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GeneArt™ Gibson Assembly® HiFi Cloning Kits

USER GUIDE

For highly-efficient, simultaneous, and seamless *in vitro* assembly of up to 5 DNA fragments plus a vector in a pre-determined order

for use with any of these products:

- GeneArt™ Gibson Assembly® HiFi Cloning Kit, Chemically Competent Cells (Cat. No. A46624)
- GeneArt™ Gibson Assembly® HiFi Cloning Kit, Electrocompetent Cells (Cat. No. A46626)
- GeneArt™ Gibson Assembly® HiFi Master Mix (Cat. No. A46627, A46628, and A46629)

Catalog Numbers A46624, A46626, A46627, A46628 and A46629

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

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Revision	Date	Description
C.0	28 September 2020	Corrected the storage temp for Master mix in contents and storage topic.
B.0	06 April 2020	Adding several edits.
A.0	13 February 2020	New document.

Contents

1 Product information

Product description

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. 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. The resulting products can be used for a variety of downstream applications including transformation, PCR and rolling circle amplification (RCA).

Engineering DNA molecules by homologous in vitro recombination presents a seamless alternative to traditional methods using restriction endonucleases and ligases. GeneArt™ Gibson Assembly® HiFi Master Mix contains a proprietary mixture of enzymes and reagents optimized to facilitate one-step assembly of overlapping DNA fragments. As a result, products assembled with GeneArt™ Gibson Assembly® demonstrate low rates of junction error and high sequence fidelity.

Key features of the GeneArt™ Gibson Assembly® HiFi Cloning Kits are:

- One-step reaction
- Accurate
- Seamless
- Optimal for 1-5 inserts
- Suitable for fragments ranging from 0.5-32 kb

To seamlessly assemble up to 5 DNA fragments plus a vector into a single recombinant DNA molecule using the GeneArt™ Gibson Assembly® HiFi Cloning Kits:

1. Combine your own linearized *E. coli* vector and the DNA fragments to assemble in a microcentrifuge tube.
2. Add appropriate amounts of the GeneArt™ Gibson Assembly® HiFi Master Mix and incubate the tube for 15-60 minutes at 50°C.
3. Transform the assembled DNA molecule into chemically or electrocompetent *E. coli*.

Contents and storage

Contents are shown for the individual kits including:

- GeneArt™ Gibson Assembly® HiFi Cloning Kit, Chemically Competent Cells (Cat. No. A46624)
- GeneArt™ Gibson Assembly® HiFi Cloning Kit, Electrocompetent Cells (Cat. No. A46626)
- GeneArt™ Gibson Assembly® HiFi Master Mix (Cat. No. A46627, A46628, A46629)

Component	A46624 10 reactions	A46626 10 reactions	A46627 10 reactions	A46628 50 reactions	A46629 200 reactions	Storage Temp.
GeneArt™ Gibson Assembly® HiFi Master Mix	100 µL	100 µL	100 µL	500 µL	2 mL	–70°C (or –20°C for up to 2 months)
Positive Control	50 µL	50 µL	50 µL	50 µL	50 µL	–20°C
One Shot™ TOP10 Chemically Competent *E. coli*	11 × 50 µL	—	—	—	—	–80°C
ElectroMAX™ DH10B Cells	—	5 × 100 µL	—	—	—	–80°C
S.O.C. Medium	6 mL	2 × 6 mL	—	—	—	4°C

The Positive Control consists of a mixture of 10 ng of a 1.5 kb insert (kanamycin cassette) and 30 ng of a 2.7 kb vector containing an ampicillin resistance gene. Select for the 4.2 kb assembled construct on LB agar plates with 100 µg/mL ampicillin or 50 µg/mL kanamycin.

Required materials not supplied

- DNA fragments for the GeneArt™ Gibson Assembly® HiFi cloning reaction
- Thermocycler
- Luria-Bert (LB) plates with appropriate antibiotic

If a Master Mix kit is purchased, the following materials are also required:

- S.O.C. medium
- High efficiency competent cells. We recommend One Shot™ TOP10 Chemically Competent E. coli or ElectroMAX™ DH10B cells from Thermo Fisher Scientific.

Additional optional materials

- DNA polymerase (e.g., SuperFi™ II DNA Polymerase, Thermo Fisher Scientific) for producing fragments to be assembled with the GeneArt™ Gibson Assembly® HiFi kit
- Restriction enzymes for producing linearized vector
- PureLink™ PCR Purification Kit (Thermo Fisher Scientific) or GeneJET™ Gel Extraction Kit (Thermo Fisher Scientific)
- Thermoblock or electroporator plus electroporation cuvettes

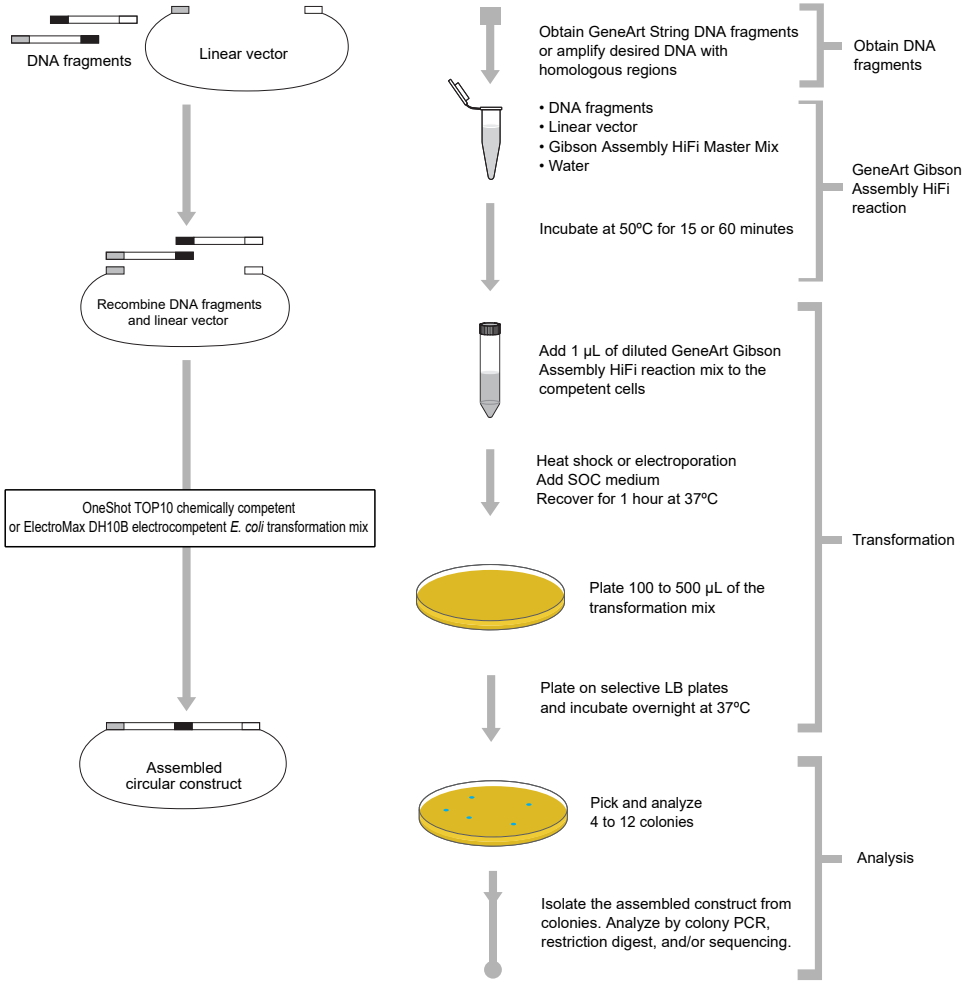
Experimental outline

DNA fragments can be obtained from a variety of methods. Building a fragment de novo, such as a GeneArt™ String™ or High-Q String™ DNA fragment allows direct design of the homologous region for Gibson Assembly® cloning. It is also possible to obtain a DNA fragment via amplification.

The table below describes the major steps required to assemble your recombinant DNA molecule using the GeneArt™ Gibson Assembly® HiFi Cloning Kit. Refer to the specified pages for details to perform each step.

Step	Action	Page
1	Design your primers to amplify your fragments	"DNA fragment design" on page 9
2	Amplify your DNA fragments with DNA Polymerase (SuperFi™ II mix)	"Guidelines for PCR" on page 12
3	Prepare a linearized vector (by using DNA Polymerase or restriction digestion)	"Guidelines for generating a linearized E. coli cloning vector" on page 12
4	Perform the GeneArt™ Gibson Assembly® HiFi reaction	"GeneArt™ Gibson Assembly® HiFi cloning reaction" on page 14
5	Transform chemically competent One Shot™ TOP10 cells - or - electrocompetent ElectroMAX™ DH10B *E. coli* cells	"Transform One Shot™ TOP10 Chemically Competent E. coli cells" on page 15 or "Transform ElectroMAX™ DH10B electrocompetent E. coli cells" on page 16
6	Analyze colonies by colony PCR, restriction analysis and/or sequencing	"Analyze transformants" on page 18

Workflow


DNA fragments
Linear vector
Recombine DNA fragments and linear vector
OneShot TOP10 chemically competent or ElectroMax DH10B electrocompetent *E. coli* transformation mix
Assembled circular construct
Obtain GeneArt String DNA fragments or amplify desired DNA with homologous regions
Obtain DNA fragments
• DNA fragments
• Linear vector
• Gibson Assembly HiFi Master Mix
• Water
Incubate at 50ºC for 15 or 60 minutes
GeneArt Gibson Assembly HiFi reaction
Add 1 µL of diluted GeneArt Gibson Assembly HiFi reaction mix to the competent cells
Heat shock or electroporation
Add SOC medium
Recover for 1 hour at 37ºC
Plate 100 to 500 µL of the transformation mix
Plate on selective LB plates and incubate overnight at 37ºC
Transformation
Pick and analyze 4 to 12 colonies
Isolate the assembled construct from colonies. Analyze by colony PCR, restriction digest, and/or sequencing.
Analysis

2 Methods

Prepare DNA inserts

DNA fragment design

GeneArt™ Gibson Assembly® technology relies on homologous recombination to assemble adjacent DNA fragments sharing end-terminal homology. The optimal length of the homologous fragment ends region depends on the number and length of the fragments in the assembly reaction. The following table lists the suggested length of the end-terminal homology.

Number of Fragments	Fragment Size	Length of Overlap Regions
1-3	≤ 8 kb	20-40 bp
	> 8 kb	40-100 bp
4-5	≤ 8 kb	40 bp
	> 8 kb	40-100 bp

- For higher order assembly, longer overlap regions will result in higher efficiency.
- You may need to optimize PCR amplification reactions when using PCR primers with long homologous overlap regions.

The GeneArt™ Gibson Assembly® HiFi reaction requires that each DNA fragment (including the cloning vector) shares a 20-40 bp terminal homology with the adjacent fragment. When obtaining de novo DNA fragments such as GeneArt™ Strings™ DNA fragments, simply include homologies directly the sequence design. If fragments are generated from existing DNA templates via PCR, primers used for generating your inserts must contain the 20-40 nt overhangs on their 5' ends to provide this homology with the adjacent fragments; however, this homology may be split between the primers used for adjacent PCR-amplified DNA fragments (see below).

Guidelines for designing PCR primers

- Design your PCR primers such that each DNA fragment to be assembled is between 0.5-5 kb in length.

  Note: Large fragments (>5 kb) are more susceptible to damage in a gel extraction procedure. Furthermore, many PCR enzymes are not processive enough to amplify fragments >5 kb.

Therefore, we recommend that you assemble multiple fragments of ≤5 kb in one reaction rather than a single large fragment.

- The 5' ends of each primer pair (forward and reverse) must contain a 20-40 nt sequence (i.e., an overhang) that is homologous to the 20-40 bp at one end of the adjacent DNA fragment (i.e., the vector or another insert) while the 3' end of each primer must be specific to your DNA element you want to assemble.
- If you are recombining the insert to the linearized vector, all 20-40 nt providing the requisite homology must be on the 5' end of the primer. To recombine two adjacent inserts, you may split the 20-40 bp homology between the fragments (e.g., 20 bp on the reverse primer of fragment 1 and 20 bp on the forward primer of fragment 2).

  Note: You can split the 20-40 bp homology between adjacent fragments in any combination (e.g., 20+20 as in the example above or 10+30, 35+5, etc.).

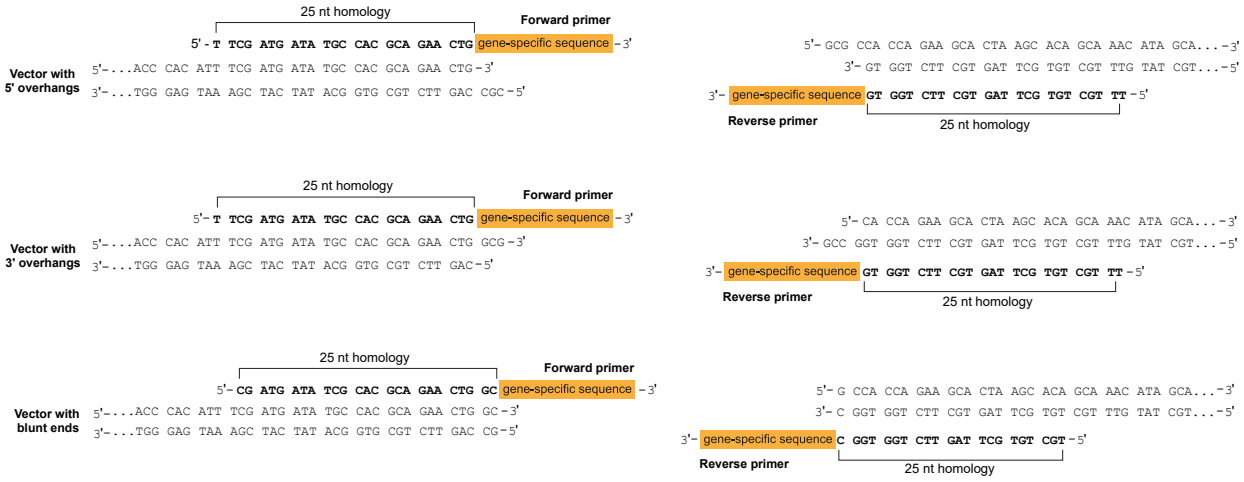
- PCR primers should be up to 65 nt in length (20-40 nt to provide the requisite homology at the 5' end and 18-25 nt specific to your DNA element).

  Note: If you are splitting the required 20-40 bp homology between adjacent fragments, one PCR primer may have as few as 18 nt and no overhang whatsoever. For example, in a 0+40 homology split, reverse primer of fragment 1 would not contribute to the homology and will consist of only 18-25 gene-specific nucleotides for fragment 1, while the forward primer of fragment 2 would contribute the entire 40 bp for the homology in addition to having 18-25 gene-specific nucleotides for fragment 2 (for a total length of 58-65 nt).

- We recommend splitting the homology between the primers to avoid long primers however.
- You may also design your PCR primers to provide specific restriction enzyme sites or to re-construct the restriction sites used for linearizing your cloning vector. Note that these sites will not be a part of the 20-40 nt homology.
- Prepare each primer pair at a concentration of 100 µM in DNase- and RNase- free water.

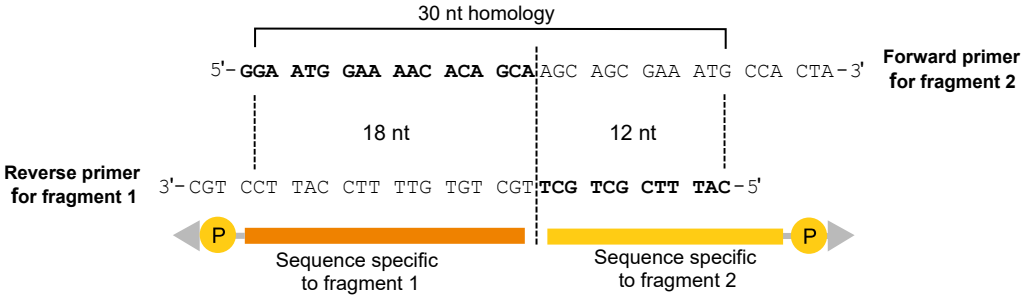
PCR primers for attaching the DNA fragment to the vector

The figure below shows examples of PCR primers used for preparing the DNA inserts for assembly in linear vectors with 5' overhangs, 3' overhangs, and blunt ends. The primer sequences providing the homology (in this example 25 nt) are shown in bold. Even though 5' and 3' overhangs produce slightly different lengths of homology to the amplified PCR product, we recommend to disregard the overhangs to the overall homology. The remaining sequences of each primer, i.e., the 3' end, are specific to the DNA element to assemble.



PCR primers for attaching adjacent DNA fragments

The figure below shows examples of PCR primers used for preparing adjacent DNA inserts for their assembly into a larger construct. When connecting two adjacent inserts, you may split the homology between the fragments in any combination. In the example below, the reverse primer of fragment 1 provide 12 nt and the forward primer of fragment 2 contribute 18 nt of a 30 bp homology for recombination. The primer sequences adding to the homology are shown in bold; the remaining sequences of each primer, i.e., the 3' end, are specific to the DNA element to assemble.



Note: While primers can be easily designed by hand, we recommend using appropriate software for primer design to avoid human error. Today, most molecular biology cloning programs support design of Gibson Assembly® experiments.

Guidelines for PCR

When using plasmid DNA, 5-20 ng of plasmid DNA is usually sufficient as a PCR template in a reaction volume of 100 µL. When using *E. coli* or human genomic DNA, you can increase the amount of template DNA to 20-200 ng.

- We recommend using Platinum™ SuperFi II PCR Master Mix (Cat. No. 12368050) for achieving the best results during PCR amplification of your DNA fragments of interest.

  Note: The processivity of the polymerase is crucial for obtaining full-length PCR products

- Use PCR cycling parameters that are appropriate for your template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- PCR products may be used directly in assembly reactions without additional purification, although results may be improved by purifying the DNA fragments through a PCR cleanup kit. We recommend that you use the PureLink™ PCR Purification Kit (Cat. No. K310001).
- After preparing your DNA fragments by PCR, verify the PCR products by gel electrophoresis. If you obtain multiple bands, we recommend gel purification of your DNA fragments. We recommend using the GeneJET™ Gel Extraction Kit (Cat. No. K0691) for best results.

Generate a linearized *E. coli* cloning vector

The GeneArt™ Gibson Assembly® technology relies on homologous recombination to assemble adjacent DNA fragments sharing end-terminal homology. Because of the mechanism of action of the enzyme mix, the cloning vector and DNA fragments used with the GeneArt™ Gibson Assembly® Kit must be linear.

This section provides guidelines for generating a linearized *E. coli* cloning vector for use in the GeneArt™ Gibson Assembly® reaction.

Vector considerations

- For small inserts (<10 kb) or non-toxic gene(s), use a high copy number vector such as pUC19.
- For large insert(s) or toxic gene(s), use a low copy number or inducible vector.

Guidelines for generating a linearized *E. coli* cloning vector

- You can prepare the linearized *E. coli* cloning vector using restriction enzymes (single or double digest) or using PCR amplification.
- When generating the linearized vector by PCR amplification, we recommend treating the PCR ampflication product with DpnI to reduce circular template carryover.
- When generating the linearized vector by restriction digest, we recommend that you digest the vector with two restriction enzymes rather than a single enzyme to reduce the amount of background. A double digest followed by PCR amplification of your linear vector virtually eliminates any background.
- You can use restriction enzymes that leave 3' protruding, 5' protruding, or blunt ends to linearize your cloning vector.

- It is very important to have a complete digest (i.e., very low background of uncut vector). Therefore, we recommend you increase the enzyme digestion time (2-3 hours to overnight) and the reaction volume.
- Analyze your restriction digestion products using agarose gel electrophoresis to verify that the digest is complete and then purify the digested vector using the PureLink™ PCR Purification Kit or equivalent.
- For a small scale digest, we recommend that you digest 2-5 µg of vector using 30-50 units of enzyme in a reaction volume of 100-200 µL.
- If you are planning on using the same linearized vector in multiple reactions, we recommend that you digest 20-50 µg of vector using 150-300 units of each restriction enzyme in a reaction volume of 400-800 µL. After purification, aliquot linearized vector and store at -20°C.

GeneArt™ Gibson Assembly® HiFi reaction

Before you set up your GeneArt™ Gibson Assembly® HiFi cloning reaction, be sure you have:

- Devised your DNA assembly strategy and possibly verified it by performing *in silico* cloning using an appropriate software tool.
- Prepared your DNA fragments (i.e., inserts) according to the guidelines in “Prepare DNA inserts” on page 9.
- Generated your linear cloning vector according to the guidelines in “Generate a linearized E. coli cloning vector” on page 12.

Materials required:

- DNA fragments to assemble
- Your own linearized *E. coli* cloning vector
- Positive Control
- GeneArt™ Gibson Assembly® HiFi Master Mix
- Sterile deionized water

Guidelines for GeneArt™ Gibson Assembly HiFi cloning reactions

- For maximum cloning efficiency we recommend using all fragments (vector and insert(s)) at equimolar ratio.
- Determine the concentration of your DNA insert solutions by OD_{260} or fluorescence and use the concentrations to calculate the volume required to achieve the required molar ratio of insert to vector.
- Use the following formula to calculate molarities:

$$pmols \approx \frac{(weight\ in\ ng) \times 1000}{(fragment\ length\ in\ bp) \times 660}$$

- Keep GeneArt™ Gibson Assembly® HiFi Master Mix on ice at all times and promptly return to freezer after use.
- When assembling multiple fragments, create a master mix of fragments in the proper ratios to minimize pipetting error.

GeneArt™ Gibson Assembly® HiFi cloning reaction

1. Thaw GeneArt™ Gibson Assembly® HiFi Master Mix on ice.
2. Vortex GeneArt™ Gibson Assembly® HiFi Master Mix immediately before use.
3. In a microcentrifuge tube on ice, set up the GeneArt™ Gibson Assembly® cloning reaction as described in the table below:

	1-3 Inserts Assembly	4-5 Inserts Assembly	Positive Control [1]
Recommended DNA molar ratio	vector:insert = 1:1	vector:insert = 1:1	—
Amount of each fragment	0.08 pmol vector 0.08 pmol each insert X µL	0.08 pmol vector 0.08 pmol each insert X µL	10 µL
GeneArt™ Gibson Assembly® HF Master Mix	10 µL	10 µL	10 µL
Deionized water volume	(10 – X) µL	(10 – X) µL	—
Total volume	20 µL	20 µL	20 µL
Incubation time at 50°C	15 minutes	60 minutes	15 minutes

[1] The positive control reagents contain all necessary fragments.

(Optional) For the Positive Control, combine 10 µL of the Positive Control and 10 µL of GeneArt™ Gibson Assembly® HiFi Master Mix in a tube on ice. Mix the reaction by vortexing.

4. Mix the reactions by vortexing, spin down and incubate at 50°C for the recommended time. For Positive Control, use 15 minutes incubation time.
5. After incubation, place the reaction mix on ice and immediately proceed to the transformation step.

Note: Reactions can also be stored at –20°C for later use.

Note: For more complex assemblies (e.g., > 5 inserts) we recommend increasing the reaction volume and using ElectroMAX™ DH10B electrocompetent cells for transformation.

Note: When using the GeneArt™ Gibson Assembly® cloning kit for the first time, we strongly recommend that you perform the positive control reactions in parallel with your samples to verify that the kit components are performing properly.

Transform One Shot™ TOP10 Chemically Competent *E. coli* cells

Materials required

- GeneArt™ Gibson Assembly® reaction mix
- One Shot™ TOP10 Chemically Competent *E. coli* cells
- S.O.C. medium
- 42°C heat block
- LB plates containing the appropriate selection antibiotic
- 37°C shaking and non-shaking incubator
- *(Optional)* pUC19 Control DNA for the transformation control reaction

Prepare reaction

1. Warm the heat block to 42°C.
2. Warm the vial of S.O.C. medium to room temperature.
3. Warm selective plates at 37°C for 30 minutes.
4. Thaw on ice 1 vial of One Shot™ Chemically Competent *E. coli* cells for each transformation.

Transform One Shot™ TOP10 Chemically Competent *E. coli* cells

1. Dilute the GeneArt™ Gibson HiFi cloning reaction ("GeneArt™ Gibson Assembly® HiFi cloning reaction" on page 14) 1:5 in nuclease-free water (e.g., 12 µL nuclease-free water and 3 µL GeneArt™ Gibson cloning reaction). Mix the reaction by vortexing, then spin down and keep diluted reaction on ice.
2. Add 1 µL of the dilution into a vial of One Shot™ TOP10 Chemically Competent *E. coli* cells and mix gently.

   IMPORTANT! Do not mix by pipetting up and down or vortexing.

   Optional: If you are performing a transformation control, add 2.5 µL of pUC19 Control DNA into a separate vial of One Shot™ TOP10 Chemically Competent *E. coli* cells and follow the transformation procedure.
3. Incubate the transformation mix on ice for 20-30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice and incubate on ice for 2 minutes.
6. Add 450 µL of room temperature S.O.C. medium to the transformation mix.
7. Cap the tube tightly and shake at 300 rpm at 37°C for 1 hour to allow the cells to recover.

8. After incubation, spread 100 µL from each transformation on a pre-warmed selective plate. Before spreading on selective plates, mix suspension by pipetting up and down. If you have performed an assembly with 4-5 fragments, plate 100 µL on one selective plate and the remaining transformation on a second selective plate.

   (*Optional*) For the positive control, plate 100 µL volume of the transformed reaction onto LB plates containing 100 µg/mL ampicillin or 50 µg/mL kanamycin.

9. Incubate the plates overnight at 37°C.

10. The next day, pick individual colonies and isolate the plasmid DNA or screen for the presence of the insert(s) by colony PCR, miniprep and restriction analysis or direct sequencing.

Transform ElectroMAX™ DH10B electrocompetent *E. coli* cells

Materials required

- Gibson Assembly® cloning reaction mix
- ElectroMAX™ DH10B electrocompetent *E. coli* cells
- S.O.C. medium
- Electroporation cuvettes
- Electroporator
- LB plates containing the appropriate selection antibiotic
- 37°C shaking and non-shaking incubator
- *(Optional)* pUC19 Control DNA for the transformation control reaction

Electroporate ElectroMAX™ DH10B cells

1. Chill microcentrifuge tubes and electroporation cuvettes on ice.

2. Thaw ElectroMAX™ DH10B cells on ice.

3. Mix the reactions by vortexing and spin down before proceeding with transformation.

4. Dilute the GeneArt™ Gibson Assembly® HiFi cloning reaction 1:5 in nuclease-free water (e.g., 12 µL nuclease-free water and 3 µL GeneArt™ Gibson Assembly® HiFi cloning reaction). Keep diluted reactions on ice.

5. When cells are thawed, mix cells by tapping gently. Pipet 20 µL of cells to each chilled microcentrifuge tube

6. Add 1 µL of the dilution, into the microcentrifuge tube containing ElectroMAX™ DH 10B, mix gently and return the tube to ice.

   Note: If you are performing transformation control, add 1 µL of pUC19 Control DNA into a separate microcentrifuge tube containing ElectroMAX™ DH10B and follow the transformation procedure.

7. Incubate the cell/reaction mix on ice for one minute.

8. After the incubation, carefully pipette the cell/reaction mixture into a chilled 0.1 cm electroporation cuvette. Gently tap the cuvette to ensure that the cell/reaction mixture makes contact all the way across the bottom of the cuvette chamber. Avoid formation of bubbles.

9. Insert the cuvette into the electoroporator and press the pulse button. If you are using the BTX™ ECM™ 630 or Bio-Rad™ Gene Pulser™ II electroporator, we recommend using the following electroporator conditions: 2.0 kV, 200 Ω, 25 µF. In case of arcing, repeat electroporation using higher dilutions (1:10 to 1:50) or desalt the GeneArt™ Gibson Assembly® HiFi cloning reaction (e.g., by ethanol precipitation) prior to electroporation.

10. Add 800 µL of S.O.C. medium to the cuvette immediately after the end of the pulse and thoroughly pipet the mixture up and down. Transfer the solution back to the microcentrifuge tube. Repeat steps 7-8 for the remaining tubes.

11. Cap the tube tightly and shake at 300 rpm at 37°C for 1 hour to allow the cells to recover.

12. After incubation, spread 50-100 µL from each transformation on a pre-warmed selective plate. Before spreading on selective plates, mix suspension by pipetting up and down.

    (Optional) For the positive control, plate a 100 µL volume of the transformed reaction onto LB plates containing 100 µg/mL ampicillin or 50 µg/mL kanamycin.

13. Incubate the plates overnight at 37°C.

14. The next day, pick individual colonies and isolate the plasmid DNA or screen for the presence of the insert(s) by colony PCR, miniprep and restriction analysis, or direct sequencing.

Analyze transformants

Once you have performed the GeneArt™ Gibson Assembly® cloning reaction and the transformation procedure, screen for "positive" colonies containing your assembled recombinant DNA molecule by isolating the plasmid DNA and sequencing or by performing restriction analysis followed by agarose gel electrophoresis. You can also screen for the presence of the insert(s) by colony PCR. Calculate cloning efficiency using the following formula:

$$Cloning\ efficiency\ (\%) = \frac{(number\ of\ positive\ colonies)}{(number\ of\ total\ colonies)} \times 100$$

Typically, positive control yields cloning efficiencies >90%. Colony output is dependent on several factors, including transformation efficiency. Note that low colony output is not necessarily indicative of low cloning efficiency.

Analyze positive clones

1. Pick 4-10 colonies and culture them overnight in LB medium containing the appropriate selection antibiotic for your cloning vector.

   Note: Pick 10 colonies for an assembly reaction involving 4-5 insert fragments plus the vector. For assembly reactions involving less than 4 insert fragments, 4-5 colonies should suffice.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits.

3. Analyze the plasmids by restriction analysis and/or by sequencing. We highly recommend that you perform sequence analysis when assembling fragments that were PCR amplified to rule out any errors made by the DNA polymerase or introduced by PCR primers during amplification.

Analyze transformants by colony PCR

1. For each sample, aliquot 19.2 µL PCR master mix into a PCR tube. Add 0.4 µL of each forward and reverse PCR primer.

2. Pick 4-10 colonies with a pipet tip and resuspend them individually in 20 µL of the PCR cocktail from step 1.

3. Using the same pipet tip, streak each colony on an LB plate containing the appropriate selection antibiotic to save for preparing glycerol stocks (“Long-term storage of positive clones” on page 19).

4. Incubate the reaction for 2-3 minutes at 94°C to lyse the cells and to inactivate the nucleases.

5. Amplify your samples for 20-30 cycles using the amplification conditions you have determined.

6. Store the reactions at 4°C.

7. Visualize the results by agarose gel electrophoresis.

Materials required

- Platinum™ Direct PCR Universal Master Mix
- Appropriate forward and reverse PCR primers (10 µM each)
- LB plates containing the appropriate selection antibiotic

Long-term storage of positive clones

After you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. Keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB agar plate containing the appropriate selection antibiotic for your cloning vector. Incubate the plate at 37°C overnight.

2. Isolate a single colony and inoculate with 1-2 mL of LB containing the appropriate selection antibiotic for your cloning vector.

3. Grow the cells until the culture reaches stationary phase (OD_{600} = 1-2).

4. Mix 0.85 mL of the culture with 0.15 mL of sterile glycerol and transfer the mix to a cryovial.

5. Store the glycerol stocks at –80°C.

References

Gibson, D.G., *et al*. 2009. *Nat Methods*. 6:343-345.

Gibson, D.G., *et al*. 2010. *Science*. 329:52-56.

Troubleshooting

Observation	Possible cause	Recommended action
No colonies after transformation with DNA inserts and the transformation control did not work.	Low transformation efficiency.	Perform the transformation procedure exactly as described.
	Competent *E. coli* cells were handled incorrectly.	Competent *E. coli* cells are very fragile. Handle the cells gently and resuspend them by pipetting up and down gently.
		Do not vortex the competent *E. coli* cells.
		Do not freeze/thaw competent *E. coli* cells. They can only be thawed once without dramatic loss in competency.
		Store competent *E. coli* cells at –80°C.

Observation	Possible cause	Recommended action
No colonies after transformation with DNA inserts and the transformation control did not work. *(continued)*	Insufficient amount of *E. coli* cells plated	Increase the amount of *E. coli* cells plated.
	Transformants plated on selective plates containing the wrong antibiotic.	Use the appropriate antibiotic for selection.
No colonies after transformation with DNA inserts, but transformation with the control assembly reaction is successful.	PCR products were not pure enough.	Repeat PCR amplification and purify product using a different method of purification.
	DNA fragments do not share the required end-terminal homology.	Make sure that your DNA fragments and the linearized cloning vector share the required 20-40 bp end-terminal homology. Refer to "Guidelines for designing PCR primers" on page 9 for the requirements on PCR primer design.
	DNA fragment ends generated by PCR were damaged.	Employ extra caution to minimize any potential damage to the ends of your DNA fragments by leaving the gel on the gel tray when exposing to UV light, using low UV power, and minimizing the time the gel is exposed to UV light. Note that an additional isopropanol precipitation after gel purification might be required to obtain the best results.
	Incorrect amounts of DNA fragments and/or vector were used.	Make sure that you use the correct amounts of DNA fragments and/or vectors for cloning. For maximum cloning efficiency, use a 1:1 molar ratio of vector:insert(s).
	The GeneArt™ Gibson Assembly® HiFi Enzyme Mix was handled incorrectly.	Quickly thaw the Enzyme Mix on ice, and immediately return to –20°C after use. The enzyme mix may be subjected to 20 freeze/thaw cycles without a loss in activity.
		Do not leave the Enzyme Mix at room temperature or on ice for extended periods of time.
Large number of the transformants contain no insert.	Cloning vector was incompletely linearized.	It is crucial that your cloning vector is fully linearized and any uncut vector is removed prior to the cloning and assembly reaction. If necessary, recut your vector and gel purify.
	Plates were too old or contained incorrect antibiotic.	Make sure to use freshly prepared LB plates containing the selection antibiotic appropriate for your cloning vector.
	The GeneArt™ Gibson Assembly® HiFi cloning reaction was not set up correctly.	Make sure to set up the reaction according to the guidelines in "Guidelines for GeneArt™ Gibson Assembly HiFi cloning reactions" on page 13.

Observation	Possible cause	Recommended action
Large number of the transformants contain no insert. *(continued)*	Incubation time was too short or too long.	Make sure that you incubate the cloning and assembly reaction mix for 13 or 60 minutes, depending on the number of fragments according to the table in "Guidelines for GeneArt™ Gibson Assembly HiFi cloning reactions" on page 13. After incubation, immediately proceed to transformation.
	Cloning and assembly reaction was not performed at the correct temperature.	Make sure to perform the seamless cloning and assembly reaction at 50°C.
Large number of the transformants contain incorrect insert.	PCR products were not pure enough.	If your PCR product is not a single distinct band, then it may be necessary to gel purify the PCR product to ensure cloning of the correct insert.

A Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

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  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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