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Utilizing both homology and oligonucleotide stitching techniques to build large constructs

High-efficiency cloning with the GeneArt Gibson Assembly HiFi Cloning Kit and GeneArt Strings DNA Fragments

Abstract

The Gibson Assembly® method is an established DNA assembly reaction that allows multiple overlapping DNA fragments to be seamlessly linked in a one-step, single-tube, isothermal reaction (Invitrogen™ GeneArt™ Gibson Assembly® HiFi Cloning Kit), or a two-step reaction in the case of the GeneArt™ Gibson Assembly® EX Cloning Kit. DNA fragments of different lengths are uniformly assembled using complementary overlaps between fragments. The inherent flexibility of this approach is suitable for small and large DNA constructs and includes both single and multiple inserts. Thermo Fisher Scientific offers two types of kits: the GeneArt Gibson Assembly HiFi Cloning Kit for assembly of up to 6 fragments and the GeneArt Gibson Assembly EX Cloning Kit for assembly of up to 15 fragments.

In this paper, we demonstrate how the GeneArt Gibson Assembly HiFi Cloning Kit can be used with multiple Invitrogen™ GeneArt™ Strings DNA Fragments to produce large constructs in highly efficient reactions. Also, we demonstrate how oligonucleotide stitching can be used to effectively create homologous regions between the insert and vector, thus allowing the use of previously generated DNA fragments.

Basis of the technology

The GeneArt Gibson Assembly HiFi Cloning Kit enables the one-step assembly of single or multiple DNA fragments into any desired vector (Figure 1). Through the concerted action of DNA exonuclease, polymerase, and ligase, fragments sharing short overlapping regions are efficiently joined without scars (seamlessly). After 15 to 60 minutes, isothermal reactions create the assembly product (a covalently bonded DNA construct) that can be directly transformed into chemically competent or electrocompetent cells [1-3].

The required homology regions can be designed into the fragments for *de novo* synthesis or added to an existing fragment by PCR amplification using custom oligonucleotides. Homology regions can be as short as 15 bp, but we recommend extending them to 20–40 bp in order to achieve the best performance.

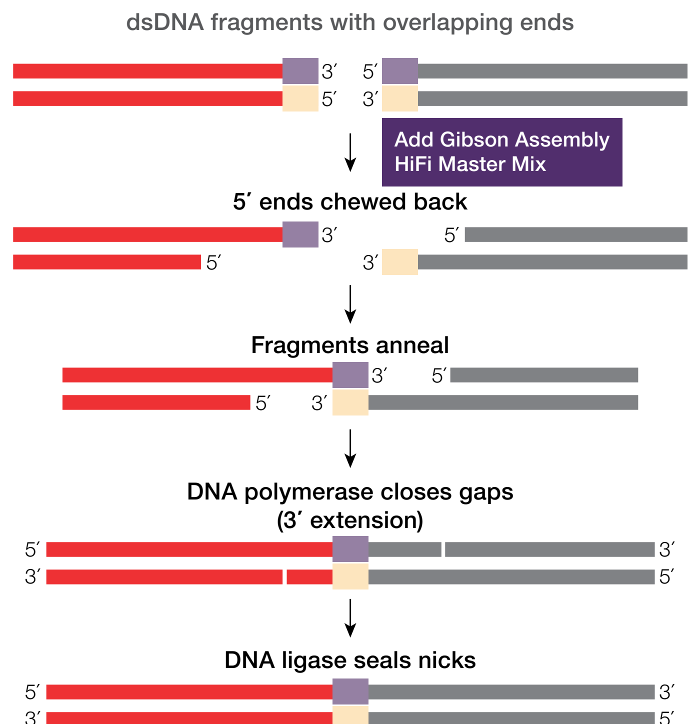

Figure 1. Overview of the reaction. The GeneArt Gibson Assembly HiFi Cloning Kit employs a one-step isothermal reaction that can join up to 6 inserts in any vector with high reliability. The combined action of DNA exonuclease, polymerase, and ligase creates a scar-free, covalently bound product that can be efficiently transformed into chemically competent or electrocompetent cells.

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Assembly of multiple GeneArt Strings DNA Fragments

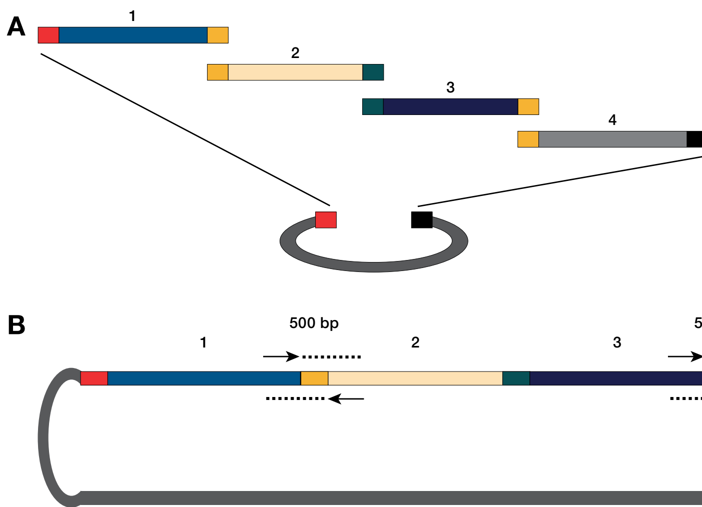
Linear double-stranded synthetic DNA fragments, such as PCR amplicons or GeneArt Strings DNA Fragments, can be efficiently assembled into any desired vector using GeneArt Gibson Assembly technology. Homology regions between 20 and 40 bp are required at the terminal ends to join adjacent fragments to each other and to the linearized vector.

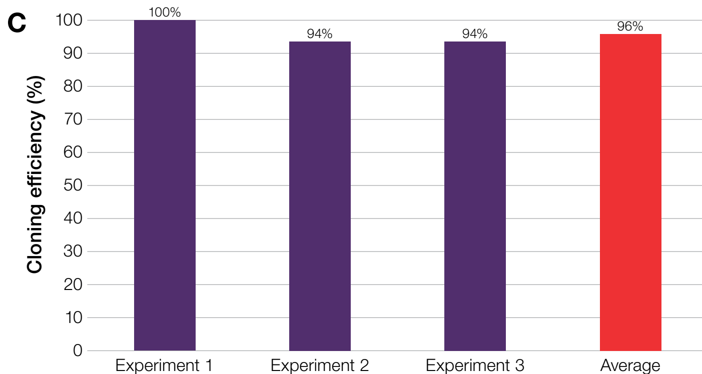
The GeneArt Gibson Assembly HiFi Cloning Kit enabled high-efficiency, simultaneous seamless assembly of four 2 kb fragments in the pUC19L vector (Figure 2A). Each fragment featured a 30 bp overlap with the adjacent one. Three independent assembly reactions were set up using 0.04 pmol of each fragment and of the linearized vector in 1X GeneArt Gibson Assembly HiFi Master Mix (20 µL total reaction volume).

After a 1 hour incubation at 50°C, 1 µL of diluted assembly product was used to electroporate Invitrogen™ ElectroMAX™ DH10B competent cells as described in the user guide (Pub. No. MAN0019062; electroporation parameters: 2.0 kV, 200 Ω, 25 µF). After addition of 800 µL SOC medium and recovery at 37°C for 1 hour, 50 µL of transformed cells were plated on LB agar plates containing ampicillin and incubated overnight at 37°C.

Eight colonies were picked from each plate and analyzed by colony PCR using Invitrogen™ Platinum™ PCR SuperMix and primers to amplify the transition regions between fragments 1 and 2 and fragments 3 and 4 (Figure 2B). Cloning efficiency was calculated as the ratio between full-length clones and analyzed clones.

The average cloning efficiency of three independent assembly experiments was 96% (Figure 2C), with an average of 1,617 colony forming units (CFUs, Figure 2D). These data demonstrate that assembly of four inserts and a vector using the GeneArt Gibson Assembly HiFi Cloning Kit works with very high reliability.





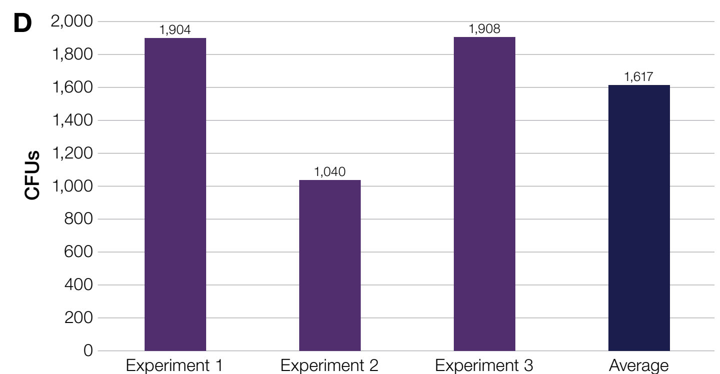


Figure 2. Seamless assembly of four 2 kb fragments (8 kb total insert size) in the pUC19L vector using the GeneArt Gibson Assembly HiFi Cloning Kit. (A) Adjacent fragments share 30 bp overlap. **(B)** Reactions were done in triplicate, and the resulting 11 kb covalently bonded plasmid was transformed via electroporation into ElectroMAX DH10B competent cells. Recombinant clones were selected on LB agar plates containing ampicillin, and PCR analysis was performed using custom primers to amplify 500 bp transition regions between fragments 1 and 2 and fragments 3 and 4. **(C)** Cloning efficiency corresponds to the ratio between full-length clones and analyzed clones and is expressed as a percentage. **(D)** CFUs were obtained by plating 1/16 of the total cell suspension.

Assembly of one or more fragments using stitching oligonucleotides

The availability of overlap regions between two fragments to be joined is the basis of Gibson Assembly technology. The required overlap regions are usually added at the fragment termini by PCR; alternatively, they can be introduced using stitching oligonucleotides that share half of the sequence with one fragment and the other half with the adjacent fragment. In this way, the oligonucleotides work like a bridge between two DNA fragments.

The Gibson Assembly method using stitching oligonucleotides offers high flexibility and enables virtually any possible combination between DNA fragments that do not originally share any homology. For example, it can be used to carry out a cloning strategy without generating a new PCR amplicon or to clone the same PCR amplicon in multiple vectors without repeating PCR amplification of this fragment. Stitching oligonucleotides also enable the transfer of a cloned sequence from one vector to another without the need for PCR amplification. This is particularly useful when working with large (>10 kb) or complex DNA inserts, such as those with high GC content and secondary structures, which make PCR amplification challenging. In this case, the insert can be cut using restriction enzymes, purified, and then added to the assembly reaction in the presence of the recipient vector and suitable stitching oligonucleotides.

In this example, we designed 30 nt stitching oligonucleotides to perform single-fragment and two-fragment cloning into the pUC19L vector. Each stitching oligonucleotide contained evenly split homology between adjacent fragments, as shown in Figure 3A. For each example, we assembled two independent constructs and performed each reaction in duplicate using the GeneArt Gibson Assembly HiFi Cloning Kit.

Inserts and vector were produced by PCR using Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase and purified using the Thermo Scientific™ GeneJET™ Gel Extraction Kit. The reaction was set up according to the instruction manual using GeneArt Gibson Assembly HiFi Master Mix, equimolar amounts of inserts and vector (0.04 pmol), and different concentrations of stitching oligonucleotides (20 nM, 30 nM, 45 nM, and 120 nM). After 15 minutes of incubation at 50°C, the assembly product was used to transform Invitrogen™ One Shot™ TOP10 chemically competent cells, and recombinant clones were selected on LB agar plates containing ampicillin.

Eight colonies were picked for each construct and analyzed by colony PCR using Platinum PCR SuperMix. Cloning efficiency is the ratio between full-length clones and analyzed clones.

The method shows high reliability with one and two fragments using a wide range of oligonucleotide concentrations (20—120 nM). For optimal cloning efficiency with 30 nt stitching oligonucleotides, we recommend using a concentration between 30 nM and 45 nM (Figure 3B, 3C).

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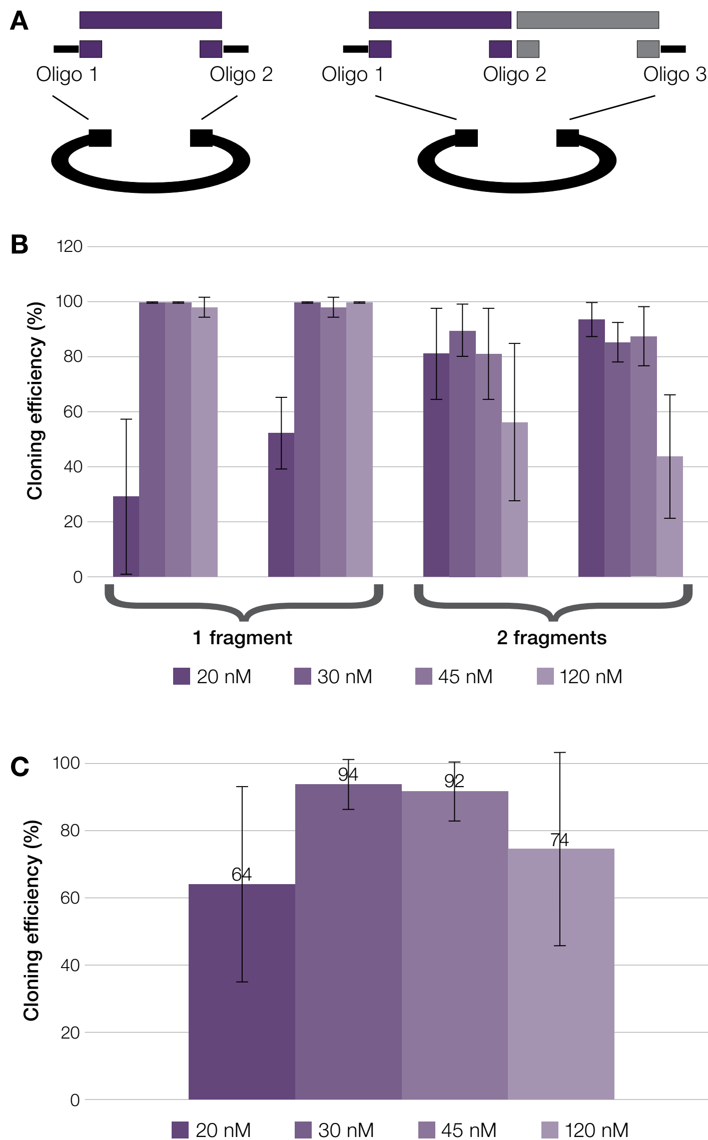

Figure 3. Stitching oligonucleotides for assembly of fragments that do not share homologous regions. (A) Stitching oligonucleotides work as a bridge between the DNA fragments to be joined, and share half of the sequence with each fragment. One-fragment and two-fragment cloning require two and three stitching oligonucleotides, respectively. **(B)** Assembly of a single fragment and two fragments in pUC19L using different concentrations of stitching oligonucleotides. For each example, two independent constructs were generated, and each GeneArt Gibson Assembly reaction was performed in duplicate. **(C)** Overall cloning efficiency obtained from one- and two-fragment cloning using different oligonucleotide concentrations. The optimum concentration is between 30 nM and 45 nM when using 30 nt oligonucleotides.

Conclusions

In summary, GeneArt Gibson Assembly HiFi Cloning technology works reliably in combination with stitching oligonucleotides and offers maximal flexibility for cloning of PCR amplicons, *de novo* synthesized fragments, and restriction fragments in any desired vector.

References

1. Gibson DG (2011) Enzymatic assembly of overlapping DNA fragments. *Meth Enzymol* 498:349–361.
2. Gibson DG, Benders GA, Andrews-Pfannkoch C et al. (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 319:1215–1220.
3. Gibson DG, Young L, Chuang R-Y et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345.

GeneArt Strings DNA Fragments are a fast and affordable alternative to traditional cloning and full gene synthesis. To order GeneArt Strings DNA Fragments, go to **thermofisher.com/strings**

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