USER GUIDE

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GeneArt® Seamless Cloning and Assembly Kit

For highly-efficient, simultaneous and seamless *in vitro* assembly of up to 4 DNA fragments plus a vector in a pre-determined order

Catalog Number A13288

Document Part Number A13333
Publication Number MAN0003222
Revision A.0

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Contents

About this guide

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Revision history

Revision	Date	Description
A.0	November 2014	• Updated link to web-based oligo designer tool. • Updated user guide template with associated updates to covers, legal, document support, and safety sections.

Product information

Product description

GeneArt® Seamless Cloning and Assembly Technology

The GeneArt® Seamless Cloning and Assembly Technology is a highly efficient, vector-independent system for the simultaneous and seamless assembly of up to 4 DNA fragments and a vector, totaling up to 13 kb in length. The system allows the cloning of the DNA fragments into virtually any linearized *E. coli* vector, requires no pre-existing recombination sites or extra DNA sequences, and eliminates the need for extensive enzymatic treatments of the DNA such as restriction and ligation. The enzyme mix provided with the GeneArt® Seamless Cloning and Assembly Kit recognizes and precisely assembles the DNA fragments sharing a 15-base pair (bp) end homology that you can create by PCR-amplification.

Note: Although we recommend using the GeneArt® Seamless Cloning and Assembly Kit for assembling up to 4 DNA fragments plus a vector, you can use it to assemble a larger number of fragments; however, the cloning efficiency will be lower. We have successfully assembled up to 6 DNA fragments and a vector. The web tool, GeneArt® Primer and Construct Design Tool, only supports the seamless assembly of up to 4 fragments.

How the GeneArt® Seamless Cloning and Assembly Kit works

Engineering DNA molecules by homologous recombination presents an alternative to traditional methods using restriction endonucleases and ligases. The GeneArt® Seamless Cloning and Assembly Kit takes advantage of a proprietary enzyme mix to fuse together DNA fragments that share terminal end-homology in an *in vitro* cloning reaction. This kit can be used for seamlessly assembling up to 4 DNA fragments and a vector simultaneously and in a precise and pre-determined order.

To seamlessly assemble up to 4 DNA fragments into a single recombinant DNA molecule using GeneArt® Seamless Cloning and Assembly Kit:

1. Combine the linear cloning vector (pUC19L or your own linear *E. coli* vector) and the DNA fragments to assemble in a microcentrifuge tube.

   Note: We recommend that you use GeneArt® Primer and Construct Design Tool, available at **www.lifetechnologies.com/order/oligoDesigner**, to design PCR primers to create end-terminal homology between your adjacent DNA fragments.

2. Add appropriate amounts of the 5X Reaction Buffer and 10X Enzyme Mix and incubate the tube for 30 minutes at room temperature.

   Note: The 10X Enzyme Mix must be added to the reaction mix as the last component.

3. Transform the assembled DNA molecule into One Shot® TOP10 Chemically Competent *E. coli*.

GeneArt® Primer and Construct Design Tool

The GeneArt® Primer and Construct Design Tool is an intuitive, web-based tool to guide you when you are designing your cloning construct and DNA fragments. The tool minimizes the planning time required for designing the PCR primers to amplify your DNA fragments and to create the end-terminal homology required for seamless assembly, identifies potential pitfalls linked to your specific sequences, and performs *in silico* cloning using your sequences. The GeneArt® Primer and Construct Design Tool also provides you with a graphic representation of the final assembled molecule as well as a downloadable GenBank file compatible with

VectorNTI® and other software for molecular biology workflows.

The GeneArt® Primer and Construct Design Tool is available at: **www.lifetechnologies.com/order/oligoDesigner**.

Advantages of the GeneArt® Seamless Cloning and Assembly Kit

- **Speed** – Facilitates the simultaneous assembly of up to 4 DNA fragments, totaling up to 13 kb in length (including the cloning vector).
- **Simplicity** – Greatly reduces *in vitro* handling of DNA and eliminates the need for enzymatic treatments of DNA such as restriction and ligation.
- **Precision** – Enables seamless assembly of DNA fragments in a precise and pre-determined order without extra sequences.
- **Flexibility** – Facilitates the assembly of multiple DNA fragments from any source using any linearized *E. coli* cloning vector as long as adjacent fragments share 15-nucleotide end-terminal homology and allows site-specific insertion and deletion of desired sequences through fragment editing.
- **Efficiency** – Generates up to 90% positive clones depending on the number of DNA fragments assembled.

Kit contents and storage

Component	Amount (20 reactions)	Storage
10X Enzyme Mix	45 µL	–80°C
5X Enzyme Buffer	90 µL	
Linear pUC19L Vector (50 ng/µL), sufficient for 4 control reactions	8 µL	
Control insert (50 ng/µL)	5 µL	
One Shot® TOP10 Chemically Competent *E. coli*	21 × 50 µL	
pUC19 Control (10 pg/µL)	10 µL	
S.O.C. media	6 mL	

Kit components

The GeneArt® Seamless Cloning and Assembly Kit contains the following components, which are sufficient for 20 cloning reactions and one control reaction.

- 10X Enzyme Mix and 5X Enzyme Buffer sufficient for 20 *in vitro* assembly reactions.
- Linear pUC19L Vector, a 2,659 bp linearized E. coli plasmid, as a cloning control vector for the assembly of DNA fragments. The vector pUC19L can be used as a cloning vector, if desired.
- Control insert, a 1,268 bp DNA fragment sharing a 15 bp homology with the linear pUC19L vector at both ends, for use as a control in the assembly reaction.
- One Shot® TOP10 Chemically Competent *E. coli* and S.O.C. medium for large-scale plasmid preps of the assembled DNA construct for downstream applications.
- pUC19 control DNA for assessing the transformation efficiency of the One Shot® TOP10 Chemically Competent *E. coli*.

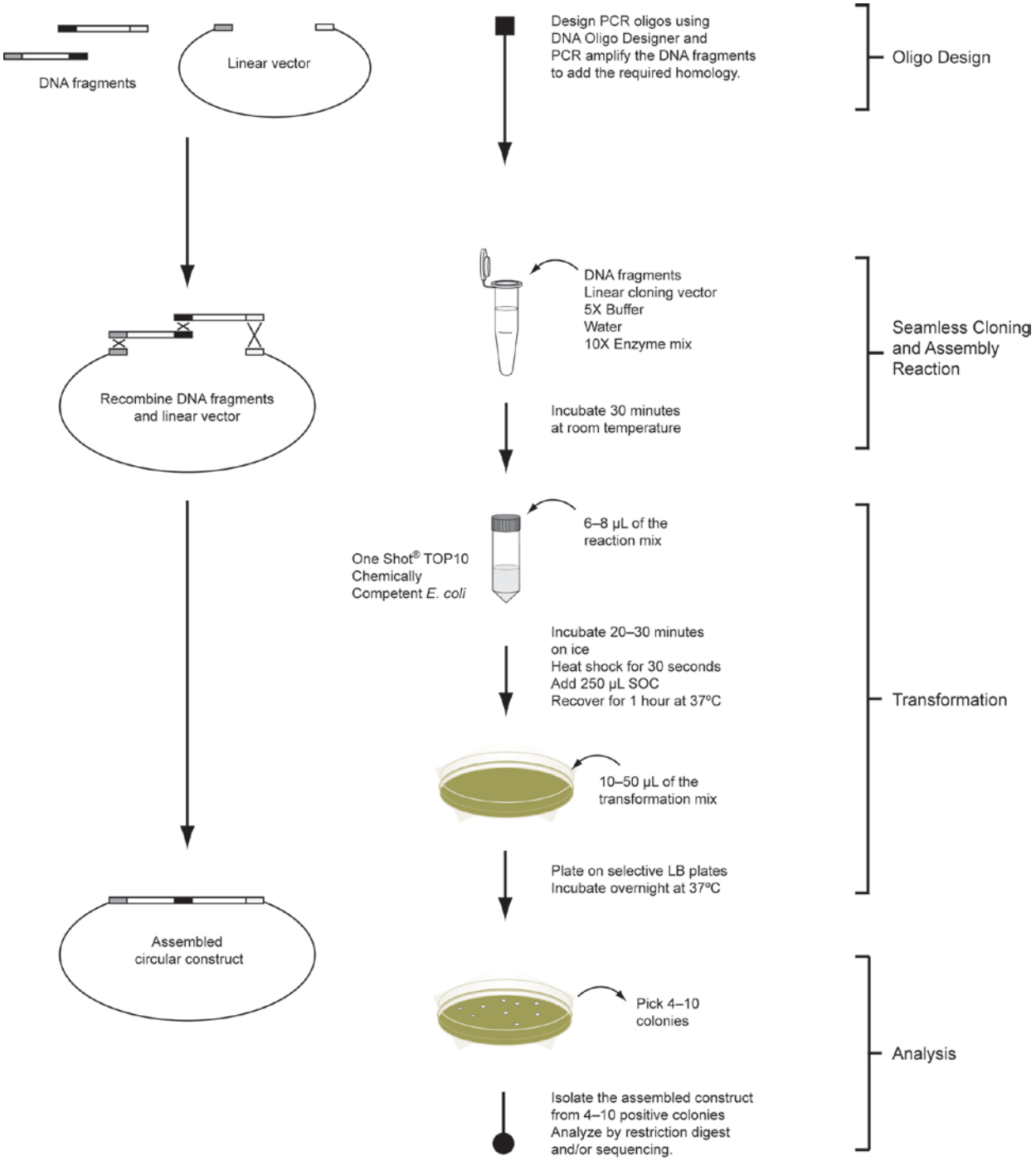
Tranformation efficiency

One Shot® TOP10 Chemically Competent *E. coli,* included in the GeneArt® Seamless Cloning and Assembly Kit, have a transformation efficiency of $\geq 1 \times 10^9$ transformants per µg plasmid DNA.

Genotype

The genotype of One Shot® TOP10 Chemically Competent *E. coli* is: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ^-

Workflow


DNA fragments
Linear vector
Recombine DNA fragments and linear vector
Assembled circular construct
Design PCR oligos using DNA Oligo Designer and PCR amplify the DNA fragments to add the required homology.
Oligo Design
DNA fragments
Linear cloning vector
5X Buffer
Water
10X Enzyme mix
Incubate 30 minutes at room temperature
Seamless Cloning and Assembly Reaction
6–8 µL of the reaction mix
One Shot® TOP10 Chemically Competent E. coli
Incubate 20–30 minutes on ice
Heat shock for 30 seconds
Add 250 µL SOC
Recover for 1 hour at 37°C
10–50 µL of the transformation mix
Plate on selective LB plates
Incubate overnight at 37°C
Transformation
Pick 4–10 colonies
Isolate the assembled construct from 4–10 positive colonies
Analyze by restriction digest and/or sequencing.
Analysis

Experimental outline

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

Step	Action	Page
1	Use the GeneArt® Primer and Construct Design Tool web-tool, to develop your DNA assembly strategy	5
2	Generate your linearized cloning vector (*optional*)	10
3	PCR amplify or synthesize your DNA fragments	11
4	Perform the *in vitro* cloning and assembly reaction	15
5	Transform One Shot® TOP10 Chemically Competent *E. coli*	17
6	Analyze positive colonies by restriction analysis and/or sequencing	19

Generate a linearized *E. coli* cloning vector

Introduction

The GeneArt® Seamless Cloning and 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® Seamless Cloning and Assembly Kit **must** be linear.

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

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 restriction digest, we recommend that you digest the vector with two restriction enzymes rather than a single enzyme to reduce the amount of background.
- You can use restriction enzymes that leave 3′ protruding, 5′ protruding, or blunt ends to linearize your cloning vector. However, we recommend that you use a restriction enzyme that produces blunt or 3′ protruding ends when possible for achieving maximum cloning efficiency.
- It is very important to have a complete digest (i.e., very low background of uncut vector). Therefore, we recommend that you increase the enzyme digestion time (2–3 hours to overnight) and the reaction volume.
- Double digestion with two restriction enzymes is the most efficient way of linearizing your cloning vector (see guidelines above). A double digest followed by PCR amplification of your linear vector virtually eliminates any background.
- Analyze your restriction digestion products using agarose gel electrophoresis to verify that the digest is complete and then purify the digested vector using PureLink® PCR Purification Kit (see page 25 for ordering information) 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.

Note: You can also use the Linear pUC19L Vector, included as a cloning control vector in the GeneArt® Seamless Cloning and Assembly Kit, for your *in vitro* cloning and assembly reaction. This ready-to-use cloning vector is also available separately as the GeneArt® Linear pUC19L Vector for Seamless Cloning (Cat. no. A13289); the ready-to-use vector provides sufficient linear vector for 20 *in vitro* seamless cloning reactions. For ordering information, see page 25.

Prepare DNA inserts by PCR

Guidelines for PCR primers

GeneArt® Seamless Cloning and Assembly reaction requires that each DNA fragment share a 15-bp (base pair) end-terminal homology with the adjacent fragment (including the cloning vector). Therefore, PCR primers used for generating your inserts must have 15-nucleotide 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).

Follow the guidelines below when designing your PCR primers:

- Design your PCR primers such that each DNA fragment to be assembled is between 100 bp and 5 kb (kilo base pairs) 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 15-nucleotide sequence (i.e., an overhang) that is homologous to the 15 bases 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 15 nucleotides providing the requisite homology must be on the 5' end of the primer. To recombine two adjacent inserts, you may split the 15-bp homology between the fragments (e.g., 7 bp on the reverse primer of fragment 1 and 8 bp on the forward primer of fragment 2).

  Note: You can split the 15-bp homology between adjacent fragments in any combination (e.g., 7+8 as in the example above or 12+3, 11+4 etc.).

- PCR primers should be up to 40 nucleotides in length (15 nucleotides to provide the requisite homology at the 5′ end and 18–25 nucleotides specific to your DNA element).

  Note: If you are splitting the required 15-bp homology between adjacent fragments, one PCR primer may have as few as 18 nucleotides and no overhang whatsoever. For example, in a 0+15 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 15 bp for the homology in addition to having 18–25 gene specific nucleotides for fragment 2 (for a total length of 33–40 nucleotides).

- 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 15-nucleotide homology.

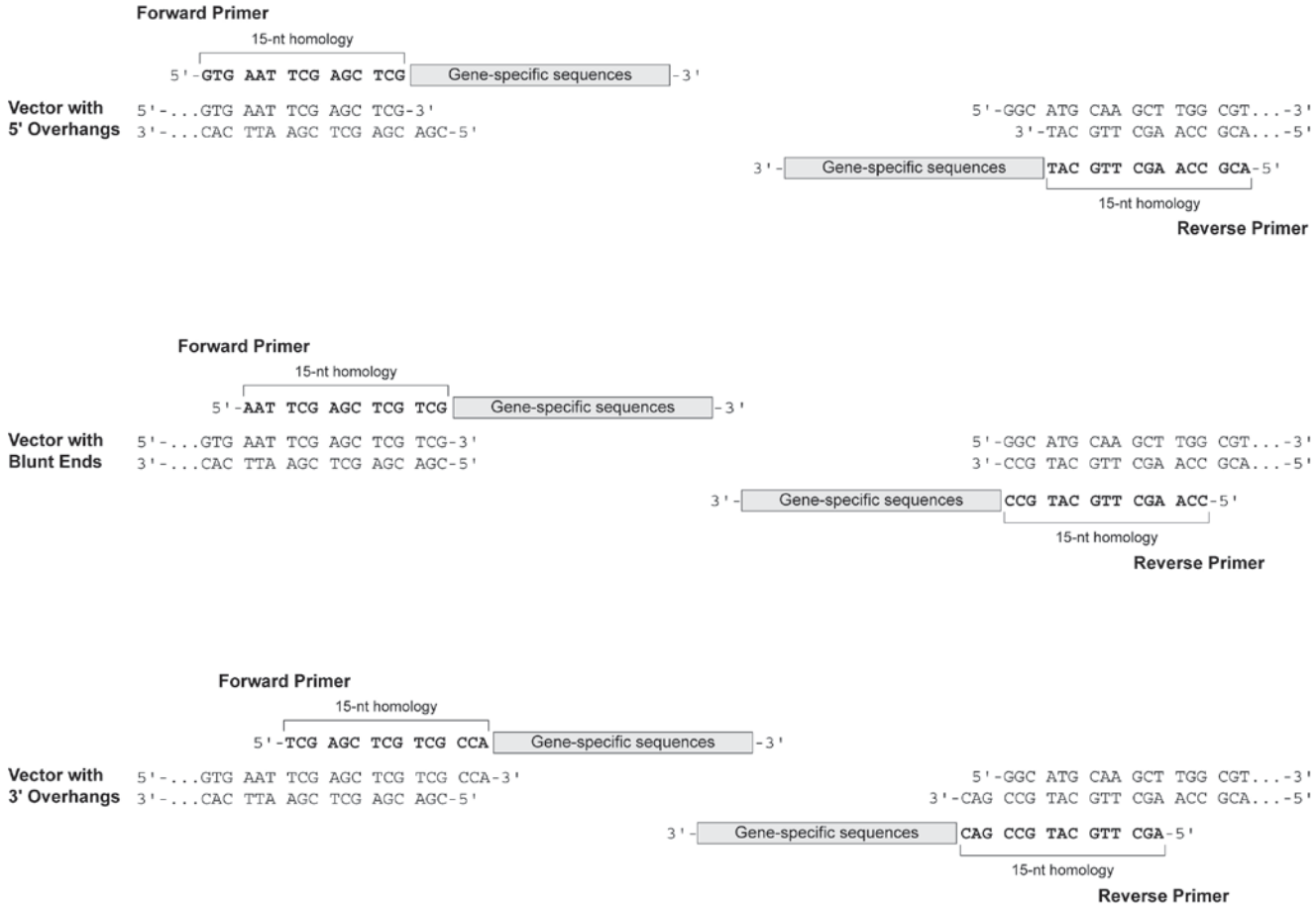
- Prepare each primer pair at a concentration of 100 µM in DNase- and RNase-free water.

Examples for PCR primers

The requisite 15-bp end-terminal homology between the vector and the insert (or between two adjacent inserts) is generated by the PCR primers used for amplifying the inserts. To determine the 15-nucleotide sequence providing the homology, start at the 3′ end of each DNA strand in the linearized vector or the adjacent insert.

PCR primers for attaching the DNA fragment to the vector

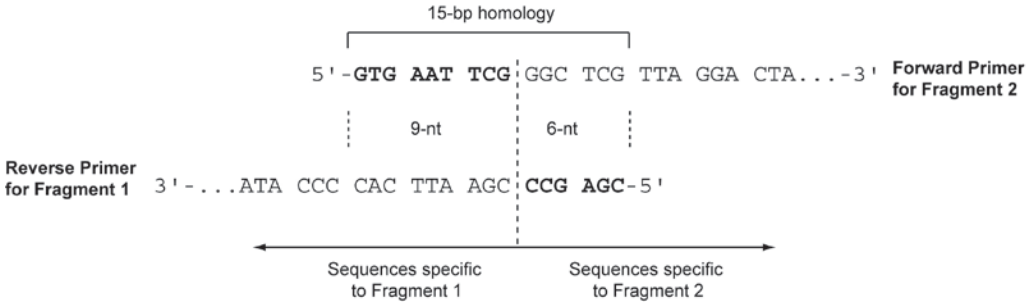
The figure below shows examples of PCR primers used for preparing the DNA inserts for assembly in linear vectors with 5′ overhangs, blunt ends, and 3′ overhangs. The primer sequences providing the 15-bp homology are shown in bold; the remaining sequences of each primer (i.e., the 3′ end) are specific 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 assembly. When connecting two adjacent inserts, you may split the 15-bp homology between the fragments in any combination.

In the example below, the reverse primer of fragment 1 and the forward primer of fragment 2 respectively provide 6 bp and 9 bp of the 15-bp homology required for recombination. The primer sequences providing 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: We highly recommend that you use the GeneArt® Primer and Construct Design Tool to verify the sequence of your DNA fragments (i.e., inserts) and your assembly strategy, and to design your PCR primers that generate the required overlap for each DNA fragment. The GeneArt® Primer and Construct Design Tool is available at: **www.lifetechnologies.com/order/oligoDesigner**.

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 AccuPrime™ *Pfx* SuperMix (see page 25 for ordering information) 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 primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- If you are using DNA fragments prepared by PCR, you must purify the DNA fragments through a PCR cleanup kit. We recommend that you use the PureLink® PCR Purification Kit (see page 25 for ordering information).
- After preparing your DNA fragments by PCR, verify the PCR products by gel electrophoresis. If you obtain multiple bands, you must gel purify your DNA fragments. We recommend using the PureLink® Quick Gel Extraction Kit for best results (see page 25 for ordering information). Elute the DNA fragments from the PureLink® column using water.
- When gel purifying your DNA fragments, employ extra caution to minimize any potential damage to the ends of the DNA fragments by leaving the gel on the gel tray when exposing it 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 solubilization might be required to obtain the best results.

  Note: Gel purifying DNA fragments will result in somewhat lower cloning efficiency.
- Large fragments (>5 kb) are more susceptible to damage in a gel extraction procedure. Therefore, we recommend that you assemble multiple fragments of ≤5 kb in one reaction rather than a single large fragment.
- You can skip the PCR purification step and use an unpurified insert in a single fragment assembly (i.e., cloning a single insert into the cloning vector) without significant decrease in cloning efficiency; however, we recommend using purified PCR inserts in a multifragment assembly.

Seamless cloning and assembly reaction

Before you begin

Before you set up your seamless cloning and assembly reaction, make sure that you have:

- Devised your DNA assembly strategy and verified it by performing *in silico* cloning using the GeneArt® Primer and Construct Design Tool, available at: **www.lifetechnologies.com/order/oligoDesigner**.
- Generated your linear cloning vector according to the guidelines on page 8.

  Note: You can also use the linear pUC19L Vector for your seamless cloning and assembly reaction. The linear pUC19L allows you to perform blue-white screening for the presence of the insert; the white colonies should contain the inserts, while the blue ones should be mostly empty vector.
- Prepared your DNA fragments (i.e., inserts) according to the guidelines on pages 11–14.

Materials needed

- DNA fragments to assemble (up to 4 DNA fragments for final construct of 13 kb in length, including the cloning vector)
- Linear pUC19L vector or your own linearized *E. coli* cloning vector
- Control insert (for assessing the efficiency of the assembly reaction)
- 5X Reaction Buffer
- 10X Enzyme mix
- Deionized, sterile water

Calculate molar ratios

For maximum cloning efficiency, use 2:1 insert:vector molar 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 2:1 molar ratio of insert to vector.

1. Determine the concentration of your insert in µg/mL.
2. Use the following formula to calculate the amount of insert needed to give a molar ratio of 2:1 between insert and linearized pUC19L. Note that the amount of pUC19L is 100 ng.

$$\text{x ng insert} = \frac{(2)\ (\text{bp insert})\ (100\text{ ng linearized pUC19L})}{(2{,}659\text{ bp pUC19L})}$$

   Note: If you are using your own linearized cloning vector, replace the divisor in the above equation with the number of base pairs in your vector.
3. Based on the calculation above, calculate the volumes needed for the seamless cloning and assembly reaction.

Note: In the case of very small inserts (≤ 200 bp), we recommend that you use at least 20 ng of insert DNA in the seamless cloning and assembly reaction.

IMPORTANT! Do not leave the 10X Enzyme Mix at room temperature, on ice, or at –20°C for extended periods of time. The enzyme mix quickly loses activity at these conditions.

- It is crucial to add the 10X Enzyme Mix to the seamless assembly and cloning reaction separately at the end after you have mixed all the other reaction components.
- After adding the 10X Enzyme Mix to the reaction mixture, promptly return it to –80°C.

Seamless cloning and assembly reaction

1. In a microcentrifuge tube, set up the seamless cloning and assembly reaction.

   IMPORTANT! It is crucial that you add the 10X Enzyme Mix as the last component (see Step 2).

Insert(s) (20–200 ng each)	x µL*
Linear pUC19L vector (100 ng)†	2 µL*
5X Reaction Buffer	4 µL
Deionized water	to 18 µL

   *For maximum cloning efficiency, use a 2:1 molar ratio of insert:vector (see previous page). The volume of the vector and insert(s) used depends on their concentrations.

   †You can also use 100 ng your own linearized cloning vector.

2. Quickly thaw the 10X Enzyme Mix on ice, add 2 µL to the reaction mix, and immediately return it back to –80°C.

   IMPORTANT! Do not leave the 10X Enzyme Mix at room temperature, on ice, or at –20°C for extended periods of time.

3. Mix the reaction components by gently tapping the sides of the centrifuge tube and incubate at room temperature for 30 minutes.
4. After 30 minutes of incubation, place the reaction mix on ice and immediately proceed to the transformation step.

Note: Do not let the samples stay on ice for more than 5 minutes before transformation.

Transform One Shot® TOP10 Chemically Competent *E. coli*

Materials needed

- Seamless cloning and assembly reaction mix (from step 4, previous page)
- One Shot® TOP10 Chemically Competent *E. coli*

  Note: Do **not** use electrocompetent *E. coli* for transformation. Only chemically competent cells result in successful seamless cloning and assembly of DNA fragments.
- S.O.C. medium
- 42°C water bath
- LB plates containing the appropriate selection antibiotic. If you have used the linear pUC19L vector for cloning, use LB plates containing 50–100 µg/mL ampicillin and X-Gal (see Recipes, page 23).

  Note: The linear pUC19L allows you to perform blue-white screening for the presence of the insert; most of the white colonies should contain the vector inserts, while the blue ones should mostly have the empty vector.
- 37°C shaking and non-shaking incubator
- *Optional*: pUC19 Control DNA for the transformation control reaction

Preparation

Each transformation requires one vial of competent cells and two selective plates.

1. Equilibrate a water bath to 42°C.
2. Warm the vial of SOC 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* for each transformation.

One Shot® TOP10 Chemical transformation

1. Add 6–8 µL of the seamless cloning and assembly reaction from step 4, page 16, into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently.

   IMPORTANT! Do not mix by pipetting up and down.

   Note: If you are performing transformation control, add 2.5 µL of pUC19 Control DNA into a separate vial of One Shot® TOP10 Chemically Competent *E. coli* and follow the transformation procedure.
2. Incubate the transformation mix on ice for 20 to 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice and incubate on ice for 2 minutes.
5. Add 250 µL of room temperature S.O.C. medium to the transformation mix.
6. Cap the tube tightly and shake it horizontally (200 rpm) at 37°C for 1 hour.
7. After incubation, dilute the transformations 1:10 in S.O.C. medium and spread 10–50 µL from each transformation on a pre-warmed selective plate. If you have performed a 4-fragment assembly, plate the transformations undiluted. We recommend that you plate two different volumes to ensure that at least one plate has well-spaced colonies.
8. Incubate the plates overnight at 37°C.
9. The next day, pick individual colonies (pick white colonies if you have used the linear pUC19L vector) and isolate the plasmid DNA or screen for the presence of the insert(s) by colony PCR.

Control experiment

Control reactions

When using the GeneArt® Seamless Cloning and Assembly Kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your seamless cloning and assembly reaction to verify that the kit components are performing properly.

The Linear pUC19L Vector, a 2.7 kb linearized *E. coli* plasmid, and the Control Insert, a 1,268 bp DNA fragment sharing a 15 bp homology with the linear pUC19L vector at both ends, are included in the GeneArt® Seamless Cloning and Assembly Kit for use in the control reactions.

Note: The GeneArt® Seamless Cloning and Assembly Kit contains sufficient reagents (5X Enzyme Buffer, 10X Enzyme Mix, and One Shot® TOP10 competent cells) for 20 cloning and assembly reactions plus one control reaction. If you wish to perform both the positive and negative control reactions, the remaining reagents are sufficient for only 18 cloning and assembly reactions.

1. Set up the positive and negative control reactions as described below.

   IMPORTANT! It is crucial that you add the 10X Enzyme Mix last.

Component	Positive control	Negative control
Deionized water	10 µL	12 µL
Linear pUC19L Vector (50 ng/µL)	2 µL	2 µL
Control insert (50 ng/µL)	2 µL	2 µL
5X Enzyme Buffer	4 µL	4 µL
10X Enzyme Mix	2 µL	–
Total volume	20 µL	20 µL

2. Mix the reaction components by gently tapping the sides of the centrifuge tube and incubate at room temperature for 30 minutes.
3. After 30 minutes of incubation, place the reaction mix on ice and immediately proceed to the transformation step (page 17).

   Note: Do not let the samples stay on ice for more than 5 minutes before transformation.
4. Plate the control transformations on LB plates containing 50–100 µg/mL ampicillin and 30–100 µg/mL X-Gal for blue-white screening.

Expected results

You can expect >90% white colonies on your positive control and mostly blue colonies on your negative control plates. Note that you may still get white (i.e., positive) colonies on your negative control plates at a very low frequency. The transformation efficiency of One Shot® TOP10 Chemically Competent *E. coli* should be $\geq 2 \times 10^8$ transformants per µg pUC19 control plasmid.

Analyze transformants

Introduction

Once you have performed the seamless cloning and assembly 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.

Analyze positive clones

1. Pick 4–10 colonies and culture them overnight in LB medium containing the appropriate selection antibiotic for your cloning vector (100 µg/mL ampicillin if you have used the linear pUC19L vector for cloning).
   Note: Pick 10 colonies for an assembly reaction involving 4 fragments plus the vector. For assembly reactions involving less than 4 fragments, 4–6 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 (see page 25 for ordering information). Refer to **www.lifetechnologies.com** or contact Technical Support for more information on a large selection of plasmid purification columns.
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 during amplification.

Analyze transformants by colony PCR

You may use PCR to directly analyze positive transformants.

- For PCR primers, use a pair of "diagnostic primers" (forward and reverse) so that the colony PCR products would span the insert.
- If your insert is too large to amplify in a single PCR, you may pick a diagnostic primer on the vector and another on one of the inserts.
- You will have to determine the amplification conditions.
- If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that the PCR gives you the correct result.

Materials Needed

- PCR SuperMix (see page 25 for ordering information)
- Appropriate forwards and reverse PCR primers (20 µM each)
- LB plates containing the appropriate selection antibiotic

Procedure

1. For each sample, aliquot 48 µL PCR SuperMix into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 10 colonies and resuspend them individually in 50 µL of the PCR cocktail from step 1, above.

Note: If you have used the linear pUC19L vector for the seamless cloning and assembly reaction and plated the transformed cells on LB plates containing X-Gal, pick white colonies.

3. Streak each colony on an LB plate plates containing the appropriate selection antibiotic to save for preparing glycerol stocks (see Long Term Storage, below).
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 to 30 cycles using the amplification conditions you have determined.
6. For the final extension, incubate the reaction at 72°C for 10 minutes. Store at the reactions at 4°C.
7. Visualize the results by agarose gel electrophoresis.

Long-term storage

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.
   Note: If you have used the linear pUC19L vector for cloning, use LB plates containing 100 µg/mL ampicillin.
2. Isolate a single colony and inoculate with it 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.

Troubleshooting

The table below lists some potential problems and solutions that help you troubleshoot your experiments using the GeneArt® Seamless Cloning and Assembly Kit.

Symptom	Cause	Solution
No colonies after transformation with DNA inserts and the transformation control did not work	Low transformation efficiency	Perform the transformation procedure exactly as described on page 17.
	One Shot® TOP10 Chemically Competent *E. coli* 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 the One Shot® TOP10 Chemically Competent *E. coli*. Competent *E. coli* can only be thawed once without dramatic loss in competency. • Store One Shot® TOP10 Chemically Competent E. coli at –80°C.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection. If you are using the linear pUC19L cloning vector, use 50 µg/mL ampicillin.
No colonies after transformation with DNA inserts, but transformation with control assembly reaction is successful	PCR products not pure enough	• Repeat PCR amplification and purify product using a different method of purification. • Alternatively, perform phenol:chloroform extraction on your original PCR product, followed by ethanol precipitation.
	DNA fragments do not share the required end-terminal homology	Make sure that your DNA fragments and the linearized- cloning vector share the required 15-bp end-terminal homology. Refer to page 11 for the requirements on PCR primer design.
	Ends of the DNA fragments 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 vector for cloning. • For maximum cloning efficiency, use a 2:1 molar ratio of insert:vector.

Symptom	Cause	Solution
No colonies after transformation, but the control transformation is successful	10X Enzyme Mix handled incorrectly	• Quickly thaw the 10X Enzyme Mix on ice, and immediately return to –80°C after use. The enzyme mix may be subjected to 20 freeze/thaw cycles without a loss in activity. • Do **not** leave the 10X Enzyme Mix at room temperature, on ice, or at –20°C for extended periods of time. • Use the reaction cocktail and the enzyme mix promptly; do **not** keep them for any extended period of time before starting the cloning reaction.
Large number of the transformants contain no insert	Cloning vector 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. • Alternatively, use the Linear pUC19L Vector for your cloning reaction.
	Plates too old or contained incorrect antibiotic	Makes sure to use freshly prepared LB plates containing the selection antibiotic appropriate for your cloning vector.
	10X Enzyme Mix was not added last	It is crucial that you add the 10X Enzyme Mix to the reaction mix as the last component.
	Incubation time was too short or too long	Make sure that you incubate the cloning and assembly reaction mix for 30 minutes. After incubation, immediately proceed to transformation.
	Cloning and assembly reaction was not performed at room temperature	Make sure to perform the seamless cloning and assembly reaction at room temperature.
Large number of the transformants contain incorrect insert	PCR products 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.

Appendix

Recipes

LB (Luria-Bertani) medium and plates

LB medium:

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL of deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave the solution on liquid cycle for 20 minutes at 15 psi. Allow the solution to cool to 55°C and add the appropriate antibiotics, if needed.
4. Store the medium at room temperature or at 4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave the medium plus agar on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool the medium to ~55°C, add the appropriate antibiotics, and pour into 10 cm plates.
4. Let the agar harden, then invert the plates and store them at 4°C, in the dark.

X-Gal stock solution

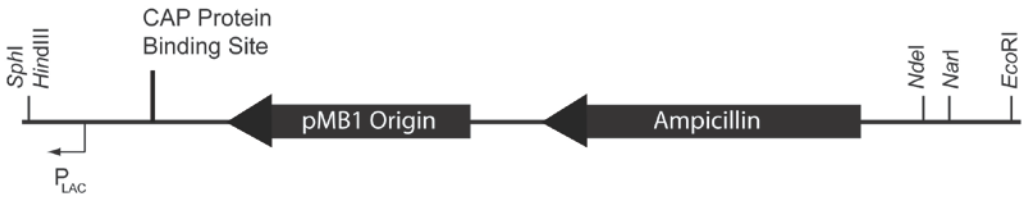
1. To prepare a 40 mg/mL X-Gal stock solution, dissolve 400 mg of X-Gal in 10 mL of dimethylformamide. Protect the X-Gal solution from light by storing it in a brown bottle at –20°C.
2. To add X-Gal to previously made agar plates, warm the plate to 37°C. Add 40 µL of the 40 mg/mL X-Gal stock solution onto the plate, spread it evenly, and let it dry for 15 minutes. Protect the plates from light.

pUC19L

Map of pUC19L

The figure below summarizes the features of the pUC19L cloning vector (2,659 bp). The vector is supplied linearized

The complete sequence and the restriction map for pUC19L is available for downloading at **www.lifetechnologies.com** or by contacting Technical Support (see page 26).



Comments for pUC19L:
2,659 nucleotides

P_{LAC} promoter: bases 75–104 (c)
CAP protein binding site: bases 124–136
pMB1 origin of replication: bases 428–1,016 (c)
Ampicillin (bla) resistance gene: bases 1,187–2,047 (c)

(c) = complementary strand

Features of pUC191L

The linear pUC19L (2,659 bp) vector contains the following elements. All features have been functionally tested.

Feature	Benefit
CAP protein site	Site where CAP (cAMP binding protein) binds to activate transcription
pMB1 origin	Allows high-copy replication and growth in *E. coli*
P_{LAC} promoter	Drives the expression of *lacZ*α (for alpha complementation)
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*
bla promoter	Allows the expression of ampicillin resistance gene
*Hin*dIII, *Eco*RI	Rare restriction enzyme recognition sites that could be used for restriction mapping of the final assembled construct

Accessory products

GeneArt® DNA assembly products

Some of the components of the GeneArt® Seamless Cloning and Assembly Kit are also available separately. These products are listed below. In addition, other GeneArt® products can be used for seamless *in vivo* assembly of up to 10 DNA fragments and vector and for site directed mutagenesis. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 26).

Product	Amount	Cat. no.
GeneArt® Seamless Cloning and Assembly Kit	1 kit	A13288
GeneArt® Linear pUC19L Vector for Seamless Cloning	20 reactions	A13289
GeneArt® High-Order Genetic Assembly System	1 kit	A13285
GeneArt® High-Order Genetic Assembly System *(with Yeast Growth Media)*	1 kit	A13286
GeneArt® High-Order Linear pYES1L Vector	10 reactions	A13287
GeneArt® High-Order Vector Conversion Cassette	10 reactions	A13291
CSM Media for MaV203 Yeast Cells	1 kit	A13292
GeneArt® Site-Directed Mutagenesis System	1 kit	A13282

Additional products

The products listed below may be used with the GeneArt® Seamless Cloning and Assembly Kit. For more information, refer **www.lifetechnologies.com** or contact Technical Support (see page 26).

Product	Amount	Cat. no.
AccuPrime™ *Pfx* SuperMix	200 reactions	12344-040
PCR SuperMix	100 reactions	10572-014
PCR SuperMix High Fidelity	100 reactions	10790-020
Platinum® *Taq* DNA Polymerase	100 reactions 500 reactions	10966-018 10966-034
Platinum® PCR SuperMix High Fidelity	100 reactions	12532-016
PureLink® PCR Purification Kit	50 preps	K3100-01
PureLink® Quick Gel Extraction Kit	1 kit	K2100-12
PureLink® HiPure Plasmid Miniprep	25 preps 100 preps	K2100-02 K2100-03
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
LB Broth	500 mL	10855-021
LB Agar	500 g	22700-025
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10

Documentation and support

Customer and technical support

Visit **www.lifetechnologies.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  – Product FAQs
  – Software, patches, and updates
- Order and web support
- Product documentation, including:
  – User guides, manuals, and protocols
  – Certificates of Analysis
  – Safety Data Sheets (SDSs; also known as MSDSs)
  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies web site at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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