Evaluation of GeneArt Gibson Assembly EX Cloning technology to build large and complex assemblies

Comparison with other seamless cloning technologies

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 EX Cloning Kit can be used to make very large constructs using up to 10 inserts. We also compare this kit to the Invitrogen™ GeneArt™ Seamless Cloning Kit and another supplier's product.

Basis of the technology

The GeneArt Gibson Assembly EX Cloning Kit is optimal for highly reliable, simultaneous assembly of multiple DNA fragments, as well as for construction of large DNA molecules or even entire genomes. Using a proprietary enzyme mix, up to 15 DNA fragments featuring complementary overlap regions can be assembled in a two-step reaction (Figure 1). Homologous ends are chewed back, exposing complementary single strands that are annealed. After extension and ligation, the assembly product is ready to be transformed into chemically competent or electrocompetent cells [1-3]. For more complex assemblies, electrocompetent cells are recommended due to their higher transformation efficiency.

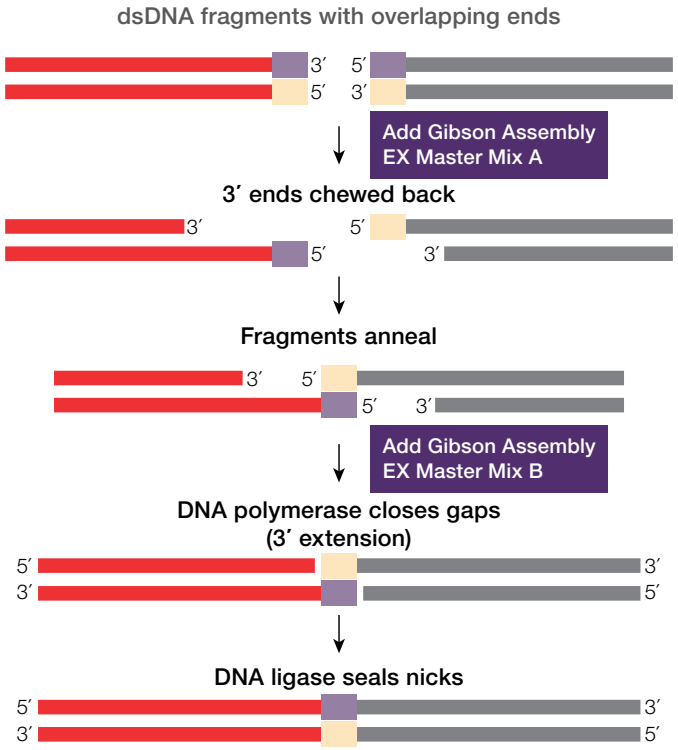

Figure 1. Overview of the reaction. The GeneArt Gibson Assembly EX Cloning Kit can assemble up to 15 inserts with high reliability in a two-step reaction. In the first step, a 3′ DNA exonuclease chews back fragment ends to allow for annealing of homologous segments. In the second step, DNA polymerase fills the gaps and DNA ligase seals the nicks to give rise to a covalently bound DNA backbone, which can be transformed into chemically competent or electrocompetent cells.

The success rate of fragment assembly is influenced by two main factors: the size and the number of fragments. Large fragments are sometimes difficult to prepare in sufficient quantity and purity. In addition, assembly efficiency of multiple fragments decreases with increasing number of fragments. This is intuitive, as every additional fragment adds two more homologous ends to be processed. As a consequence, a successful cloning strategy balances the size and number of fragments combined.

Here we show how GeneArt Gibson Assembly EX technology delivers high cloning efficiency both when joining numerous short fragments and when joining a few large fragments, thus offering maximal flexibility.

Assembly of 3, 4, and 5 kb inserts from multiple fragments

In this example, we used the GeneArt Gibson Assembly EX Cloning Kit to assemble 6, 8, and 10 fragments into the Invitrogen™ pcDNA™ 3.4 vector to create inserts of 3, 4, and 5 kb, respectively. We also compared its performance with the GeneArt Seamless Cloning Kit and In-Fusion™ HD cloning kit (Takara Bio), both of which offer seamless cloning of DNA fragments containing end-terminal homology.

Each individual fragment was 0.5 kb in length and contained 30 bp homologous with the adjacent end. The inserts and vector were produced by PCR using Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase (Cat. No. 12361010) and purified using the Thermo Scientific™ GeneJET™ Gel Extraction Kit (Cat. No. K0691). Each reaction was set up in triplicate using 0.04 pmol of each insert and of the linearized vector, and assembly was performed according to each manufacturer's instructions using the recommended competent cells. Assembly products for the In-Fusion cloning kit were transformed into Stellar™ competent cells (Takara Bio), and assembly products for the GeneArt Gibson Assembly EX Cloning Kit and GeneArt Seamless Cloning Kit were transformed into Invitrogen™ One Shot™ TOP10 competent cells. Recombinant clones were selected on LB agar plates containing ampicillin. Eight colonies were picked for each construct and analyzed by colony PCR using Invitrogen™ Platinum™ PCR SuperMix. Cloning efficiency corresponds to the ratio between full-length clones and analyzed clones.

The average cloning efficiency for a 6-fragment assembly is very high (98%) for the GeneArt Gibson Assembly EX Cloning Kit and the GeneArt Seamless Cloning Kit in comparison to the In-Fusion kit (10%) (Figure 2). For 8- and 10-fragment assemblies, the GeneArt Gibson Assembly EX Cloning Kit shows the highest performance and only a moderate drop in efficiency (maximum of 13%) when increasing the number of fragments from 6 to 10. The In-Fusion cloning kit was unable to assemble 8 and 10 fragments in this experiment.

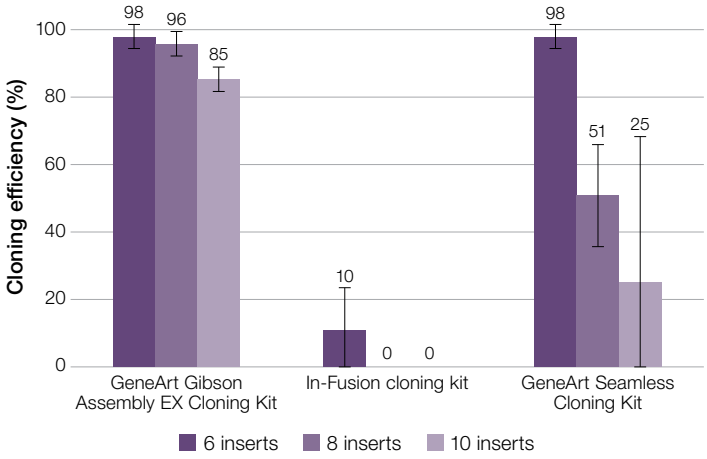

Figure 2. Comparison of multiple-fragment cloning efficiency. GeneArt Gibson Assembly EX, In-Fusion, and GeneArt Seamless cloning kits were evaluated for the ability to assemble 6, 8, and 10 fragments into the pcDNA 3.4 vector (the total length of the assembled insert was up to 5 kb). Each kit was used in combination with the recommended chemically competent cells, and each reaction was done in triplicate. After transformation, 8 colonies were analyzed. Cloning efficiency represents the ratio of colonies containing a full-length construct to analyzed colonies. The GeneArt Gibson Assembly EX Cloning Kit in combination with One Shot TOP10 chemically competent cells gave the highest cloning efficiency.

In contrast to GeneArt Seamless and In-Fusion cloning technologies, GeneArt Gibson Assembly cloning technology generates covalently bonded assembly products that can be transformed into electrocompetent cells. The products obtained from assemblies of 6, 8, and 10 fragments using the GeneArt Gibson Assembly EX Cloning Kit were transformed into Invitrogen™ ElectroMAX™ DH10B competent cells. As expected, cloning efficiency and transformation efficiency were higher compared to what was obtained with One Shot TOP10 chemically competent cells (Figure 3).

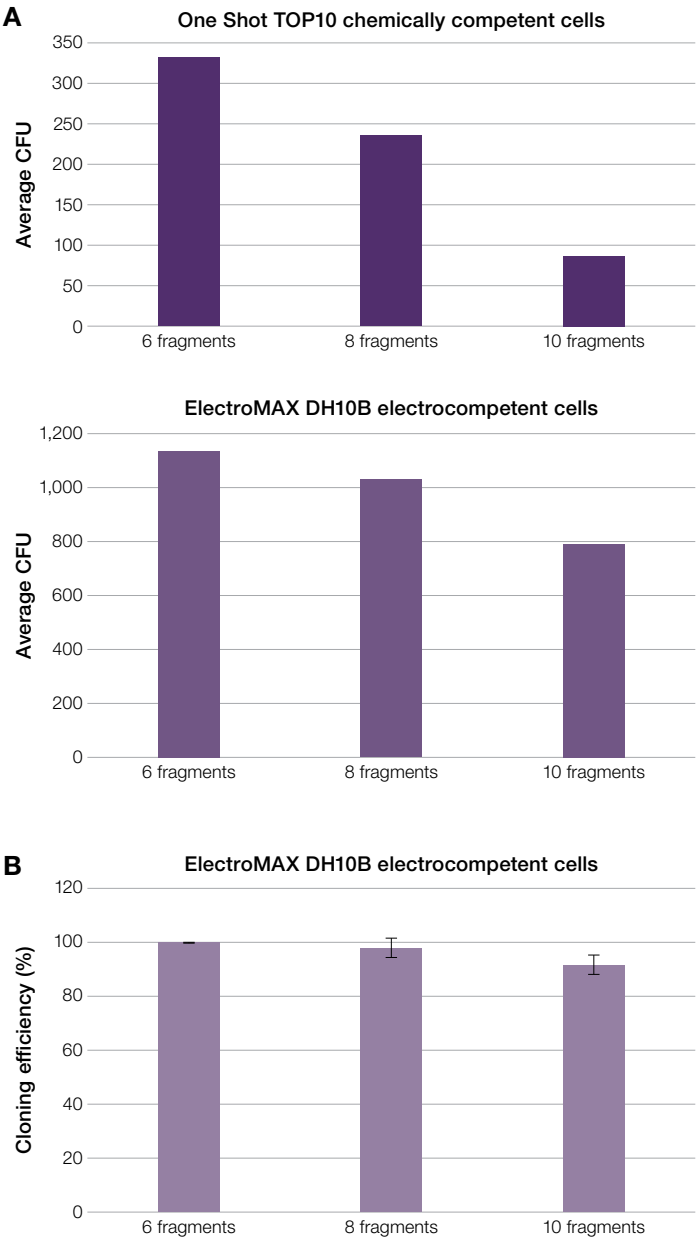

Figure 3. Higher transformation and cloning efficiencies for multiple-fragment constructs using electrocompetent cells. **(A)** The products obtained from assemblies of 6, 8, and 10 fragments into the pcDNA 3.4 vector using the GeneArt Gibson Assembly EX Cloning Kit (same products as in Figure 2) were transformed into either One Shot TOP10 chemically competent cells or ElectroMAX DH10B electrocompetent cells. As expected, average colony-forming units (CFUs) decreased with increasing number of fragments. Electroporation results in up to 10x more CFUs than heat-shock transformation. **(B)** Electroporation of ElectroMAX DH10B competent cells also resulted in higher cloning efficiency compared to that obtained from heat shock of One Shot TOP10 competent cells (see Figure 2).

Assembly of a 10 kb insert from 5 fragments

The efficiency of Gibson Assembly technology is generally more affected by an increasing number of fragments in a reaction than by increasing fragment size. Therefore, when building larger molecules, it is recommended to keep the number of fragments as small as possible. In the following example, we assembled a 10 kb insert in the pUC19L vector using five 2 kb fragments. As in the previous experiment, the inserts and vector were prepared by PCR with Platinum SuperFi II DNA Polymerase and purification using the GeneJET Gel Extraction Kit. Adjacent fragments shared 30 bp of homology. Three independent assembly reactions were set up using 0.04 pmol of each fragment (including the vector).

Each assembly product was subsequently transformed into One Shot TOP10 chemically competent cells and ElectroMAX DH10B electrocompetent cells. Average cloning efficiency was again calculated as the ratio of full-length to analyzed clones (Figure 4).

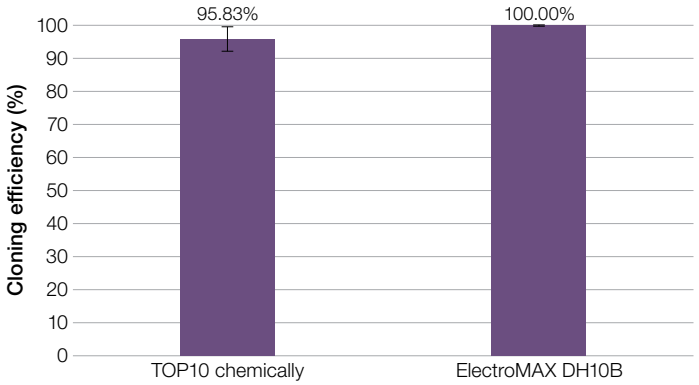

Figure 4. Assembly of a 10 kb insert with high cloning efficiency. The assembly product from the GeneArt Gibson Assembly EX Cloning Kit is a covalently bonded DNA molecule that can be transformed into chemically competent or electrocompetent cells. High cloning efficiency between 95% and 100% was obtained in each case.

Assembly of large DNA constructs

When assembling large DNA constructs, such as those coding for pathways or representing entire genomes, it is preferable to keep the number of fragments as low as possible to maximize cloning efficiency. The size of the fragments also plays an important role—the larger the fragments, the more difficult it is to prepare them in sufficient amount and purity for Gibson Assembly cloning.

When working with fragments larger than 15 kb, PCR amplification also becomes challenging; therefore, restriction digest of cloned fragments may be more appropriate to prepare the DNA segments to be joined.

Independent from the method chosen to prepare the DNA fragment, we recommend the use of ElectroMAX DH10B electrocompetent cells to obtain a high transformation efficiency. A high colony number increases the chance of finding a correct clone.

invitrogen

Another important consideration is the use of the correct cloning vector. *E. coli* typically displays a low tolerance for high-copy plasmids containing large DNA constructs. As a result, *E. coli* uses DNA repair mechanisms to shrink the plasmid, usually eliminating parts of the insert and keeping the elements needed for growth of the plasmid, such as the replication origin and selective markers. To avoid this, it is recommended to use low-copy plasmids that allow for propagation of the full-length construct without hampering the *E. coli* DNA replication cycle.

Taking into consideration all of these aspects, in the following example we used the GeneArt Gibson Assembly EX Cloning Kit to build large DNA constructs in the low-copy pASE101 vector [4]. The inserts and vector with 50 bp of homology at each end were amplified using Platinum SuperFi II DNA Polymerase and purified with the GeneJET Gel Extraction Kit. Two and four fragments were assembled into the pASE101 vector to build 25 kb and 50 kb inserts, respectively (Figure 5A and 5B). To maximize transformation efficiency, the assembly product was precipitated with 100% ethanol and resuspended in nuclease-free water, then 10 µL was used to transform ElectroMAX DH10B electrocompetent cells. Sixteen colonies were analyzed to calculate cloning efficiency (Figure 5C). While cloning efficiency was low (between 6 and 20%), high colony numbers made it possible to identify a colony containing the full-length insert in both cases.

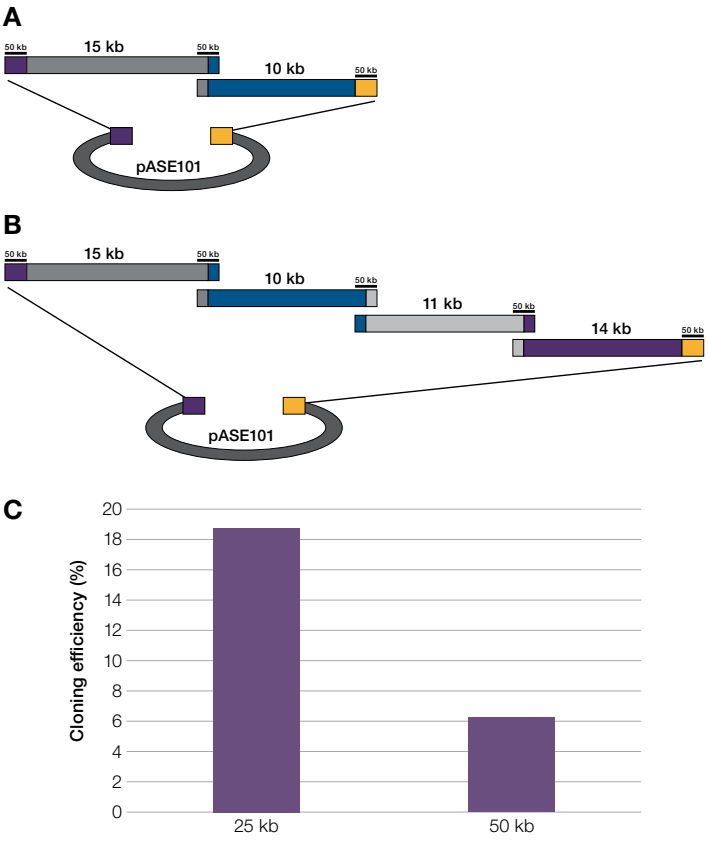

Figure 5. Large-fragment DNA assembly using the GeneArt Gibson Assembly EX Cloning Kit. (A) 25 kb and **(B)** 50 kb inserts were assembled starting from 2 and 4 DNA fragments, respectively, and cloned into the low-copy pASE101 vector. **(C)** 3 out of 16 picked colonies contained the full-length 25 kb construct, and 1 out of 16 picked colonies contained the full-length 50 kb construct.

Conclusions

In this white paper, we have shown that GeneArt Gibson Assembly EX Cloning technology can be used to assemble large and complex DNA fragments in the vector of your choice.

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.
4. Baek C-H, Chesnut J, and Katzen F (2015) Positive selection improves the efficiency of DNA assembly. *Anal Biochem* 476:1–4.

Invitrogen™ 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**

Find out more at **thermofisher.com/gibsonassembly**

ThermoFisher SCIENTIFIC