GeneArt™ Gibson Assembly® HiFi Cloning Kits

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

Catalog Numbers A46624, A46626, A46627, A46628 and A46629

Pub. No. MAN0019062 **Rev.** D

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Gibson Assembly® method allows seamless, one-step, single-tube assembly of multiple overlapping DNA fragments in an isothermal reaction. This flexible assembly methodology works for both small and large DNA constructs, accommodating single or multiple inserts. The resulting products are suitable for various downstream applications, including transformation, PCR, rolling circle amplification (RCA), synthetic biology, in vitro cell-free workflows, and mutagenesis.

GeneArt™ Gibson Assembly® HiFi Master Mix, with its proprietary enzyme blend, ensures high-fidelity assembly with a low rate of junction errors, offering a seamless alternative to traditional restriction-ligation methods.

Features of GeneArt™ Gibson Assembly® HiFi Cloning Kits

- **One-step, single-tube reaction with scarless fragment assembly**
  Simplifies the cloning process by using complementary overlaps to join DNA fragments without the need of restriction enzymes and ligases.
- **High accuracy and fidelity**
  Precise assembly with low rates of junction errors, providing high sequence fidelity.
- **Multiple insert assembly for a wide range of fragment size**
  Capable of assembling up to five DNA fragments plus a vector in one reaction, suitable for assembling DNA fragments ranging from 0.5 to 32 kb.

Contents and storage

Component	A46624 [1] 10 reactions	A46626 [2] 10 reactions	A46627 [3] 10 reactions	A46628 [3] 50 reactions	A46629 [3] 200 reactions	Storage
GeneArt™ Gibson Assembly® HiFi Master Mix	100 µL	100 µL	100 µL	500 µL	2 mL	–20°C
Positive Control [4]	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

[1] GeneArt™ Gibson Assembly® HiFi Cloning Kit, Chemically Competent Cells
[2] GeneArt™ Gibson Assembly® HiFi Cloning Kit, Electrocompetent Cells
[3] GeneArt™ Gibson Assembly® HiFi Master Mix
[4] 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.

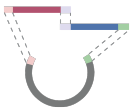
Experimental outline

The following steps provide an overview of the procedure used to assemble a recombinant DNA molecule using the GeneArt™ Gibson Assembly® HiFi Cloning Kit.

1 Obtain DNA fragments

1. Design homologous ends for DNA fragment assembly (see page 3).
2. Obtain GeneArt™ Strings™ DNA fragments or amplify the fragment using PCR (see page 3).
3. Linearize vector by PCR amplification or by restriction digestion (see page 6).

2 Perform Gibson Assembly® reaction



1. Combine DNA fragments (either purified or directly PCR product) with GeneArt™ Gibson Assembly® HiFi Master Mix.
   - Direct from PCR fragment assembly (see page 7).
   - Purified fragment assembly (see page 8).
2. Incubate at 50°C for 15 to 60 minutes.
3. (*Optional*) *In vitro* cell-free fragment assembly and amplification using RCA (see page 13).

   Note: If performing RCA, transforming competent cells and screening colonies is not required.

3 Transform competent cells

1. Add diluted reaction mix to the competent cells.
   - Use chemically competent cells:
     - Rapid protocol (see page 9).
     - High efficiency protocol (see page 10).
   - Use electrocompetent cells (see page 11).
2. Plate transformation mix on LB agar plates with selective antibiotic and incubate overnight at 37°C.

4 Screen colonies for construct

1. Pick 4 to 12 colonies to screen for insert.
2. Analyze plasmid DNA using colony PCR, restriction enzymes or sequencing (see page 12).

Obtain DNA fragments

DNA fragments can be obtained by the following methods.

- Build a fragment *de novo* using GeneArt™ Strings™ DNA fragment synthesis services.

  Note: Strings™ sequences can be optimized using GeneArt™ GeneOptimizer™ software to improve downstream mRNA stability and protein expression.

- Use PCR amplification with primers that introduce homologous regions for directional assembly (see page 3).

Guidelines for DNA fragment design

- Small 0.1-0.5 kb fragments can be cloned but have less efficiency (as low as 40%), depending on factors such as the number of fragments being assembled.
- 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, include homologies directly in the sequence design. If fragments are generated from existing DNA templates via PCR, primers used for generating inserts must contain the 20-40 nt overhangs on their 5'-ends to provide homology with the adjacent fragments.
- The optimal length of the homologous fragment overlap 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.

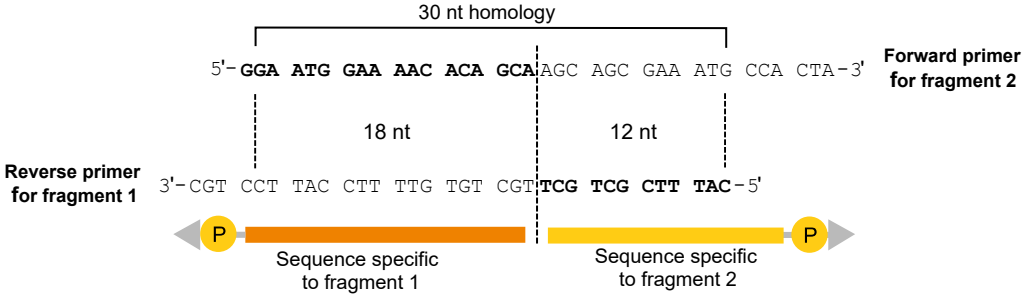
Number of fragments	Fragment size	Length of overlap regions
1–5 [1]	≤8 kb	20–40 bp
	>8 kb	40–100 bp

[1] For higher order assembly, longer overlap regions can result in higher efficiency. PCR amplification conditions may need optimization when using PCR primers with long homologous overlap regions.

Prepare DNA inserts by PCR

Guidelines for designing PCR primers

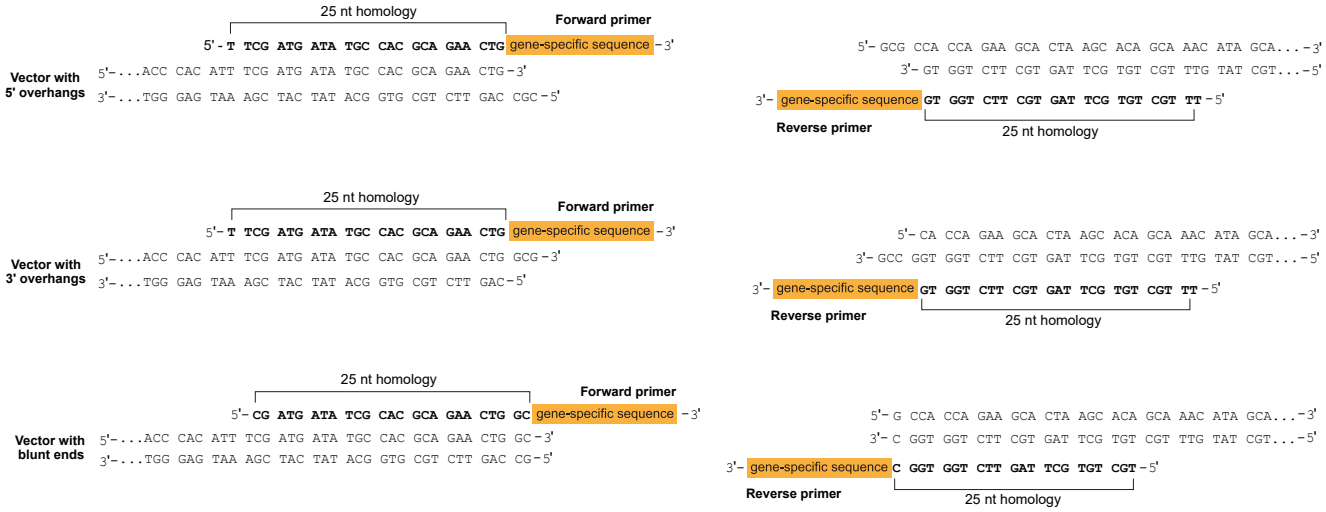
- While primers can be easily designed by hand, using appropriate software for primer design is recommended. Most modernn molecular biology cloning programs support design of Gibson Assembly® experiments (e.g., SnapGene, Benchling, VectorBee, or the open source Python pydna package).
- Use PCR primers ~65 nt in length (20-40 nt for the requisite homology at the 5' end, and 18-25 nt specific to the DNA element).
- Ensure the 5'-ends of each primer pair (forward and reverse) include a 20-40 nt overhang that is homologous to the 20-40 bp sequence at one end of the adjacent DNA fragment (i.e., the vector or another insert).
- Selecting overlapping regions of homology with T_m >50°C can improve efficiency.
- For higher-order assembly, longer overlap regions (40 bases) can result in improved efficiency.
- Ensure the 3'-end of each primer is specific to the DNA element you want to amplify.
- The primer should not include tandem repeats, homopolymers, or sequences with high secondary structure. Avoid GC content below 40% or above 60%.
- If recombining the insert to a linearized vector, all 20-40 nt requisite homology sequences must be on the 5'-end of the primer.
- When recombining two adjacent inserts, the 20-40 bp homology can be split between the fragments in any combination (e.g., for a a 30 bp region of homology, the sequences can be split 12+18, 15+15, 25+5, etc.).
  In the following figure, the reverse primer of fragment 1 provides 12 nt and the forward primer of fragment 2 contributes 18 nt of a 30 bp region of homology. The primer sequences that are part of the homologous regions are shown in bold; the remaining sequences of each primer, i.e., the 3'-ends, are specific to the DNA element to be assembled.



Note: If the required 20-40 bp homology between adjacent fragments is split, one PCR primer can have as few as 18 nt with no overhang whatsoever. For example, in a 0+40 homology split, the reverse primer of fragment 1 will not contribute to homology and

consists of only 18-25 gene-specific nucleotides for fragment 1, while the forward primer of fragment 2 would contribute the entire 40 bp for homology in addition to having 18-25 gene-specific nucleotides for fragment 2 (for a total length of 58-65 nt).

- Splitting homology between the primers to avoid long primers is recommended.
- (*Optional*) Incorporate a restriction enzyme site into the primers between the overlap region flanking insert that will allow subsequent release of the insert from the vector (for example using a Type IIs restriction enzymes for Golden Gate cloning). Ensure that the restriction enzyme site is not present in the insert itself.
- (*Optional*) Prepare DNA inserts for assembly in linear vectors created by linearization of the vector by restriction digestion.



This figure shows PCR primers used for preparing DNA inserts for assembly in linear vectors with 5' overhangs, 3' overhangs, and blunt ends generated by restriction digestion. The primer sequences that are part of the homologous regions (in this example 25 nt) are shown in bold. The remaining sequences of each primer, i.e., the 3' ends, are specific to the DNA element to be assembled.

Guidelines for PCR

- If using circular plasmid DNA as template, use 0.1–10 ng of plasmid DNA to minimize background colonies after transformation. Treat the PCR product with DpnI before setting up the GeneArt™ Gibson Assembly® HiFi cloning reaction is recommended.
- If using *E. coli* or human genomic DNA, increase the amount of template DNA to 5–100 ng.
- PCR products can be used directly in assembly reactions without additional purification (see page 7), although results may be improved by purifying the DNA fragments through a PCR cleanup kit (e.g., the PureLink™ PCR Purification Kit, Cat. No. K310001).
- After preparing DNA fragments by PCR, verify the PCR products by gel electrophoresis (e.g., using the E-Gel™ Power Snap Plus Electrophoresis System, Cat. No. G9301). If multiple bands are observed, purification of the DNA fragments is recommended (e.g., using E-Gel™ CloneWell™ II Agarose Gels, Cat. No. G661818, or the GeneJET™ Gel Extraction Kit, Cat. No. K0691).

Required materials not supplied

- Platinum™ SuperFi II PCR Master Mix (Cat. Nos. 12368050, 12369050)
- PCR primers (100 µM each in nuclease-free water)
- Sterile nuclease-free water
- FastDigest™ DpnI (Cat. No. FD1703)
- Thermocycler

Perform PCR

1. Thaw PCR reagents on ice.

2. Set up the PCR reagents in microcentrifuge tubes on ice, as described in the following table:

	20 µL reaction	50 µL reaction	Final concentration
2X Platinum™ SuperFi II PCR Master Mix	10 µL	25 µL	1X
Forward primer	*x* µL	*x* µL	0.2–0.5 µM
Reverse primer	*x* µL	*x* µL	0.2–0.5 µM
Template DNA	*x* µL	*x* µL	0.1–10 ng (plasmid) 5–100 ng (gDNA)
Nuclease-free water	fill to 20 µL	fill to 50 µL	—

3. Incubate reactions in a thermocycler using the following parameters.

Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing [1] Extension	98°C 60°C 72°C	5–10 seconds 10 seconds 15–30 seconds per kb	25–35
Final extension	72°C	5 min	1
Hold	4°C	—	1 (Hold)

[1] Universal annealling temperature if using Platinum™ SuperFi II DNA Polymerase.

4. (*For PCR from plasmid DNA templates*) Digest PCR template with DpnI restriction endonuclease.
   a. Add 1 µL FastDigest™ DpnI to the PCR mixture.
   b. Mix thoroughly and incubate at 37°C for 15 minutes.
   c. Incubate at 80°C for 5 minutes to inactivate the restriction enzyme. Hold at 4°C.

5. Verify PCR product purity by gel electrophoresis (e.g., the E-Gel™ Power Snap Plus Electrophoresis System, Cat. No. G9301).

   If multiple bands are observed, purifiy the DNA fragments (e.g., using E-Gel™ CloneWell™ II Agarose Gels, Cat. No. G661818, or the GeneJET™ Gel Extraction Kit, Cat. No. K0691).

6. Proceed to “GeneArt™ Gibson Assembly® HiFi reaction (Direct from PCR)” on page 7.

7. (*Optional*) Purify the DNA fragments using a PCR cleanup kit (e.g., the PureLink™ PCR Purification Kit, Cat. No. K310001), then proceed to “GeneArt™ Gibson Assembly® HiFi reaction (Purified fragments)” on page 8.

Prepare 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.

- 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

- 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, treating the PCR ampflication product with DpnI to reduce circular template carryover is recommended.
- When generating the linearized vector by restriction digestion, use two different restriction enzymes rather than a single enzyme to reduce the amount of background. A double digest followed by PCR amplification of the linear vector is recommended.
- Use restriction enzymes that leave 3' protruding, 5' protruding, or blunt ends to linearize the cloning vector.
- It is very important to have complete digestion (i.e., very low background of uncut vector). Therefore, increasing the enzyme digestion time (2-3 hours to overnight) and the reaction volume is recommended.
- Analyze 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, using 2-5 µg of vector with 30-50 units of enzyme in a reaction volume of 100-200 µL is recommended.
- If the same linearized vector is used for multiple reactions, digest 20-50 µg of vector using 150-300 units of each restriction enzyme in a reaction volume of 400-800 µL. After purification, make aliquots of the linearized vector and store at -20°C.

Perform Gibson Assembly® reaction

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

- Devised a DNA assembly strategy and possibly verified it by performing *in silico* cloning using an appropriate software tool.
- Prepared DNA fragments (i.e., inserts) according to the guidelines in "Prepare DNA inserts by PCR" on page 3.
- Generated a linear cloning vector according to the guidelines in "Prepare linearized E. coli cloning vector" on page 6.

GeneArt™ Gibson Assembly® HiFi reaction (Direct from PCR)

When using circular plasmid DNA as a template, it is important to use a minimal amount of DNA to reduce the template background after transformation. Treating the PCR product with DpnI restriction endonuclease to remove the plasmid template before setting up the GeneArt™ Gibson Assembly® HiFi DNA assembly reaction is necessary to minimize background colonies.

Required materials

- PCR product
- Linearized *E. coli* cloning vector
- Positive Control
- GeneArt™ Gibson Assembly® HiFi Master Mix
- Sterile nuclease-free water
- Thermocycler and/or water bath

Perform GeneArt™ Gibson Assembly® HiFi cloning reaction direct from PCR

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

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 (PCR) Assembly	Positive Control [1]
Amount of each PCR product	up to 1 µL vector [2] up to 1 µL each insert [2]	10 µL
GeneArt™ Gibson Assembly® HiFi Master Mix	10 µL	10 µL
Nuclease-free water	fill to 20 µL	—

[1] The positive control reagents contain all necessary fragments.
[2] Adding 1 µL of each PCR product is recommended. The total amount of PCR products (for vector and inserts) must not exceed 4 µL.

4. Mix the reactions by vortexing, briefly centrifuge, and incubate at 50°C for 15 minutes.
5. After incubation, place the reaction mix on ice and immediately proceed to "Transform competent cells" on page 9.

   Note: Reactions can also be stored at –20°C for later use, but transformation efficiency decreases with prolonged storage.
6. (*Optional*) Proceed to "In vitro DNA fragment assembly and Rolling Circle Amplification (RCA)" on page 13.

GeneArt™ Gibson Assembly® HiFi reaction (Purified fragments)

Note: These guidelines only apply when performing assembly using purified DNA fragments with measured concentrations.

- 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.

Required materials

- DNA fragments
- Linearized *E. coli* cloning vector
- Positive Control
- GeneArt™ Gibson Assembly® HiFi Master Mix
- Sterile nuclease-free water
- Thermocycler and/or water bath

Perform GeneArt™ Gibson Assembly® HiFi cloning reaction

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

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]
Amount of each fragment (A 1:1 molar ratio of vector to fragment is recommended)	0.08 pmol vector, 0.08 pmol each fragment up to a total of 10 µL combined		10 µL
GeneArt™ Gibson Assembly® HiFi Master Mix	10 µL		10 µL
Nuclease-free water	fill to 20 µL		—
Incubation time at 50°C	15 minutes	60 minutes	15 minutes

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

4. Mix the reactions by vortexing, spin down and incubate at 50°C for the recommended time.
5. After incubation, place the reaction mix on ice and immediately proceed to “Transform competent cells” on page 9.

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

   Note: For complex assemblies (e.g., >5 inserts) increasing the reaction volume and using ElectroMAX™ DH10B electrocompetent cells for transformation is recommended.
6. (*Optional*) Proceed to “In vitro DNA fragment assembly and Rolling Circle Amplification (RCA)” on page 13.

Transform competent cells

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

Required materials

- GeneArt™ Gibson Assembly® reaction product
- One Shot™ TOP10 Chemically Competent *E. coli*
- (*for high efficiency protocol*) S.O.C. medium
- (*for high efficiency protocol*) 42°C water bath or heat block

  Note: Better results are obtained using a water bath because of better heat transfer properties of liquid.
- Luria-Bertani (LB) plates with appropriate antibiotic (e.g., One Shot™ LB Agar Plates with 100 µg/mL Ampicillin)
- 37°C shaking and non-shaking incubator
- *(Optional)* pUC19 Control DNA for the transformation control reaction

Prepare reaction

1. Warm the water bath or heat block to 42°C.
2. Warm the vial of S.O.C. medium to room temperature.
3. Warm selective plates at 37°C until the plates are warm to the touch.

   Note: It is especially important for plates reach 37°C to obtain the best results.
4. Thaw One Shot™ Chemically Competent *E. coli* cells on ice immediately before transformation.
   - Thaw one vial for each transformation.
   - Do not hold vials in hand or expose to warmth, as higher temperatures significantly decreases cell competency.
   - Centrifuge vials briefly after thawing to collect cell suspension droplets.
5. Proceed to “Transform cells (Rapid protocol)”, or “Transform cells (High efficiency protocol)” on page 10.

Transform cells (Rapid protocol)

Note: This protocol is only recommended for transformation using ampicillin selection.

1. Dilute the GeneArt™ Gibson HiFi cloning reaction (from “GeneArt™ Gibson Assembly® HiFi reaction (Direct from PCR)” on page 7 or “GeneArt™ Gibson Assembly® HiFi reaction (Purified fragments)” on page 8) with nuclease-free water.
   a. Use a 1:4 (v/v) dilution of the assembly reaction with nuclease-free water (e.g., 3 µL GeneArt™ Gibson cloning reaction and 12 µL nuclease-free water)

      Note: A 5-fold dilution is optimal. Higher DNA input does not necessarily equal higher CFU output.
   b. Mix the reaction by vortexing.

      IMPORTANT! Do not mix by pipetting up and down.
   c. Keep the diluted reaction on ice until ready to add to the competent cells.
2. Add 1 µL of the dilution into a vial of One Shot™ TOP10 Chemically Competent *E. coli* cells and mix gently by stirring with a clean pipette tip.

   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 5 minutes.
4. Spread 50 µL from each transformation on a prewarmed ampicillin selective LB agar plate.

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

5. Incubate the plates overnight at 37°C.

6. 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 cells (High efficiency protocol)

1. Dilute the GeneArt™ Gibson HiFi cloning reaction (from "GeneArt™ Gibson Assembly® HiFi reaction (Direct from PCR)" on page 7 or "GeneArt™ Gibson Assembly® HiFi reaction (Purified fragments)" on page 8) with nuclease-free water.
   a. Use a 1:4 (v/v) dilution of the cloning reaction with nuclease-free water (e.g., 3 µL GeneArt™ Gibson cloning reaction and 12 µL nuclease-free water)

      Note: A 5-fold dilution is optimal. Higher DNA input does not neccesarily equal higher CFU output.

   b. Mix the reaction by vortexing.

      IMPORTANT! Do not mix by pipetting up and down.

   c. Keep the diluted reaction on ice until ready to add to the competent cells.

2. Add 1 µL of the dilution into a vial of One Shot™ TOP10 Chemically Competent *E. coli* cells and mix gently by stirring with a clean pipette tip.

   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 at 42°C for 30 seconds 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 prewarmed selective plate. Before spreading on selective plates, mix suspension by pipetting up and down. If you have performed an assembly using 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 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

Required materials

- GeneArt™ Gibson Assembly® reaction product
- ElectroMAX™ DH10B Cells
- S.O.C. medium
- Electroporation cuvettes
- Electroporator
- Luria-Bertani (LB) plates with appropriate antibiotic (e.g., One Shot™ LB Agar Plates with 100 µg/mL Ampicillin)
- 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 immediately before transformation.
3. Mix the reactions by vortexing then centrifuge briefly before proceeding with transformation.
4. Dilute the GeneArt™ Gibson Assembly® HiFi cloning reaction 1:4 (v/v) 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. Mix thawed 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.

   Note: Ensure there is no water condensate on the exterior of the chilled cuvette.
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 (1:9 to 1:49 v/v) or desalt the GeneArt™ Gibson Assembly® HiFi cloning reaction (e.g., by ethanol precipitation) before 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.

Screen colonies for construct

Once the GeneArt™ Gibson Assembly® cloning reaction and the transformation procedure are complete, 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 (e.g., using the E-Gel™ Power Snap Plus Electrophoresis System, Cat. No. G9301). Alternatively, screen for the presence of the insert(s) by colony PCR. Calculate cloning efficiency using the following formula:

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

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 the PureLink™ HQ Mini Plasmid DNA Purification Kit (Cat. No. K210001) or the PureLink™ HiPure Plasmid Miniprep Kit (Cat. No. K210002).

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, add 19.2 µL Platinum™ Direct PCR Universal 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 12).

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 (e.g., using the E-Gel™ Power Snap Plus Electrophoresis System, Cat. No. G9301).

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.

In vitro DNA fragment assembly and Rolling Circle Amplification (RCA)

Since the product of Gibson Assembly® cloning is circular, it can be amplified using Rolling Circle Amplification (RCA), an efficient isothermal method for amplification of circular nucleic acid templates. A strand-displacing polymerase like EquiPhi29™ DNA Polymerase produces high yields of long concatemeric products within just 2 hours from a minimal amount of template. These amplified products are suitable for various downstream applications, including sequencing, cell-free protein expression, and restriction digestion.

Assemble your fragments as described in "Perform Gibson Assembly® reaction" on page 7. Purifying fragments for Gibson Assembly® is recommended if they were amplified using PCR.

Note: Similar RCA product yield can be achieved after 15-, 35- or 60-minute Gibson Assembly® reactions. For more information see the **"From DNA construction to protein in 1 day"** application note at **thermofisher.com**.

Required materials not supplied

- EquiPhi29™ DNA Amplification Kit (Cat. No. A65393)
- GeneArt™ Gibson Assembly® reaction product
- Sterile nuclease-free water (Cat. No. R0581)

Perform RCA

1. Thaw RCA reagents on ice.
2. Set up the RCA reagents in microcentrifuge tubes on ice, as described in the following table:

	20 µL reaction	Final concentration
10X EquiPhi29™ DNA Polymerase Reaction Buffer	2 µL	1X
100 mM DTT	0.2 µL	1 mM
500 µM Exo-Resistant Random Primer	2 µL	50 µM
10 mM dNTP mix	2 µL	1 mM each
Template	1 µL	10X diluted Gibson Assembly® reaction product or Nuclease-free water as non-template control (NTC)
Nuclease-free water	fill to 18 µL	—

3. Incubate the prepared reaction mix in a thermal cycler at 95°C for 3 minutes to denature the DNA, then immediately place the tube back on ice.
4. Gently vortex the mixture, then briefly centrifuge the tube to collect the components from the walls of the tube.
5. Add polymerase and pyrophosphatase to the reaction.

	20 µL reaction	Final concentration
EquiPhi29™ DNA Polymerase	1 µL	0.5 U/µL
Pyrophosphatase	1 µL	0.005 U/µL

6. Incubate the reaction at 42°C for 2 hours.
7. Heat for 10 minutes at 65°C to inactivate the polymerase.
8. Analyze product: RCA reaction yield can be quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Cat. No. P7589); quality can be assessed by analyzing intact and digested RCA product using restriction enzymes by gel electrophoresis with E-Gel™ EX Agarose Gels, 1% (Cat. No. G401001).

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.
	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. See "Guidelines for designing PCR primers" on page 3 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.	The enzyme mix may be subjected to 20 freeze/thaw cycles without a loss in activity.
		Enzyme Mix is stable at room temperature for at least two months.
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 "GeneArt™ Gibson Assembly® HiFi reaction (Purified fragments)" on page 8.
	Incubation time was too short or too long.	Make sure that you incubate the cloning and assembly reaction mix for 15 or 60 minutes, depending on the number of fragments according to the table in "Perform GeneArt™ Gibson Assembly® HiFi cloning reaction" on page 8. 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.

Limited product warranty

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Revision	Date	Description
D	6 June 2025	Change of publication format. Update of transformation procedure.
C	28 September 2020	New document for GeneArt™ Gibson Assembly® HiFi Cloning Kits.

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