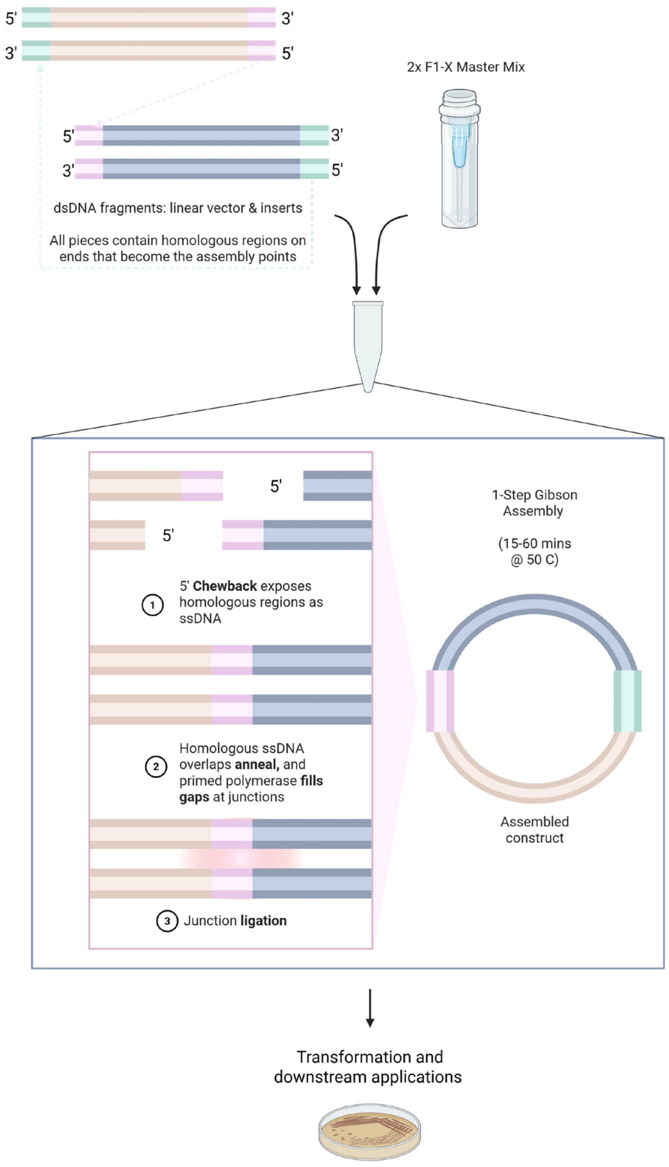


Figure 1. Gibson Assembly Method Overview. Schematic showing the three enzymatic activities (exonuclease, polymerase, and ligase) working together to join DNA fragments with homologous overlaps in a single isothermal reaction. Note that overhang design dictates the circularity or linearity of the final assembly product. Reaction efficiency is influenced by DNA quality, design, concentration, and reaction time/ temperature.

F1-X™ Workflow Overview

The F1-X™ workflow is engineered for simplicity, precision, and speed:

- **Fragment preparation:** Generate DNA fragments with homologous overlaps (typically 20-40 bp for simple assemblies or up to 80 bp for complex constructs). Fragments can be generated by PCR or restriction digestion, or sourced from DNA synthesis providers. Quality control, outlined in this manual, is an essential step of the process
- **Assembly:** Combine mixture of fragments with F1-X™ Master Mix (2×) and incubate at 50°C for 15-60 minutes
- **Downstream applications:** Use assembled DNA directly for transformation without purification. Alternatively, use the assembled construct as a template for traditional PCR or rolling circle amplification (Figure 2)

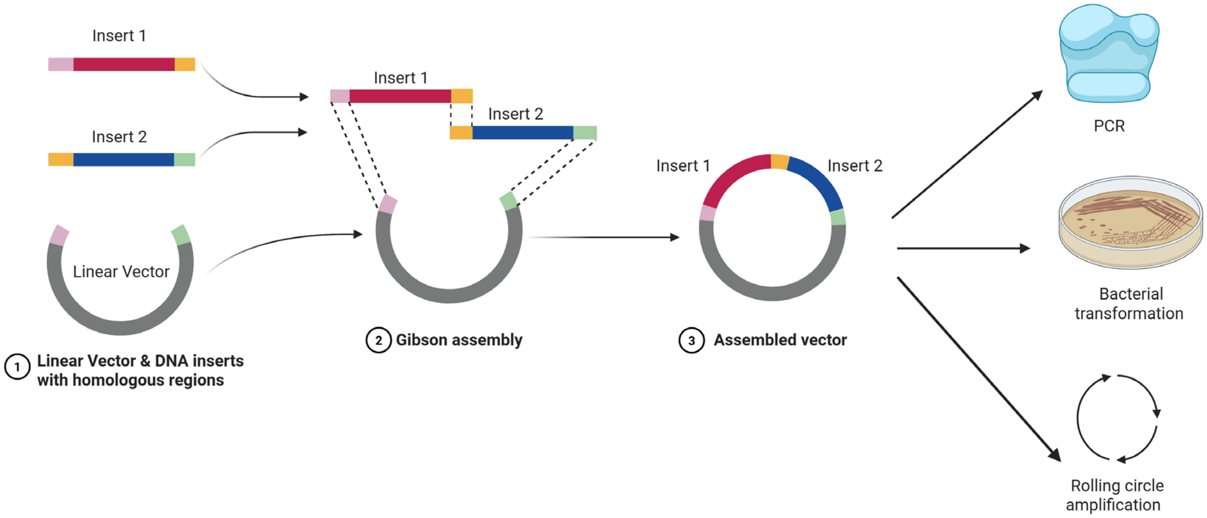


Figure 2. F1-X™ Next-Gen Gibson Assembly® Workflow. DNA fragments with homologous overlaps are assembled in 1 Step using the F1-X™Master Mix. The resulting construct can be directly used downstream

DNA Fragment Preparation and Quality Control

DNA quality is one of the most critical factors for Gibson Assembly® success. Even DNA from commercial providers should undergo quality control before use in assembly reactions.

Essential Quality Metrics and Measurement Methods

All DNA fragments require assessment of these three parameters:

Parameter	Purpose	Example Methods	Acceptance Criteria
Concentration	Accurate quantification for proper stoichiometry	Qubit™, NanoDrop™	Sufficient for target amounts
Integrity	Verify full-length products	Gel electrophoresis, TapeStation™	>80% full-length product
Purity	Assess contamination levels in solution	A260/280, A260/230 ratios	A260/280 ≥1.8, A260/230 ≥2.0

Concentration Measurement Methods

Method	Spectrophotometry (e.g. Nanodrop™)	Fluorometry (e.g. Qubit™)
Principle	UV absorbance at 260nm	Fluorescent dye specific to dsDNA
Speed	< 10 seconds per sample	2-3 minutes per sample
Sample Volume	1-2 µL	1-20 µL (assay dependent)
Key Strengths	• Provides purity ratios (260/280, 260/230) • No consumables required • Immediate results • Retains sample	• Highly specific for dsDNA • Accurate at low concentrations • Unaffected by RNA/protein • Works with unpurified PCR products
Considerations	• May overestimate due to contaminants • Less accurate at << 10 ng/µL • Cannot distinguish ssDNA and dsDNA • Not suitable for unpurified PCR	• Requires dyes and standards • Consumable costs • Calibration needed • Sample not recoverable

Integrity Measurement Methods

Method	Gel Electrophoresis	Capillary electrophoresis (e.g. Tapestation)
Output Type	Visual bands on gel	Digital electropherogram
Resolution	Qualitative	Quantitative
Sample Required	5-10 µL	1 µL
Key Strengths	• Direct visual confirmation • Detects degradation/smearing • Multiple sample comparison • Cost-effective	• Automated analysis • Precise sizing • Quantification included • Data automatically in CSV form
Considerations	• Manual interpretation often needed • Lower resolution for large fragments • More sample consumed	• Higher cost per sample • Equipment investment • Kit limitations by size range
Ideal Use Case	Routine quality checks	High-throughput or publication data

Common DNA Purification Methods

While F1-X™ tolerates unpurified PCR products (up to 20% v/v), purification is recommended for complex assemblies where 3+ fragments are present in the reaction.

Method	Description	Best Use Case
Column-Based	Silica membrane columns bind DNA under high-salt conditions	Routine PCR cleanup, general purification
Bead-Based	Magnetic beads reversibly bind nucleic acids	High-throughput, automation-compatible workflows
Gel Extraction	DNA separated by electrophoresis, target band recovered	Size selection, removal of specific contaminants
Precipitation	Alcohol and salts precipitate nucleic acids	Bulk cleanup, concentration of large constructs

Assessing DNA Purity

Spectrophotometric Analysis:

- **A260/A280 ratio:** Assesses protein contamination. Pure DNA: 1.8-2.0
- **A260/A230 ratio:** Detects organic compounds and salts. Pure DNA: >2.0
- **RNA contamination:** Increases A260/A280 ratio >2.0 and inflates concentration estimates

Fragment Preparation Methods

PCR Amplification (Recommended)

PCR is the most versatile method for adding homologous overlaps to an existing piece of DNA. The overall workflow includes the following:

1. Design primers with correct binding region and homologous overlap region
2. Assemble PCR reactions using high-fidelity polymerase (Platinum™ SuperFi™ II, Phusion®, Q5®, KOD Xtreme™, for example)
3. Digest residual template by adding DpnI + rCutSmart™ buffer; incubate 30 minutes-2 hours at 37°C
4. Analyze by electrophoresis and gel extract if low molecular weight contaminants are present
5. Purify if needed, or use directly with F1-X™

Recommended PCR Cycling Conditions

We always recommend reviewing the manufacturer's guidelines for best use of PCR products. Below is an illustrative example.

Step	Temperature	Time	Cycles	Notes
Initial Denaturation	98°C	30 sec	1	Fully denatures template
Denaturation	98°C	10 sec	30	
Annealing	Tm°C*	10 sec	30	Use Tm for gene-specific region only
Extension	72°C	15-30 sec/kb	30	Adjust for amplicon length
Final Extension	72°C	5 min	1	Completes full-length products

*Calculate based on polymerase vendor's Tm calculator

Restriction Enzyme Digestion

For vector linearization or fragment excision:

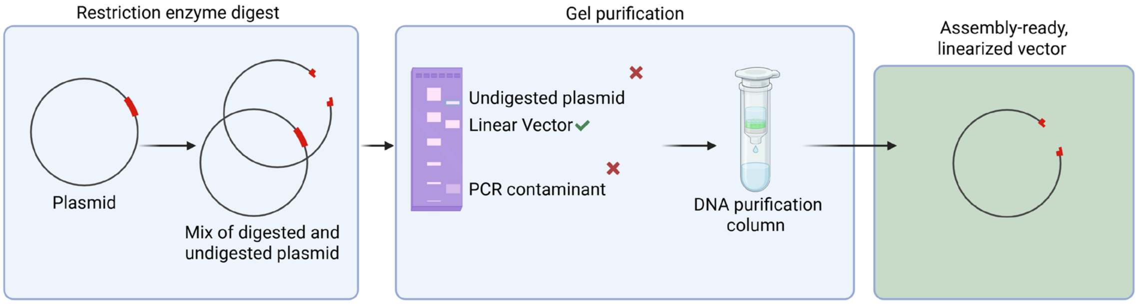
1. Digest with appropriate restriction enzyme(s) according to manufacturer's instructions
2. Treat with CIP (Calf Intestinal Phosphatase) to prevent self-ligation
3. Heat inactivate enzymes per manufacturer's recommendations
4. Gel purify linearized vector to remove uncut plasmid and contaminants
5. Quantify and assess purity before proceeding to assembly

Vector Preparation Workflows

Vectors for F1-X™ assembly can be prepared through multiple approaches, each with specific advantages depending on your source material and throughput requirements. Figure 3 shows a comparison of restriction enzyme digest and PCR options. Optional QC for vector includes transforming linear vector directly to cells to ensure no background colonies are present, and performing a vector-only F1-X™control to ensure no self-ligation.

Method	**Advantages**
PCR Amplification	• DpnI treatment eliminates template background • Enables overlap addition during amplification • Allows sequence modifications • Compatible with automation
Restriction Enzyme Digestion	• Cost-effective for high-throughput • Suitable for large vectors (>8 kb) • Scalable process • Preserves original vector sequence
Synthetic DNA Assembly	• Complete sequence control and optimization • No existing plasmid template required • Eliminates PCR artifacts • Vendor normalization saves QC time

Best practices: preparing vector via restriction digest



Best practices: preparing vector via PCR

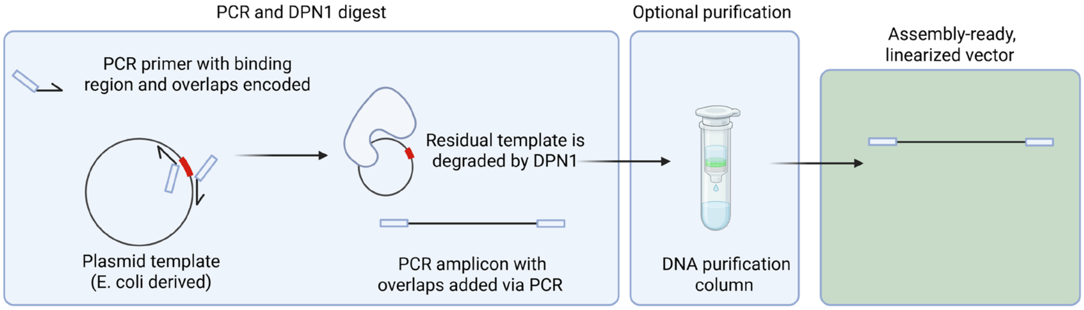


Figure 3. Vector Preparation Workflow Examples - Flowchart showing example workflows for vector preparation from plasmid DNA, including restriction enzyme digest or PCR amplification approaches.

Homologous Overlap Design Guidelines

General Design Principles

- **Recommended starting point:** 40 bp overlaps for all assemblies
- **Minimum overlap:** 20 bp (acceptable for simple 2-3 fragment assemblies)
- **Complex assemblies:** Use 40+ bp overlaps for optimal efficiency
- **Avoid:** Highly repetitive sequences, extremely low/high GC content
- **Ensure:** Overlaps are unique within the construct

Suggested Overlap Lengths by Fragment Size and Number

2-3 Fragment Assemblies

Fragment Size	Recommended Overlap
0.1-2 kb	20-40 bp
2-10 kb	30-40 bp
10-32 kb	40+ bp

4-12 Fragment Assemblies

Fragment Size	Recommended Overlap
0.1-5 kb	40 bp
5-10 kb	40-60 bp
10-32 kb	60-80 bp

Vector-Specific Recommendations

Vector Size	Overlap Length	Example
2-5 kb	30-40 bp	pUC19, pBR322
5-8 kb	40 bp	BAC vector
8-15 kb	40 bp	Lentiviral vector

Design Tools

Figure 4 shows a visual depiction of how overlaps for Gibson Assembly work. For complex constructs, consider using the **DNA Fragment Splitter for Gibson Assembly** tool, which provides:

- Automatic fragment splitting with optimized overlaps
- Synthesis-ready FASTA exports
- Built-in QC and annotations
- Expert-guided feedback in the interface

How Overlaps Work: Visual Example

Original sequence at junction (40 bp overlap in CAPS):

```
[...]gggcctcttcgctattacgccagctggcgaaagggggatgtgctgcaaggcgattaagGCACGCATC
TGGAATAAGGAAGTGCCATTCCGCCTGACCTgaagatcctttgatctcacgttgtgtctcaaaatctc[...]
```

Fragment 1: Left piece + Overlap (via PCR or synthesis)

```
[...]gggcctcttcgctattacgccagctggcgaaagggggatgtgctgcaaggcgattaagGCACGCAT
CTGGAATAAGGAAGTGCCATTCCGCCTGACCT
```

Fragment 2: Overlap + Right piece (via PCR or synthesis)

```
GCACGCATCTGGAATAAGGAAGTGCCATTCCGCCTGACCTgaagatcctttgatctcacgttgtgtctcaa
aatctc[...]
```

After Gibson Assembly® reaction:

```
[...]gggcctcttcgctattacgccagctggcgaaagggggatgtgctgcaaggcgattaagGCACGCAT
CTGGAATAAGGAAGTGCCATTCCGCCTGACCTgaagatcctttgatctcacgttgtgtctcaaaatctc[
...]
```

Color key: 🟦 Left fragment 🟩 40 bp overlap region 🟧 Right fragment

The Gibson Assembly® HiFi master mix enzyme blend chews back the 5' ends, allowing the complementary overlap regions to anneal, then extends and ligates to create a seamless junction.

Figure 4. Example of a 40 bp homologous overlap (shown in CAPS) used to join two adjacent DNA fragments. The F1-X™ Gibson Assembly reaction chews back the 5' ends to expose complementary regions, allowing fragments to anneal, extend, and ligate into a seamless construct. All sequences are shown from 5' to 3'.

F1-X™ Assembly Protocol

General Guidelines

- Keep F1-X™ Master Mix (2×) on ice at all times. Assemble reactions on ice
- Include appropriate controls
- Follow DNA QC procedures precisely

Calculating DNA Amounts and Ratios

The reaction setup depends on fragment number. DNA mixtures should be prepared at >2x of the final target concentration.

Target Ratios and Amounts (final amounts in 10 µL reaction):

Simple Assemblies (2-3 fragments)	**Complex Assemblies (4-12 fragments)**
Total DNA: 0.03-0.2 pmols	**Total DNA:** 0.2-0.4 pmols
Vector amount: 25-50 ng	**Per fragment:** 0.02-0.04 pmol
Molar ratio*: Vector:Insert = 1:2	**Molar ratio*:** Equimolar (all pieces)

** For fragments ≤100 bp, use 5× molar excess.*

Quick Reference Table

Size	ng of DNA	pmol of DNA
0.5 kb	20 ng / 40 ng	0.061 / 0.121
1 kb	10 ng / 25 ng	0.015 / 0.038
5 kb	10 ng / 25 ng	0.003 / 0.008
8 kb	25 ng / 50 ng	0.005 / 0.009
10 kb	25 ng / 50 ng	0.004 / 0.008
20 kb	50 ng / 100 ng	0.004 / 0.008
30 kb	50 ng / 100 ng	0.003 / 0.005

To determine the pmols or ngs of DNA:

pmol = [ng DNA/(660 × # of bases)] × 1000

ng = [pmol DNA × (660 × # of bases)]/1000

Controls

- ✓ F1-X™ Positive Control or DNA from a previously verified assembly (Appendix A)
- ✓ Negative Control: Vector only (no inserts) to assess background
- ✓ No-assembly Control: DNA fragments without F1-X™ Master Mix for gel analysis reference

Assembly Procedure

Materials

- F1-X™ Master Mix (2×) - store at -20°C
- DNA fragments with overlaps
- Nuclease-free water
- Thermocycler

Protocol

1. **Thaw** F1-X™ Master Mix (2×) on ice
2. **Prepare DNA fragments** by mixing in nuclease-free water according to calculated ratios
3. **Vortex** Master Mix before use
4. **Combine on ice:**

Component	Volume
F1-X™ Master Mix (2×)	5 µL
DNA Mix	X µL
Nuclease-free water	5-X µL
Total	**10 µL**

For Positive Control: add 5 µL F1-X™ Positive Control (2×) to 5 µL F1-X™ Master Mix (2×)

5. **Mix** well and spin down briefly
6. **Incubate and assemble**:
   - Simple assemblies (2-3 fragments): 50°C for 15 minutes
   - Complex assemblies (4-12 fragments): 50°C for 60 minutes
7. **Store** at -20°C or use immediately for transformation/ downstream applications
8. **Optional post-assembly analysis**: run 5-7 µL on an agarose gel to verify assembly. Include no-assembly control for reference, as assembled DNA may appear faint.

F1-X™ Transformation Protocols

Transformation Guidelines

Optimization of dilution factor and amount of cloning reaction to transform is essential for achieving robust colony counts. The suggestions below are guidelines for standard chemically and electrocompetent cell workflows.

Format	Volume and type of cells	Volume of F1-X™cloning reaction	Optimization parameters
Microcentrifuge tube	50 µL chemically competent cells	2.5-4.0 µL	• 3-5 fold dilution in NFW before transformation
96-well plate	20 µL chemically competent cells	1.0-2.0 µL	• 3-5 fold dilution in NFW before transformation
Microcentrifuge tube	30 µL electrocompetent cells	2.0 µL of diluted reaction (3-fold dilution)	• 3-10 fold dilution in NFW • Column-based desalting

Competent Cell Selection

Recommended efficiency: ≥1×10⁹ CFU/µg pUC19

- Generally, chemically competent cells are sufficiently efficient to produce colonies from cloning reactions if design and DNA quality are robust. For low efficiency or complex cloning, consider higher efficiency electrocompetent cells
- Large constructs (>10 kb): High-efficiency electrocompetent cells preferred, choose strain based on whether high density repeats or toxic elements are present

Chemically Competent Transformation Protocol

Guidelines for using DH5α™, NEB® 5-alpha in 96-well plate format are shown below. Always refer to competent cell manufacturer guidelines for more details. If using 50 µL of competent cells in microcentrifuge times, transform 2.5 µL of the cloning reaction.

1. Add 20 µL competent cells to pre-chilled tubes or deep 96 well plate on ice
2. Add 1 µL of F1-X™ assembly reaction (neat) to 20 µL cells and mix gently on ice
3. Incubate on ice for 30 minutes
4. Heat shock at 42°C for 30 seconds
5. Return to ice for 2 minutes
6. Add 200 µL SOC medium; incubate 37°C for 1 hour (with shaking for high efficiency)
7. Plate cells using patch plate, streak, or beads/ spreaders as needed. See Plating Guidelines section for more information

Electrocompetent Transformation Protocol

Guidelines for using TransforMax™ EPI300™ or equivalent is shown below. Always refer to competent cell manufacturer guidelines for details.

1. Dilute F1-X™ reaction 3-5 fold in ice-cold water
2. Add 2 µL diluted reaction to 30 µL cells on ice; mix gently
3. Transfer to pre-chilled electroporation cuvette
4. Electroporate: 1200V, 25µF, 200Ω (0.1 cm cuvette)
5. Immediately add 950 µL SOC to cells for recovery; incubate 37°C for 1 hour with shaking
6. Plate as above

Plating Guidelines

Traditional plating

Transformation Efficiency	Number of Fragments	Suggested Plating Percentage Range*†	Expected Colonies†
> 1×10^9 CFU/µg pUC19	1-5	1%-10%	>100
> 1×10^9 CFU/ µg pUC19	6-12	1%-100%‡	>100
> 1×10^8 CFU/ug pUC19	1-5	10% µL -100%‡	>100
> 1×10^8 CFU/ µg pUC19	6-12	10%-100%‡	>100

*Conservative estimates based on the guidelines above where percentage refers to % of culture rescue. For example, 1%-10% of a 200 µL transformation rescue refers to 2 µL - 200 µL plated. †Colony counts are estimates and will vary based on assembly complexity, efficiency, DNA quality, and design. ‡Centrifuge briefly and remove supernatant before streaking the pellet from a µL-scale culture

Patch Plating Protocol for High-Throughput Workflows

Since the goal of transformation in cloning is typically to obtain individual colonies for sequencing, plating serial dilutions of transformation culture helps determine optimal plating volumes before sequencing while conserving reagents.

1. In a 96-well plate, dilute each rescue culture 1:10 and 1:100 in SOC
2. Using a multichannel pipette, gently patch 2.5-5 µL of each dilution onto selective agar plates, keeping droplets as individual patches
3. Incubate plates overnight at the appropriate temperature (typically 37°C) and store transformation cultures at 4°C
4. Identify conditions with individual colonies to calculate CFU per µL, then use optimal culture volume for sequencing plates. Note that transformation cultures in SOC can be stored at 4°C overnight while patch plates are grown

Colony Screening and Verification

Colonies can be pre-screened for full length assembly product via colony PCR, miniprep/ digest, or screened directly from the agar plate depending on the desired workflow.

Colony Selection Guidelines (Full-Length Constructs to Screen)

Assembly Complexity	Colonies to Screen
Simple (2-3 fragments)	2-8 colonies
Complex (4-12 fragments)	5-10 colonies

Note: Requirements are sequence dependent. Factors include fragment sizes, fragment error rates, GC content, DNA quality, overlap length, and presence of toxic sequences. Pre-screening colonies with colony PCR or miniprep and restriction digest is recommended for complex assemblies. If sequence perfect clones are not found after screening plenty of colonies, sequence the DNA material input to the F1-X™reaction to help narrow down the root cause.

Appendices

Appendix A: Protocol and Expected Results from F1-X™ Positive Control (2×)

Each F1-X™ kit includes a pre-mixed Positive Control DNA (2×) to verify proper assembly conditions and transformation efficiency.

Positive Control Composition

The Positive Control DNA (2×) contains a 4-fragment assembly designed to produce a functional GFP expression construct in a pUC vector. The mixture includes three insert fragments of ~0.7 kb and one linearized pUC19 vector fragment (2.7 kb), present at approximately equimolar ratios (1:1:1:1). All fragments contain 40 bp homology regions for seamless assembly. Upon successful assembly, the fragments combine to form an approximately 4.6 kb circular plasmid containing an intact GFP gene under constitutive expression, which produces green fluorescent colonies upon transformation.

Protocol

1. Assembly Reaction Setup

Prepare a 10 µL reaction as follows:

Component	Volume
F1-X™ Positive Control DNA (2×)	5 µL
F1-X™ Master Mix (2×)	5 µL
Total Volume	**10 µL**

2. Mix gently and incubate at 50°C for 15 - 60 minutes.

3. Transform according to guidelines above alongside experimental samples.

Expected Results (refer to Figure 5)

- **Colony count:** >100 colonies per transformation
- **Appearance:** Green fluorescent colonies visible under ambient light
- **Efficiency:** >75% of colonies should be green

Note: If fluorescence is difficult to visualize under room light, illuminate plates with blue light or use colony count as the primary success metric.

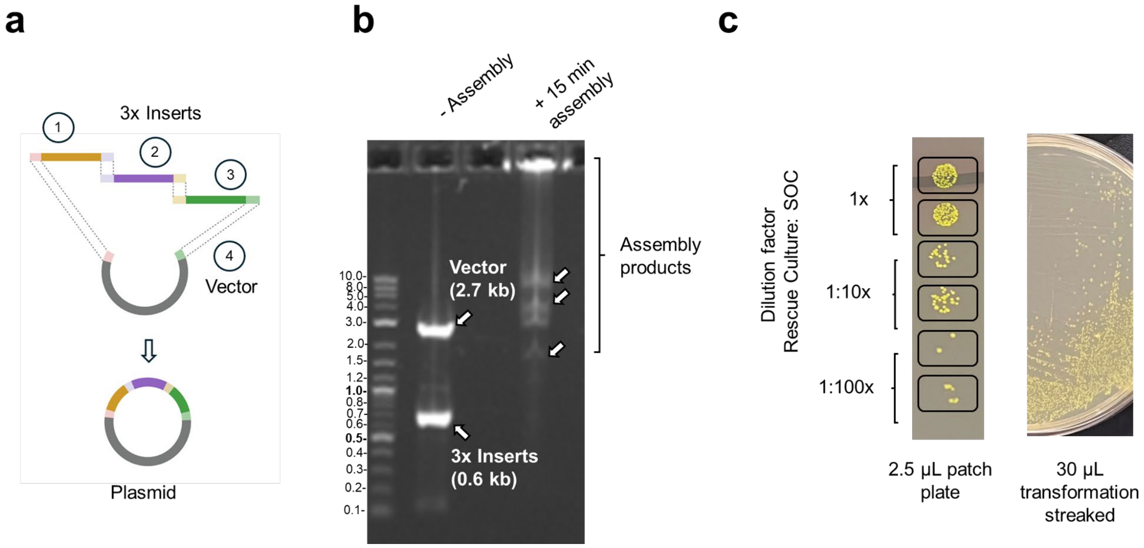


Figure 5. ***(a)*** *Schematic of the 4-fragment assembly. Three insert fragments (1, 2, 3) and one linearized vector fragment (4) are assembled into a circular plasmid containing an intact GFP gene.* ***(b)*** *Agarose gel electrophoresis showing assembly products. Without F1-X™ (left lane), distinct bands are visible for the vector (2.7 kb) and three insert fragments (~0.6 kb each). With F1-X™ assembly at 50°C for 15 minutes (right lane), high molecular weight products (~4 kb) are observed.* ***(c)*** *Transformation results showing green fluorescent colonies. Serial dilutions from rescue culture (left) demonstrate efficient assembly, with visible green colonies at dilutions up to 1:100. A standard 30 µL transformation (right) produces a lawn of green colonies, confirming >50 colonies with >90% assembly efficiency.*

Appendix B: Seamless Removal of Restriction Enzyme Seams with the Gibson Assembly® Chemistry

Gibson Assembly chemistry can eliminate restriction enzyme seams during assembly. The 5′ exonuclease activity removes restriction site overhangs, while polymerase and ligase activities create seamless junctions between fragments with homologous overlaps.

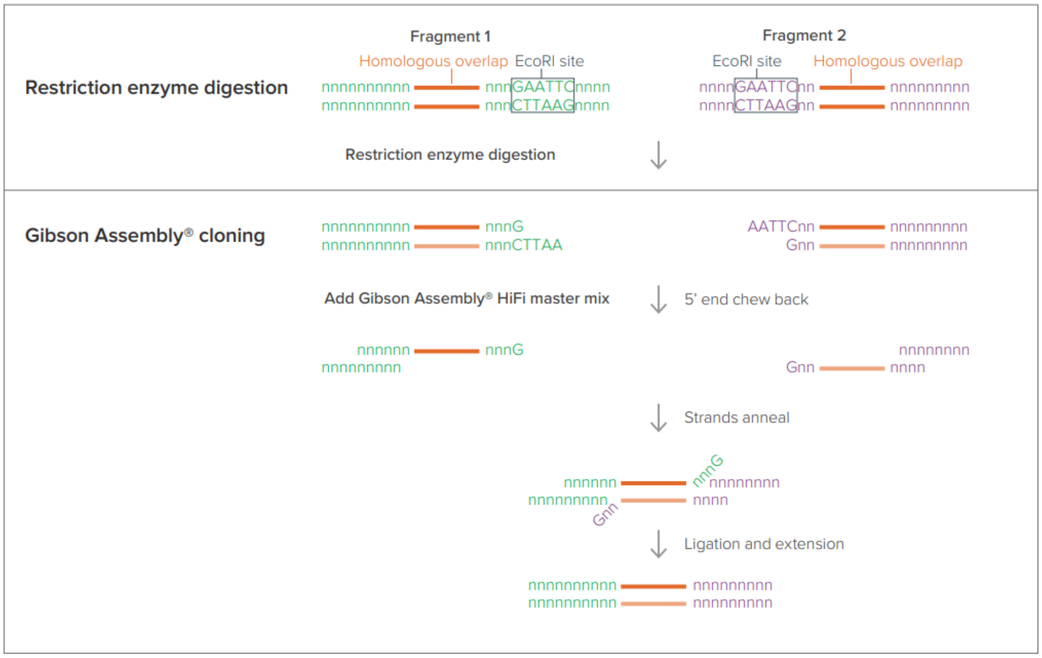


Figure 6. Elimination of EcoRI Seam During Gibson Assembly. Diagram depicting two fragments with homologous overlap regions (shown in orange) and an external EcoRI site. Following restriction enzyme digestion with EcoRI, the fragments may be assembled using the Gibson Assembly reaction. The restriction enzyme digestion seam is eliminated by the 5′ chew back and ligation activities of the F1-X™ Master Mix.

Troubleshooting Guide

Common Assembly Issues

Issue	Likely Cause	Solution
No colonies from positive control	Competent cell issue	• Use high-efficiency cells (≥10^9 CFU/µg) • Handle cells carefully (no vortex, keep cold) •Add a plasmid control to transformation
	Improper F1-X™ handling	• Reduce freeze-thaw cycles • Thaw on ice • Vortex 15 sec before use
	Selection issue	• Confirm antibiotic concentration • Check plate freshness
No colonies from experimental samples	Primer design issue leads to bad overlaps	• Verify overlap length (~40 bp) • Check for secondary structures • Confirm primer specificity • Run assembly on gel to confirm change in molecular weight (if not, re design)
	Low assembly efficiency	• Column purify PCR products • Check DNA quality (concentration, purity) • Verify fragment integrity by gel and gel extract if needed
	DNA molar ratios are off	• Use Qubit™ for quantification • Check A260/280 ratio (≥1.8) • Remove contaminants and repeat
High vector background	Background template from vector prep	• Treat with DpnI for PCR vectors • Gel purify to remove uncut plasmid • Consider CIP treatment • Transform vector directly to cells (no assembly) to gauge where the background is coming from (carryover from prep vs. self-ligation)
	Self ligation of vector	• Use double digest for restriction vectors • Increase overlap length (>20 bp, target 40 bp)
Incorrect assemblies	Input DNA errors	• If suspected, sequence fragments before assembly • Use high-fidelity polymerase and constrain PCR cycles
	Assembly conditions	• Increase overlap length (40+ bp) • Raise reaction temperature to 55°C • Extend incubation time • Ensure molar ratios are correct
	Recombination in bacterial host	• Run a no-Master Mix control with DNA fragments and ensure that no colonies appear. If unwanted recombination is an issue, split assembly into 3+ DNA pieces or reduce overlap length to <25 bp
Low colony numbers	Transformation efficiency	• Use fresh, high-efficiency cells • Optimize dilution factor of F1-X™• Column purify assembly reaction to desalt before transformation • Try electroporation
	Insert toxicity	• Use low-copy vector • Reduce incubation temperature (30° C) • Try different E. coli strains
Inconsistent results	Reaction setup	• Ensure that reaction setup is happening on ice; even short room temperature incubations are enough to get inconsistent results • Ensure thermal cycler has reached the intended temperature before putting reactions in • Avoid pipetting ultra small volumes
	Equipment calibration	• Ensure thermal cycler temperature is correct or run a temperature gradient

Common DNA Preparation Issues

Issue	Likely Cause	Solution
Multiple PCR bands	Non-specific amplification	• Optimize annealing temperature • Redesign primers • Use touchdown PCR
Low PCR yield	Poor primer design	• Check for secondary structures • Optimize Mg^{2+} concentration • Increase primer concentration
Gel smearing	DNA degradation	• Use fresh reagents • Check for nuclease contamination • Keep samples cold • Purify samples before storage in TE buffer • Heat kill enzymes used in preps
Low A260/230 ratio	Organic contamination	• Re-purify with cleanup kit • Use ethanol precipitation • Check buffer carryover

Optimization Strategies

For Difficult Assemblies

- Increase reaction temperature to 55-56°C for higher stringency and extend incubation time up to 1-2 hours
- Use longer overlaps (60-80 bp for complex assemblies)
- Consider two-stage assembly for >12 fragments
- Consider whether the comp cell strain being used is appropriate

For Low Efficiency

- Verify all fragments are full-length by gel
- Use higher DNA concentrations within recommended range
- Ensure proper molar ratios (equimolar for complex assemblies)
- Column purify all DNA fragments before assembly
- Column purify assembly reaction to desalt before transformation
- Consider electroporation vs. heat shock

For Repetitive Sequences

- Design overlaps to avoid repetitive regions
- Use longer overlaps to increase uniqueness
- Consider fragments with repetitive sequences internalized
- Perform sequential assembly stages

FAQ: General Questions

What are the advantages of Gibson Assembly®?

- 1-Step, seamless cloning without restriction site dependence
- Scarless junctions with no unwanted sequences
- Versatile for genes, plasmids, pathways, and whole genomes
- Direct use for transformation, PCR, or rolling circle amplification

Can I amplify the assembled product directly? Yes. The covalently joined DNA can be used as a PCR template or for rolling circle amplification without purification. As a starting point, we recommend testing 1 µL of F1-X™ reaction in a 20 µL PCR or rolling circle amplification, reaction following manufacturer guidelines for reaction setup.

Can I combine ssDNA oligonucleotides with dsDNA fragments? Yes. Use 45 nM as starting concentration for each oligonucleotide. Oligonucleotides >90 bases may have interfering secondary structures.

Can I assemble linear fragments without a vector? Yes. F1-X™ can assemble multiple linear fragments into complete plasmids, including entire constructs from synthetic DNA. Note that the orientation of overlap design dictates the final construct sequence.

How large can assembled fragments be? For individual fragments: up to 32 kb. For total construct size: up to 100 kb. Larger constructs are possible with multi-stage assembly.

How many fragments can I assemble? While we have verified up to 14 fragments in a single assembly, for robust results, use up to 12 fragments recommended for single-stage assembly. It is critical that overlaps are 40 bp or more for multi-fragment assembles. More fragments may be possible with multi-stage approaches.

Will this work with repetitive sequences? Yes. Design fragments with repetitive sequences internalized rather than at overlap regions, or use longer overlaps for uniqueness.

Can small fragments (≤200 bp) be assembled? Yes. F1-X™ handles fragments as small as 100 bp. Use ≥5-fold molar excess for optimal results.

Do I need to use PCR for vector preparation? No. Vectors can be linearized by restriction digest. All end types (blunt, 5′ overhang, 3′ overhang) are compatible. Vectors can also be prepared synthetically as gene blocks or fragments.

What are the shortest/longest overlaps I can use? We recommend 40 bp as a starting point for multi-fragment assembles . For simple assemblies of 1-3 fragments, use as little as 20 bp overlaps. For complex constructs of 4+ fragments, use 40+ bp.

How should I store the kit? Store at -20°C in stable temperature location (not frost-free freezer). Limit to 5 freeze-thaw cycles or aliquot for frequent use.

What DNA concentrations should I use? Use the amounts specified in the protocol section. Lower concentrations can be tested if materials are limiting.

Can I use different incubation times? Use a minimum 15 minutes for simple assemblies; for more complex assemblies, longer times (1 hour) are acceptable. Follow protocol recommendations for consistency.

Can I use different incubation temperatures? We recommend 50°C as a starting point for all assembly reactions. F1-X™ contains a proprietary blend of high-fidelity enzymes that are active between 50°C-56°C. If your construct requires additional optimization for enhanced annealing stringency, run the assembly at 53°C-56°C and extend the reaction time between 1-2 hours.

Do I need to inactivate restriction enzymes used to prep my DNA? Yes. Residual restriction enzyme can cause star activity and DNA degradation.

Is gel purification necessary? It is not required if PCR yields >80% full-length product. Low molecular weight contaminants caused by off-target amplification in PCR have a detrimental effect on cloning efficiency.

What sequencing protocol is recommended? Sequence entire insert plus ~500 bp of vector at junctions. If PCR was used for vector preparation, consider sequencing entire construct using NGS or Sanger sequencing. We recommend consulting with your sequencing vendor to find the right workflow for your needs.

I don’t see full-length product when I visualize assemblies via agarose gel electrophoresis. Is something wrong? No, this is normal. Agarose gel electrophoresis is a qualitative, construct-dependent indicator of assembly success. Gibson Assembly reactions often produce high molecular weight species that appear as laddering rather than discrete bands. Compare your assembly reaction to a no-assembly control and look for reduced substrate bands and higher molecular weight products. For definitive confirmation of assembly success, use transformation as your primary success metric since colony count and sequencing verification provide definitive confirmation of assembly success.