Technical Application Note

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One-Shot Assembly of a 14-Fragment Lycopene Biosynthesis Pathway Using F1-X™ Next-Gen Gibson Assembly

Racer Biosciences

Summary

This application note demonstrates successful 14-fragment assembly of a complete metabolic pathway using F1-X™ Next-Gen Gibson Assembly with standard laboratory techniques:

- **Single-step workflow:** Same protocol used for routine 2-3 fragment assemblies—no staged assemblies or specialized optimization required
- **Standard laboratory equipment:** Thermal cycler and chemically competent cells—no electroporation or specialized instruments
- **De novo construct synthesis:** Entire 9 kb plasmid assembled from synthetic fragments, eliminating vector isolation and preparation steps
- **Broad assembly range:** F1-X™ specified for 2-12 fragments, with demonstrated capability extending to 14+ fragments depending on construct complexity

Few commercially available 1-step Gibson Assembly kits have demonstrated reliable performance above 6-8 fragments in a single reaction, making F1-X™ a versatile solution for both routine cloning and complex pathway assemblies.

Background

Gibson Assembly, with tens of thousands of citations, remains the preferred method for seamless DNA assembly due to its simplicity and reliability. As synthetic DNA costs continue to decline, researchers increasingly leverage multi-fragment assembly strategies for complex pathway construction. However, synthetic DNA providers face fundamental limitations—the most robust range for single-fragment gene synthesis is typically under 2 kb. While some providers offer synthesis up to 5 kb, this often comes at the cost of reduced yield, lower sequence fidelity, and extended turnaround times (10-15 days versus 5 days for shorter fragments). For constructs exceeding these practical limits, or when assembling multiple genetic elements into a single plasmid, multi-fragment assembly becomes not just convenient, but necessary.

This application note demonstrates F1-X Next-Gen Gibson Assembly performance in a 14-fragment assembly of the lycopene biosynthesis pathway—a complete metabolic pathway synthesized entirely de novo without vector preparation. **F1-X is specified for assemblies of 2-12 fragments, with demonstrated performance up to 14+ fragments depending on**

construct complexity. This stress test validates the robustness and headroom built into the F1-X formulation, showing that F1-X Next-Gen Gibson Assembly can handle complex multi-fragment assemblies that would be impossible through traditional single-fragment synthesis—all while using the same simple workflow as routine 2-fragment cloning. **The study demonstrates that a complete plasmid can be built from concept to sequence-confirmation in under one week without vector isolation, restriction digests, PCR amplification, gel purification, column cleanup, or Sanger sequencing (Fig. 1).**

Workflow and Timeline

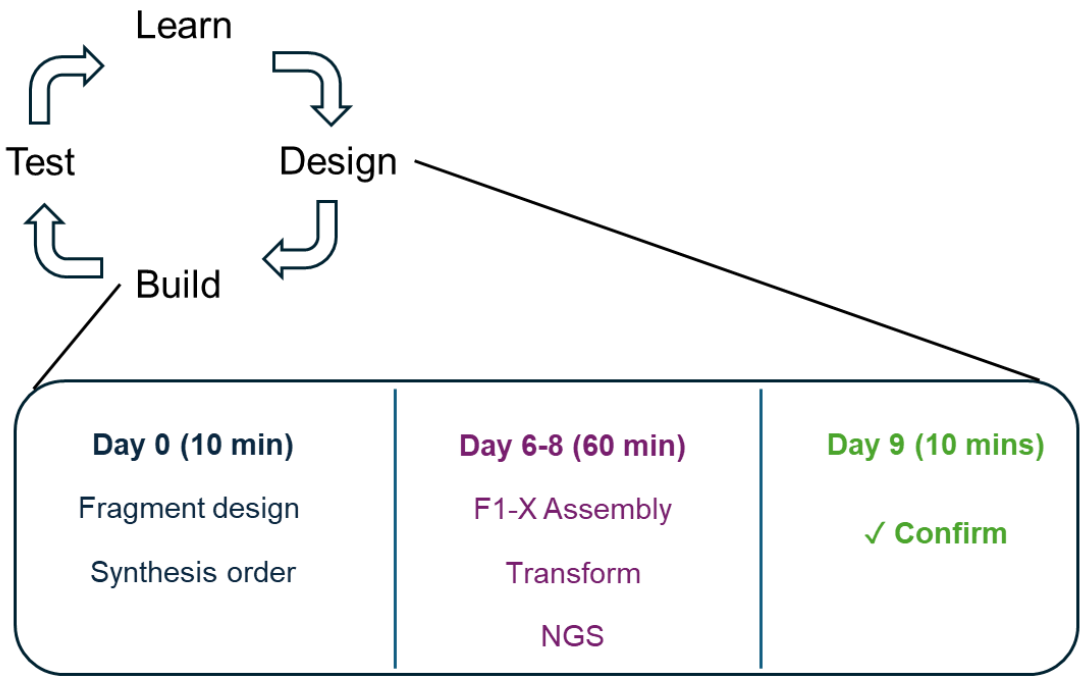

Figure 1. End-to-end workflow for 14-fragment plasmid assembly using F1-X Next-Gen Gibson Assembly. The entire process from initial design to sequence-confirmed clone requires ~80 minutes of hands-on time over 9 days. The DBTL (Design-Build-Test-Learn) cycle illustrates the iterative nature of synthetic biology workflows. The timeline shows three distinct phases with minimal hands-on intervention. Note that the majority of elapsed time is DNA synthesis turnaround and passive incubations. No special techniques required—standard chemically competent cells, heat shock transformation, and basic lab equipment only.

Methods and Results

Construct Design

The lycopene biosynthesis plasmid pAC-LYCipi (Addgene Plasmid #53279; 8,913 bp) was selected as a test case for one-shot, multi-fragment assembly. This construct exceeds the practical size limit for single-fragment gene synthesis and contains multiple genetic elements (promoters, genes, terminators) that benefit from modular assembly approaches.

The complete plasmid was divided into 14 synthetic fragments using our automated fragment splitter algorithm. The tool was run in **self-circularization mode** with 40 bp overlaps, which generates fragments with overlaps designed to circularize the final construct without requiring a separate vector backbone. The FASTA output from the splitter tool was used directly to order fragments from Twist Biosciences via their online ordering portal. The accompanying GenBank (.gb) output file, which contains the complete annotated sequence with all 14 fragment boundaries marked, was used to verify design completeness and confirm the circular topology of the intended final construct. **The fragment splitter tool is free and available on the Racer Biosciences website.** The design workflow is highlighted in Fig. 2.

Figure 2. Automated fragment design for circular plasmid assembly. The fragment splitter tool automatically divides the 9 kb pAC-LYCipi plasmid into 14 optimally-sized fragments with 40 bp overlaps for Gibson Assembly. A size of ~700 bp sub-fragments was chosen to be within the optimal range of DNA synthesis providers, and to enable testing of >10 fragment assembly in F1-X. Fragment boundaries are calculated to maintain GC content balance (40-60%). The tool outputs synthesis-ready FASTA files that can be submitted directly to DNA synthesis providers, along with an annotated GenBank file for record-keeping.

DNA QC and Assembly

Dried DNA fragments (Twist Biosciences; 5-day turnaround) were resuspended in nuclease-free water, then analyzed via absorbance and agarose gel electrophoresis for purity and concentration. All 14 fragments were present at full length as confirmed by gel electrophoresis, with 1 fragment showing low molecular weight contaminants (Fig. 3a).

An equimolar mixture of all fragments was prepared at 2× concentration (~0.02 pmol per piece, for ~120 ng of total DNA in the reaction). Gibson Assembly was performed by mixing 5 µL of the fragment mixture with 5 µL of F1-X Master Mix (2×) on ice. The reaction was briefly vortexed and incubated at 50°C for 60 minutes. Following assembly, 5 µL of the reaction was analyzed by E-gel (Thermo Fisher) to confirm formation of high-molecular-weight assembly products (Fig. 3b).

Transformation and Sequencing

One microliter of the assembly reaction was mixed with 20 µL of high-efficiency chemically competent DH5α cells (NEB) and incubated on ice for 30 minutes. Heat shock at 42°C for 30 seconds was followed by 2-minute re-incubation on ice. 200 µL of SOC medium was added, followed by recovery at 37°C for 1 hour with shaking. The entire recovery culture was plated on LB agar with 34 µg/mL chloramphenicol and incubated for 12 hours at 30°C.

Plates were then moved to darkness at room temperature for lycopene synthesis and pigment development. Functional clones produced visible red/pink pigmentation within 48 hours (Fig. 3c). For sequence confirmation, lycopene-producing colonies were screened using next-generation sequencing (Oxford Nanopore platform; sequencing service provider: Eton Biosciences). Of 11 colonies sequenced, 50% assembled to full length with various junction errors, and 20% were error-free across all 14 junctions (Fig. 3d).

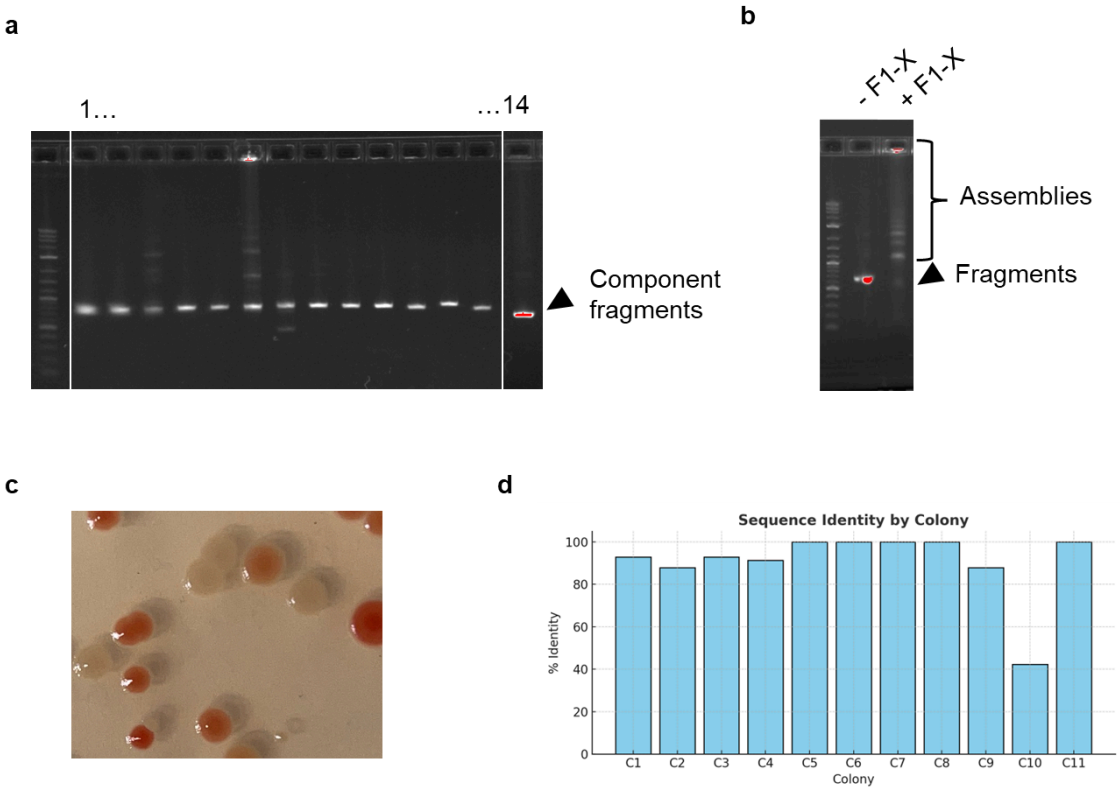

Figure 3. One-shot assembly of 9 kb plasmid from 14 synthetic fragments using F1-X™ Next-Gen Gibson Assembly. (a) Fragment QC: Agarose gel analysis confirms all 14 synthetic fragments are present at expected sizes with high purity. Ladder is 1 kb+ (NEB). (b) Post-assembly analysis: E-gel shows formation of high-molecular-weight products consistent with covalent assembly, with minimal remaining unassembled fragments. (c) Functional validation: Transformation plates showing lycopene-producing colonies (red/pink pigmentation) after 48-72 hours of pigment development. White colonies indicate assembly errors or non-functional variants. Transformation efficiency: ~15-30 CFU per plate; functional colony rate: ~15-30%. (d) Sequence verification: Next-generation sequencing analysis (Oxford Nanopore) of 11 colonies. Results show 50% full-length assemblies and 20% error-free clones (2/11 colonies with perfect sequence across all 14 junctions).

Discussion

Why Multi-Fragment Assembly Matters

Multi-fragment assembly strategies are essential for several common research applications. Metabolic pathway engineering often requires assembling multiple genes with associated regulatory elements into a single expression vector, as demonstrated here with the lycopene biosynthesis pathway. Large genomic constructs for CRISPR applications, BAC assembly, or synthetic chromosome engineering frequently exceed practical synthesis limits. Additionally, modular combinatorial cloning—where researchers need to rapidly test different promoter-gene-terminator combinations—benefits significantly from fragment-based approaches where individual elements can be swapped by reordering specific fragments rather than resynthesizing entire constructs.

The de novo synthesis approach demonstrated here eliminates vector preparation entirely. Traditional cloning workflows require isolating and preparing a vector backbone, which introduces additional steps: minipreps, restriction digests, and potential complications with low-copy or unstable plasmids. By synthesizing all components as fragments, including what would traditionally be the "vector backbone," researchers can bypass these preparatory steps and proceed directly from sequence design to assembly. This is particularly advantageous when the target vector itself contains repetitive sequences, secondary structures, or other features that complicate traditional cloning approaches.

Pushing the Boundaries of Gibson Assembly

This 14-fragment assembly represents a significant stress test for any 1-step Gibson Assembly master mix. While the absolute sequence fidelity (20% error-free) is lower than expected for simple 2-3 fragment assemblies, this result demonstrates that F1-X™ Next-Gen Gibson Assembly can successfully operate beyond typical specifications using only routine laboratory equipment. To our knowledge, few commercially available Gibson Assembly kits have demonstrated reliable performance above 6-8 fragments in a single reaction.

It should be noted that electroporation typically yields transformation efficiencies up to 2 orders of magnitude higher than chemical competence, especially for larger DNA constructs, and could produce thousands of colonies for more comprehensive fidelity analysis. However, this work was conducted using chemically competent cells, a thermal cycler, and standard laboratory equipment to demonstrate F1-X™ performance under routine cloning conditions without specialized protocols or instrumentation.

> **F1-X™ is specified for assemblies of 2-12 fragments, with demonstrated capability up to 14+ fragments.** However, performance at high fragment counts is construct-dependent and relies heavily on three critical factors: (1) high-quality fragment integrity (confirmed by gel electrophoresis or equivalent QC), (2) accurate equimolar ratios of all fragments, and (3) adherence to the F1-X user guide protocols.

> When these conditions are met, F1-X™ Next-Gen Gibson Assembly provides robust performance across a wide range of assembly complexities using standard molecular biology techniques.

These results validate F1-X™ Next-Gen Gibson Assembly as a versatile master mix capable of handling both routine cloning and complex multi-fragment assemblies with the same workflow. Users can confidently keep F1-X™ in their freezer knowing it will perform reliably for everyday 2-4 fragment joins while maintaining proven capacity for assemblies up to 12 fragments as specified, with headroom beyond when needed.

The Modern Cloning Landscape

The declining cost of synthetic DNA fundamentally changes how we approach molecular cloning. Strategies that were cost-prohibitive five years ago—synthesizing entire vectors de novo, ordering dozens of variants, avoiding all PCR and subcloning steps—are now routine. Combined with F1-X Next-Gen Gibson Assembly reliability and next-generation sequencing platforms (e.g., Oxford Nanopore plate-based methods that require no primer design), complex pathway assembly is faster and more accessible than ever.

The workflow demonstrated here is remarkably straightforward: design → synthesis → one-tube assembly → standard heat shock transformation → sequencing. No special equipment, no electroporation, no gel purification, no column cleanup. Just ~80 minutes of hands-on time and standard molecular biology techniques. Traditional approaches involving vector isolation, restriction digests, PCR amplification, gel purification, and Sanger sequencing would require significantly more time, effort, and troubleshooting.

Conclusions

F1-X Next-Gen Gibson Assembly Master Mix demonstrates robust performance across the full spectrum of assembly complexity—from standard 2-3 fragment cloning to assemblies of 2-12 fragments (product specification) and beyond. This 14-fragment stress test pushes the boundaries of current Gibson Assembly technology and validates the headroom engineered into F1-X, all while using the same single-step workflow and routine laboratory equipment as standard cloning.

Success in multi-fragment assembly depends on fragment quality, equimolar ratios, and protocol adherence. When these fundamentals are maintained, F1-X Next-Gen Gibson Assembly delivers reliable performance that eliminates the need for multiple specialized kits, vector preparation workflows, or specialized equipment.

For researchers facing DNA synthesis size limitations or building complex multi-element constructs, F1-X Next-Gen Gibson Assembly provides a proven solution with demonstrated capability up to 14+ fragments (construct-dependent). The de novo synthesis approach eliminates traditional vector preparation headaches entirely. For labs performing primarily routine cloning with occasional complex assemblies, F1-X offers the confidence of a single, freezer-stable master mix that handles both scenarios effectively with the same simple protocol.

BUILD MORE WITH F1-X

One master mix. One workflow. Routine cloning to extreme assemblies.

References

[1] Cunningham FX Jr, Lee H, Gantt E. Carotenoid biosynthesis in the primitive red alga Cyanidioschyzon merolae. *Eukaryot Cell.* 2007 Mar;6(3):533-45. doi: 10.1128/EC.00265-06. PMID: 17085635.

Publication Date: October 2025

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Product Information: racerbio.com/collections/shop

Fragment Splitter Tool: racerbio.com/pages/bioinformatics-tools

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