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GeneArt® Seamless PLUS Cloning and Assembly Kit

For highly-efficient, simultaneous, and seamless *in vitro* assembly of up to 4 DNA inserts plus any vector totaling up to 40 kb in length in a pre-determined order

Catalog Number A14603

Publication Number MAN0006685
Revision 3.0

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Contents

Kit Contents and Storage

Kit Components

The GeneArt® Seamless PLUS Cloning and Assembly Kit is shipped on dry ice, and it contains the components listed below, which are sufficient for 20 reactions at 5 µL total volume per reaction. **Store the kit components as indicated**. For convenience, you may store the entire contents of the kit at –80°C.

Box	Component	Amount	Storage
1	GeneArt® 2X Enzyme Mix	100 µL	–80°C
	Linear pYES7L Vector (50 ng/µL)	20 µL	–20°C
	Control *lacZ* insert	5 µL	–20°C
	Linear pUC19L Vector (50 ng/µL)	40 µL	–20°C
	Control insert (50 ng/µL)	5 µL	–20°C
2	One Shot® DH10B™ T1^R SA Cells	21 × 50 µL	–80°C
	pUC19 Control (10 pg/µL)	10 µL	–80°C
	Stbl3™/pRK2013 Glycerol Stock*	1 mL	–80°C
	S.O.C. medium	6 mL	4°C

*Stbl3™ *E. coli* cells containing the helper plasmid pRK2013 used in tri-parental mating for horizontal DNA transfer.

One Shot® DH10B™ T1^R SA Competent Cells

The high transformation efficiency of One Shot® DH10B™ T1^R SA Chemically Competent *E. coli* makes them ideally suited for transformation with the small, medium, and large constructs assembled GeneArt® Seamless PLUS Cloning and Assembly Kit.

Genotype of DH10B™ T1^R SA Cells

F$^-$ mcrA Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ* ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*) 7697 *galU galK* λ$^-$ *rpsL nupG tonA*

Genotype of Stbl3™ Cells

F$^-$ *mcrB mrr hsdS*20(r_B^-, m_B^-) *recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20*(Str^R) *xyl*-5 λ$^-$ *leu mtl*-1
Note: This strain is *endA1+*

Product Use

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Description of the System

GeneArt® Seamless PLUS Cloning and Assembly Kit

GeneArt® Seamless PLUS Cloning and Assembly Technology

The GeneArt® Seamless PLUS Cloning and Assembly Technology is a highly efficient, vector-independent system for the simultaneous and seamless assembly of up to 4 DNA inserts between 100 bp and 10 kb and any vector, totaling up to 40 kb in length.

The system allows the cloning of the DNA inserts 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 PLUS Cloning and Assembly Kit recognizes and precisely assembles the DNA inserts sharing an end-terminal homology of between 15 bp and 80 bp, depending on the total construct size (see Table 1, page 9).

How the GeneArt® Seamless PLUS 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 PLUS Cloning and Assembly Kit takes advantage of a proprietary enzyme mix to fuse together DNA inserts that share terminal end-homology in an *in vitro* cloning reaction.

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

1. Generate the DNA inserts you wish to assemble by PCR amplification or by excising them from a plasmid by restriction digest. You can also purchase synthesized DNA fragments at **www.lifetechnologies.com/geneart**.

   Note: We recommend that you use our web-based GeneArt® Primer and Construct Design Tool, available at **http://bioinfo.invitrogen.com/oligoDesigner**, to design the PCR primers for creating the required end-terminal homology between your adjacent DNA inserts.
2. Combine the linear cloning vector (pYES7L, pUC19L, or your own linear *E. coli* vector) and the DNA inserts to assemble in a microcentrifuge tube.
3. Add the appropriate amount of GeneArt® 2X Enzyme Mix and incubate the tube at room temperature for 15–60 minutes, depending on the final construct size and the length of the end-terminal homology between the DNA inserts.

   Note: The GeneArt® 2X Enzyme Mix must be added to the reaction mix as the last component.
4. Transform the assembled DNA molecule into One Shot® DH10B™ T1^{R} SA Cells.
5. *Optional:* If you have used the linear pYES7L vector, perform tri-parental mating to horizontally transfer your recombinant construct into a recipient strain without *in vitro* manipulating the DNA.

Continued on next page

GeneArt® Seamless PLUS Cloning and Assembly Kit, continued

Although we recommend using the GeneArt® Seamless PLUS Cloning and Assembly Kit for assembling up to 4 DNA inserts plus a vector, totaling up to 40 kb in length, you can use it to assemble a larger number of inserts or to create constructs that exceed 40 kb in length; however, the cloning efficiency and the number of transformants will be lower. We have had success with assembling up to 7 DNA inserts and a vector, as well as cloning inserts totaling up to 50 kb and a vector. Note that the GeneArt® Primer and Construct Design Tool only supports the seamless assembly of up to 4 inserts.

Components of the GeneArt® Seamless PLUS Cloning and Assembly Kit

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

- GeneArt® 2X Enzyme Mix sufficient for 20 *in vitro* assembly reactions.
- Linear pYES7L Vector, a 7,266-bp linearized, broad-range, conjugative cloning vector for the assembly of DNA inserts. You can horizontally transfer the recombinant DNA construct assembled on pYES7L into a variety of recipient organisms, if desired.
- Control *lacZ* insert, a 1.6 kb DNA insert sharing a 15-bp homology with the linear pYES7L vector at both ends, for use as a control in the assembly reaction. Use the Control *lacZ* insert with the linear pYES7L vector.
- Linear pUC19L Vector, a 2,659-bp linearized *E. coli* plasmid, as a cloning control vector for the assembly of DNA inserts. You can also use pUC19L as a repository vector for generating pre-cloned fragments or as a final cloning vector, if desired.
- Control insert, a 1.3 kb DNA insert sharing a 15-bp homology with the linear pUC19L vector at both ends, for use as a control in the assembly reaction. Use the Control insert with the linear pUC19L vector.
- One Shot® DH10B™ $T1^R$ SA Chemically Competent *E. coli* for large-scale plasmid preps of the assembled DNA construct for downstream applications. Use the S.O.C. medium, supplied with the kit, for the recovery of cells after transformation.
- pUC19 control DNA for assessing the transformation efficiency of the One Shot® DH10B™ $T1^R$ SA Cells.
- Stbl3™/pRK2013 Glycerol Stock (Stbl3™ *E. coli* strain containing the helper plasmid pRK2013) for use in tri-parental mating to facilitate the horizontal transfer of the assembled DNA construct from the donor strain into recipient cells.

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Linear pYES7L Vector

The pYES7L vector is a 7,266-bp linearized, high-capacity, broad-range conjugative cloning vector developed for the assembly of DNA inserts using the GeneArt® Seamless PLUS Cloning and Assembly Kit.

Because the pYES7L vector can mobilize and replicate in a variety of hosts thanks to the presence of RK2 broad-range host replication features (*oriV* and *trfA* gene) and yeast replication elements (ARS4/CEN5), it can be used to transfer the assembled inserts from donor *E. coli* cells into a wide range of recipient organisms such as *Agrobacterium tumefaciens*, *Rhizobium meliloti*, and *Saccharomyces cerevisiae* (see **Horizontal DNA Transfer**, page 28).

For a map and features of the linear pYES7L vector, see page 37.

Linear pUC19L Vector

The pUC19L vector is a 2,659-bp linearized *E. coli* plasmid, which is included as a cloning control vector in the GeneArt® Seamless PLUS Cloning and Assembly Kit.

The linear pUC19L Vector can also be used as a cloning vector for *in vitro* seamless assembly reactions and subsequent blue-white screening of transformants for the presence of the insert; upon transformation, most of the white colonies should contain the vector inserts, while the blue ones should mostly have the empty vector.

For a map and features of the linear pUC19L vector, see page 38.

Note: The linear pUC19L vector is also available separately from Life Technologies as the GeneArt® Linear pUC19L Vector for Seamless Cloning (Cat. no. A13289). For ordering information, see page 39.

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 inserts. The tool minimizes the time required for designing the PCR primers to amplify your DNA inserts and to create the end-terminal homology required for seamless assembly, identifies potential pitfalls linked to your specific sequences, performs *in silico* cloning using your sequences, and allows one-click online ordering of custom primers (for countries with enabled online ordering). In addition to designing the oligos used in cloning and assembly reactions, 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 **http://bioinfo.invitrogen.com/oligoDesigner**. For more information, see page 33.

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Advantages of the GeneArt® Seamless PLUS Cloning and Assembly Kit

- **Speed** – Facilitates the simultaneous assembly of up to 4 DNA inserts plus a vector, totaling up to 40 kb in length.
  Note: You can use the GeneArt® Seamless PLUS Cloning and Assembly Kit to assemble a larger number of inserts or to create constructs that exceed 40 kb in length; however, the cloning efficiency will be lower.
- **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 inserts in a precise and pre-determined order without extra sequences.
- **Flexibility** – Facilitates the assembly of multiple DNA inserts from any source using any linearized *E. coli* cloning vector as long as adjacent inserts share end-terminal homology.
- **Efficiency** – Generates up to 90% positive clones depending on the number of DNA inserts assembled.
- **Broad range** – Final assembled construct can be horizontally transferred into Gram-negative bacteria and yeast (see **Horizontal DNA Transfer**, page 28, for considerations on the capacity).

Possible Applications

Using the GeneArt® Seamless PLUS Cloning and Assembly Kit, you can clone multiple DNA inserts into a single vector without subcloning, create modular expression vectors with interchangeable parts, construct seamless fusion proteins, delete and replace DNA segments, make internal protein fusions, swap tags on a gene, add UTRs to a cDNA, insert restriction sites, and more.

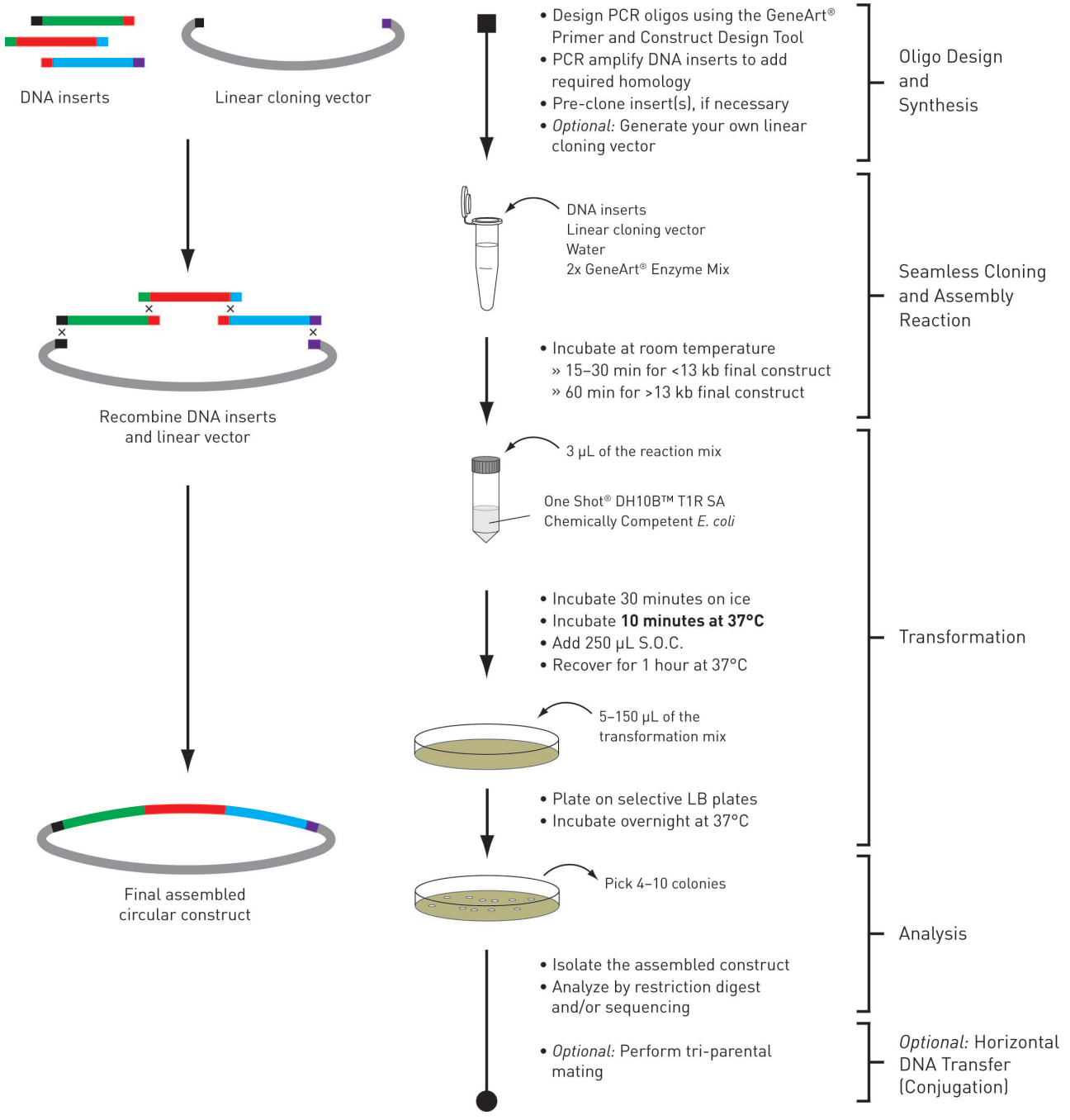
Some of the many applications that will benefit from the GeneArt® Seamless PLUS Cloning and Assembly Kit are the following:

- Cloning and protein expression, including difficult and large-inserts cloning
- Synthetic biology-related applications
- Drug discovery
- Biofuels
- Bioremediation
- Plant biotechnology
- Synthetic vaccines
- Bioproduction of chemicals and drugs
- Metabolic engineering

GeneArt® Seamless PLUS Cloning and Assembly Workflow

Workflow

The figure below summarizes the comprehensive workflow for the GeneArt® Seamless PLUS Cloning and Assembly Kit.



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GeneArt® Seamless PLUS Cloning and Assembly Workflow, continued

Experimental Outline

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

Step	Action	Page
1	Using the GeneArt® Primer and Construct Design Tool, develop your DNA assembly strategy and perform *in silico* cloning to verify it	33
2	Generate your linearized cloning vector (*optional*)	10
3	PCR amplify your DNA inserts	11
4	Pre-clone you insert(s), if necessary	16
5	Perform the *in vitro* cloning and assembly reaction	20
6	Transform One Shot® DH10B™ T1^R SA Cells	22
7	Analyze positive colonies by restriction analysis and/or sequencing	25
8	Perform tri-parental mating (i.e., conjugation) to horizontally transfer your assembled construct into Gram-negative bacteria or yeast cells (*optional*)	28

Methods

Guidelines for Successful Cloning and Assembly

Number of DNA Inserts and Final Construct Size

We recommend using the GeneArt® Seamless PLUS Cloning and Assembly Kit for assembling up to 4 DNA inserts plus a vector, totaling up to 40 kb in length. However, you can use the kit to assemble a larger number of inserts or to create constructs that exceed 40 kb in length, but the cloning efficiency and colony output will be lower. Note that the GeneArt® Primer and Construct Design Tool only supports the seamless assembly of up to 4 inserts.

Note: We have had success with assembling up to 7 DNA inserts and a vector, as well as cloning inserts totaling up to 50 kb and a vector.

Requirement for Linear Cloning Vector

While the GeneArt® Seamless PLUS Cloning and Assembly technology allows the cloning of the DNA inserts into virtually any linearized *E. coli* vector, because of the mechanism of action of the GeneArt® Enzyme Mix, the cloning vector and the DNA inserts used with the kit **must** be linear.

For guidelines on generating a linearized *E. coli* cloning vector, see **Generating a Linearized *E. coli* Cloning Vector**, page 10.

pYES7L and pUC19L as Cloning Vectors

You can also use the linear pYES7L or pUC19L vectors, included in the GeneArt® Seamless PLUS Cloning and Assembly Kit, for your *in vitro* cloning and assembly reactions.

- Use the linear pYES7L vector for cloning if you wish to horizontally transfer your assembled construct into Gram-negative bacteria or yeast cells by performing tri-parental mating (i.e., conjugation). For a note on its capacity, see page 28.
- Use the linear pUC19L vector for cloning if you wish to perform blue-white screening for the presence of the insert. Fragments up to 10 kb each may be cloned into pUC19L.

The ready-to-use linear pUC19L cloning vector is also available separately from Life Technologies as the GeneArt® Linear pUC19L Vector for Seamless Cloning (Cat. no. A13289), providing sufficient linear vector for 20 *in vitro* seamless cloning reactions. For ordering information, see page 39.

Homology Requirements for Adjacent DNA Inserts

The GeneArt® Seamless PLUS Cloning and Assembly technology relies on homologous recombination to assemble adjacent DNA inserts sharing end-terminal homology. Therefore, each DNA insert must have a 15- to 80-bp sequence overlap with the adjacent insert (including the cloning vector), with the length of the overlap depending on the size of the insert and the total size of the final construct.

Table 1, on page 9, lists the end-terminal homology (i.e., total overlap) required for successful cloning and assembly.

Continued on next page

Guidelines for Successful Cloning and Assembly, continued

Table 1 End-terminal homology (i.e., total sequence overlap) required for successful assembly

Total construct size*	Insert size	End-terminal homology required	Pre-cloning recommended
Up to 13 kb	Up to 10 kb	15 bp or more	Not necessary
Between 13 kb and 25 kb	Up to 2.5 kb	40 bp or more	Not necessary
	Between 2.5 kb and 10 kb	40 bp or more	Yes
Larger than 25 kb*	Up to 10 kb	80 bp†	Yes
	Larger than 10 kb‡	80 bp†	Yes

* Although we recommend using the GeneArt® Seamless PLUS Cloning and Assembly Kit for assembling constructs totaling up to 40 kb in length, you can use it to create constructs that exceed 40 kb in length; however, the cloning efficiency and the number of transformants will be lower. We have had success with assembling constructs up to 60 kb; however, the quality of PCR fragments used in such large assemblies is critical.

† Overlap between the insert and the vector can be 40 bp or more.

‡ Inserts larger than 10 kb may not be efficiently PCR amplified. Therefore, they must be split into smaller inserts and pre-cloned into an intermediate vector (see **Pre-Cloning**, page 17). Note that the GeneArt® Primer and Construct Design Tool does not support the assembly of inserts larger than 10 kb.

Guidelines for Generating DNA Inserts

- DNA inserts to be assembled can be synthesized, generated by PCR amplification, or by excising pre-existing inserts containing the required homologous sequences from a plasmid by restriction digest. Regardless of the method used for their generation, adjacent DNA inserts must share the required end-terminal homology for successful assembly and maximum cloning efficiency.
- You may use any combination of synthesized inserts, PCR-amplified inserts, and inserts generated by restriction digest for assembly. However, in our experience, the quality of DNA inserts synthesized or generated by restriction digest is superior to those generated by PCR amplification.
- When recombining two adjacent DNA inserts generated by PCR amplification, the required end-terminal homology may be split between the inserts. This is achieved by using reverse and forward primers that contain sequences specific to both inserts during amplification (for an example, see page 13).
  Note: You can split the homology between adjacent inserts in any combination (e.g., 20+20, 30+10, 35+5 etc. for a total of 40-bp overlap).
- Pre-clone medium- to large-size inserts (>2.5 kb) belonging to large assemblies (>13 kb) into an intermediate vector and then excise them by restriction digestion prior to performing the assembly (see **Pre-Cloning**, page 16).
- In some instances, fairly long primers are required for generating a DNA insert by PCR-amplification. We have observed that as the primer size increases, the cloning efficiency and colony output decreases. Therefore, we recommend splitting each long primer into two smaller ones, and using two pairs of primers for amplification rather than one (see **Stacked Primers Approach**, page 14).

Generating a Linearized *E. coli* Cloning 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), using PCR amplification, or using both. We recommend linearizing the vector and then PCR amplifying it, which significantly reduces the background and results in shorter primers for the first and last inserts.
- When generating the linearized vector by restriction digest, we recommend that you digest the vector with two restriction enzymes (i.e., double digest) rather than a single enzyme to reduce the amount of background. Double digestion with two restriction enzymes is the most efficient way of linearizing your cloning vector. 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. However, we recommend that you use a restriction enzyme that produces blunt or 3′ protruding ends when possible to achieve 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.
- 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 or equivalent (see page 40 for ordering information).
- 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.

When assembling large constructs (>25 kb) using the linear pYES7L cloning vector, we do **not** recommend PCR amplification of the vector to add the necessary end-terminal homology with the outermost DNA inserts. PCR amplifying the pYES7L cloning vector for large assemblies significantly reduces the colony output; however, this does not affect the cloning efficiency (i.e., most recovered colonies will contain the correctly assembled construct).

On the other hand, using a non-PCR amplified pYES7L vector for such assemblies requires the use of very long PCR primers to add the required end-homology to the outermost DNA inserts. In such cases, we recommend the "stacked primers approach" for splitting the homology requirements between two primer pairs (see page 14).

If you nevertheless decide to use the PCR-amplified pYES7L cloning vector for large assemblies, we recommend plating a larger volume (100–200 µL) from each transformation to ensure a sufficient number of colonies to screen for the presence of the insert(s) by colony PCR.

Preparing DNA Inserts by PCR

Guidelines for PCR Primers

PCR primers used for generating your inserts must have 15- to 80-nucleotide overhangs on their 5′ ends to provide the required end-terminal homology with the adjacent inserts (see Table 1, page 9, for required end-terminal homology); however, this homology may be split between the primers used for adjacent PCR-amplified DNA inserts (see guidelines for 15-bp overlaps, below).

- Design your PCR primers such that each DNA insert to be assembled is between 100 bp and 10 kb (kilo base pairs) in length.

  Note: Large inserts (>5 kb) are more susceptible to damage in a gel extraction procedure. Furthermore, many PCR enzymes are not processive enough to amplify inserts >5 kb. Therefore, we recommend that you assemble multiple inserts of ≤5 kb in one reaction rather than a single large insert.

- The 5′ ends of each primer pair (forward and reverse) must contain a 15- to 80-nucleotide sequence (i.e., an overhang) that is homologous to bases at one end of the adjacent DNA insert (i.e., the vector or another insert) while the 3′ ends must contain 18–25 nucleotides specific to the DNA element you want to assemble.

  Note: As the primer size increases, the cloning efficiency and colony output decreases. Therefore, we recommend splitting each long primer into two smaller ones, and using two pairs of primers for amplification rather than one (see **Stacked Primers Approach**, page 14).

- If you are joining the insert to the linearized vector, all of the nucleotides providing the requisite homology must be on the 5′ end of the primer. When recombining two adjacent inserts, you may split the homology between the inserts as represented on the image below. For example, for a 15-bp total homology, you can have 7 bp on the reverse primer of insert 1 and 8 bp on the forward primer of insert 2.

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

- If you are splitting the required end-terminal homology between adjacent inserts, one PCR primer may have as few as 18 nucleotides and no overhang whatsoever (i.e., contain only homologous sequences specific to the DNA element you want to assemble).

  Note: For example, in a 0+15 homology split, reverse primer of insert 1 would not contribute to the homology and will consist of only 18–25 gene specific nucleotides for insert 1, while the forward primer of insert 2 would contribute the entire 15 bp for the homology, in addition to having 18–25 gene specific nucleotides for insert 2 (for a total length of 33–40 nucleotides).

- You may also design your PCR primers to provide specific restriction enzyme sites (for pre-cloning or other applications) or to re-construct the restriction sites used for linearizing your cloning vector. Note that these sites will not be a part of the end-terminal homology.

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

Continued on next page

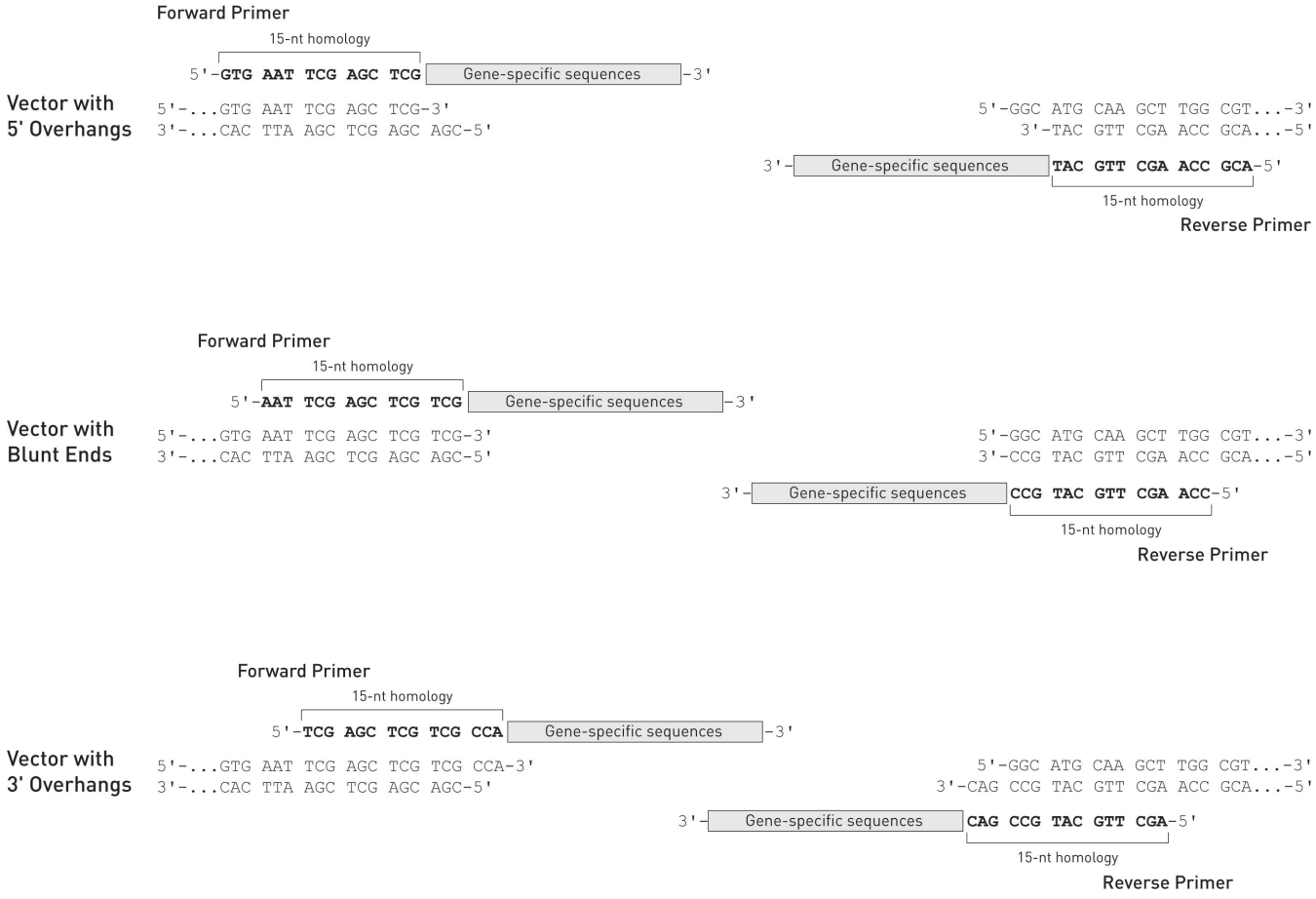
Preparing DNA Inserts by PCR, continued

Examples for PCR Primers

The requisite 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 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 Insert 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. In these examples, the required end-terminal homology is 15 bp (i.e., the hypothetical assembly involves inserts of <10 kb for a final construct of <13 kb). 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.



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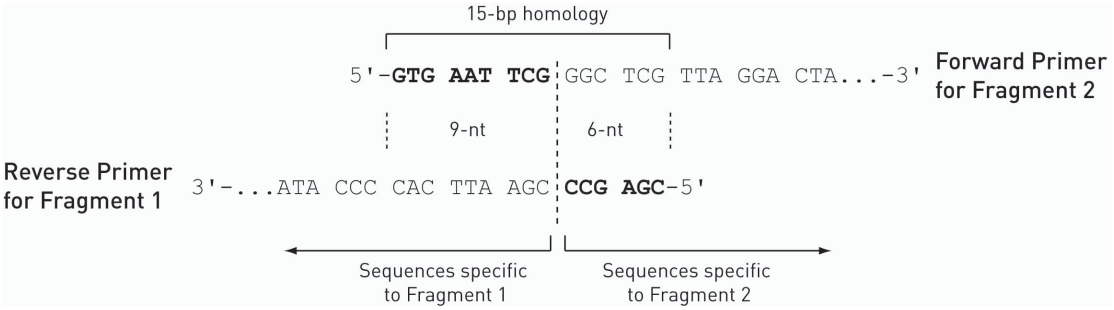
Examples for PCR Primers, continued

PCR Primers for Joining Adjacent DNA Inserts

The figure below shows examples of PCR primers used for preparing adjacent DNA inserts for assembly. When joining 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: When joining two adjacent inserts, you may split the required end-terminal homology between the inserts in any combination (e.g., 9+6 as in the example below, or 12+3, 11+4 etc. for a 15-bp homology).



We highly recommend that you use the web-based GeneArt® Primer and Construct Design Tool to verify the sequence of your DNA inserts and your assembly strategy, and to design your PCR primers that generate the required overlap for each DNA insert. For more information on the GeneArt® Primer and Construct Design Tool, see page 33.

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Preparing DNA Inserts by PCR, continued

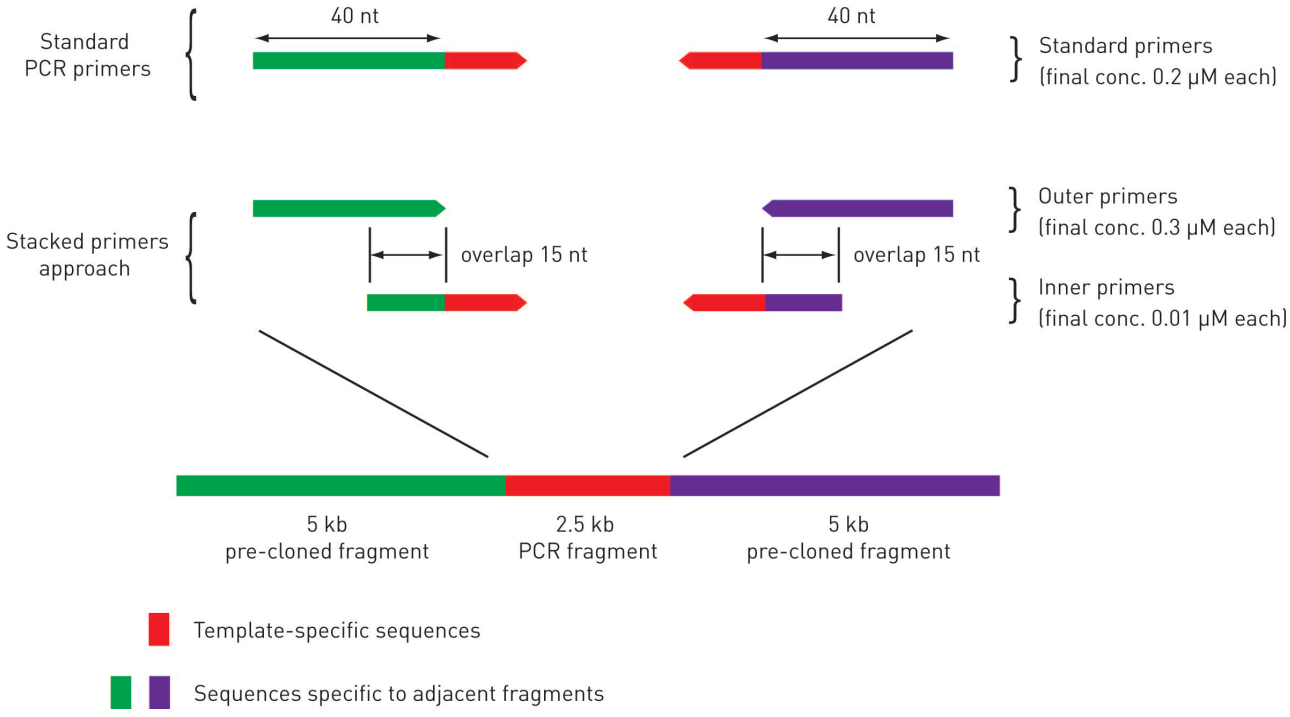
Stacked Primers Approach

The "stacked primers approach" involves splitting long PCR primers required for generating DNA inserts into two smaller ones, and using two pairs of primers for amplification rather than a single primer pair. Note that the stacked primers approach is not the same as "nested PCR".

DNA inserts used in large assemblies (>13 kb) require long primers for their generation (40–80 nucleotides to provide the sequence overlap with adjacent fragments and 18–25 nucleotides that are gene-specific). We have observed that as the primer size increases, the cloning efficiency and colony output decreases. Pre-cloning reduces the length of the primers necessary to incorporate the required homology by dividing it between the primer used for the insert and the primer used for the pre-cloning vector (see page 16); however, this additional cloning step increases the time for the overall workflow.

While we recommend pre-cloning medium- to large-size inserts (>2.5 kb) belonging to large assemblies (>13 kb), you may use the stacked primers approach to generate smaller DNA inserts of up to 2.5 kb for large assemblies.

The example below depicts the hypothetical assembly of a PCR-amplified DNA fragment (shown in red) and two pre-cloned DNA fragments is (shown in green and purple). The standard primer strategy and the stacked primers approach are shown for the red PCR fragment. Note that the sequence span for the outer and inner primers is the same as for the standard primer, which allows the use of two pairs of shorter primers in the PCR, instead of a single pair of long primers.



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Preparing DNA Inserts by PCR, continued

Guidelines for PCR

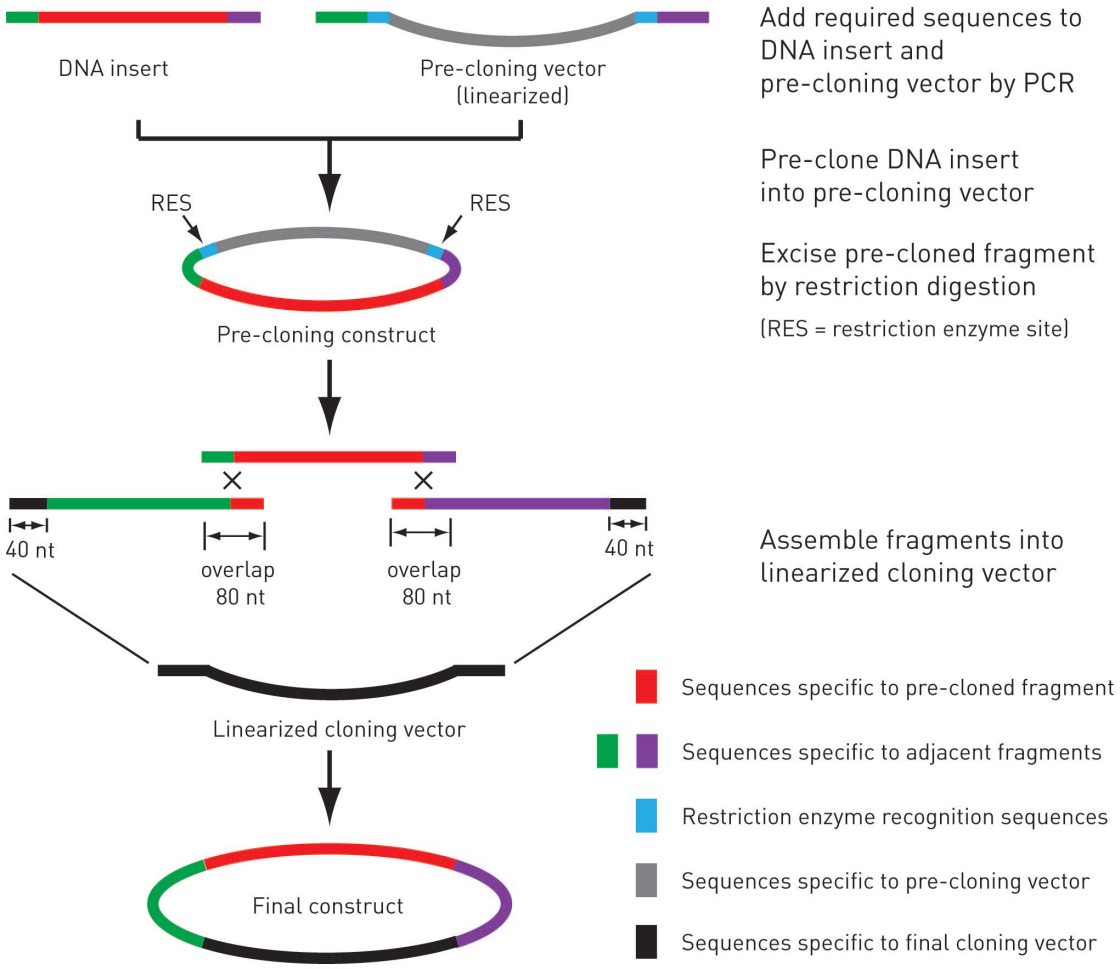
- When using plasmid DNA, 5–20 ng of plasmid DNA is usually sufficient as a PCR template in a reaction volume of 100 µL. When using *E. coli* or human genomic DNA, you can increase the amount of template DNA to 20–200 ng.
- We recommend using standard PCR primers (forward and reverse) at a final concentration of 200 nM each. If the PCR efficiency is not optimal, repeat the reaction with different primer concentrations from 100 nM to 500 nM, in 100 nM increments.

  If using the stacked primer approach (see page 14), use the outer primers (forward and reverse) at a final concentration of 300 nM each, and the inner primers at a final concentration of 10 nM each.
- The reaction mixtures and PCR protocols recommended in the GeneArt® Seamless PLUS Cloning and Assembly Kit have been optimized using the AccuPrime™ *Pfx* SuperMix (see page 40 for ordering information), because the AccuPrime™ *Pfx* DNA polymerase is ideal for high-fidelity, high-specificity amplification of DNA fragments. Reaction conditions may need to be optimized for other enzymes.
- 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 inserts prepared by PCR, you must purify the DNA inserts through a PCR cleanup kit. We recommend that you use the PureLink® PCR Purification Kit (see page 40 for ordering information).
- After preparing your DNA inserts by PCR, verify the PCR products by gel electrophoresis. If you obtain multiple bands, you must gel purify your DNA inserts. We recommend using the PureLink® Quick Gel Extraction Kit for best results (see page 40 for ordering information). Elute the DNA inserts from the PureLink® column using water.
- When gel purifying your DNA inserts, employ extra caution to minimize any potential damage to the ends of the DNA inserts 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 inserts will result in somewhat lower cloning efficiency.
- Large inserts (>5 kb) are more susceptible to damage in a gel extraction procedure. Therefore, we recommend that you assemble multiple inserts of ≤5 kb in one reaction rather than a single large insert.
- You can skip the PCR purification step and use an unpurified insert in a single insert 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 multi-insert assembly.

Pre-Cloning

Introduction

Pre-cloning involves cloning a DNA insert generated by PCR amplification into a small, intermediate vector ("pre-cloning vector", usually <3 kb in size) and then excising it by restriction digestion prior to performing the seamless assembly reaction to create the final recombinant construct. The figure below depicts the pre-cloning workflow. Note that this example applies to constructs larger than 25 kb as implied by the 80-nt overlap that is required for constructs of this size.



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Pre-Cloning, continued

Guidelines for Pre-Cloning

- We recommend pre-cloning medium- to large-size inserts (>2.5 kb) belonging to large assemblies (>13 kb) into a "pre-cloning vector" to increase the cloning efficiency and colony output during final assembly.
- PCR amplify the "pre-cloning vector" to incorporate the sequences required for pre-cloning and restriction digestion. This minimizes the size of the primers required for generating the DNA insert to be pre-cloned.
- PCR amplify the DNA insert to incorporate the sequences required for pre-cloning and final assembly.
- Refer to **Pre-Cloning Primer Design**, page 18, for guidelines on designing PCR primers used for incorporating the sequences required for pre-cloning to the DNA insert and the pre-cloning vector.
- We highly recommend that you use the GeneArt® Primer and Construct Design Tool to verify the sequence of your DNA inserts and your assembly strategy, and to design your PCR primers that generate the required overlap for each DNA insert. For more information on GeneArt® Primer and Construct Design Tool, see page 33.

In a small number of cases, the PCR-amplification of a pre-cloning vector with two identical restriction sites at both ends results in a molecule with a propensity to recircularize. This happens when the pre-cloning vector, the pre-cloning fragment, or both have ends with small stretches of homology (usually 4 or more consecutive base pairs with high GC content next to the restriction sites; this is not the case with pUC19L). For these uncommon events, which are not detected by the GeneArt® Primer and Construct Design Tool, we recommend screening a larger than usual number of colonies or replacing the restriction site of **one** of the oligonucleotides used to amplify the pre-cloning vector. The choice of the restriction site can be made by re-running the GeneArt® Primer and Construct Design Tool and picking another restriction site from the list. The list contains only those sites that are not found either in the vector or in the fragment to be pre-cloned.

Continued on next page

Pre-Cloning Primer Design

The overlapping sequences contributed by the 5′ overhangs of the primers used for amplifying the intermediate ("pre-cloning") vector and the DNA insert allow for homologous recombination to take place during pre-cloning. This recombination event joins the sequences that provide the required end-terminal homology with the adjacent fragment in the final assembly reaction, and minimizes the size of the primers necessary to incorporate this homology to the DNA insert.

Upon excision by restriction digestion, the pre-cloned DNA insert will bear residual sequences belonging to the restriction sites at its ends, forcing the recombination to occur a few nucleotides inside of the corresponding inserts. This does not interfere with the cloning efficiency and these extra sequences are readily eliminated from the final construct.

Follow the guidelines below when designing your PCR primers for pre-cloning. See **Example for Pre-Cloning Primer Design**, page 19, for a graphical representation of the PCR primers required for generating the DNA insert to be pre-cloned and for amplifying the pre-cloning vector.

PCR Primers for Generating the DNA Insert to be Pre-Cloned

- The 3′ ends of each primer pair (forward and reverse) used for generating the DNA insert must contain 18–25 nucleotides specific to the DNA element you want to assemble (i.e., gene-specific sequences; shown in red).
- The 5′ end of each primer used for the DNA insert must consist of an overhang containing part of the required homology with the adjacent fragment that will be used in the final assembly (shown in green and purple). At least 15 nucleotides of this sequence must overlap of with the sequences on the 5′ end of corresponding primer used for amplifying the pre-cloning vector.

PCR Primers for Amplifying the Pre-Cloning Vector

- The 3′ ends of the primer pair (forward and reverse) used for amplifying the pre-cloning vector must contain 18–25 nucleotides specific to the intermediate vector (shown in grey).
- The 5′ end of each primer used for amplifying the pre-cloning vector must consist of an overhang containing a portion of the homologous sequences required between the DNA insert to be pre-cloned and the adjacent fragment in the final assembled construct (shown in green and purple). At least 15 nucleotides of this sequence must overlap of with the sequences on the 5′ end of corresponding primer used for generating the DNA insert.
- These 5′ sequences providing the required end-terminal homology between the DNA insert and the adjacent fragment must be flanked by a restriction enzyme recognition sequence not present in the pre-cloned DNA construct (shown in blue) to allow for the excision of the pre-cloned fragment prior to the final seamless assembly reaction.

Continued on next page

Pre-Cloning, continued

Example for Pre-Cloning Primer Design

In the example below, the 5′ ends of the forward and reverse primers used for generating the DNA fragment each contain a 30-nt sequence (shown in green and purple), which are homologous to adjacent fragments in the final construct The 3′ ends of these primers contain gene-specific sequences (shown in red).

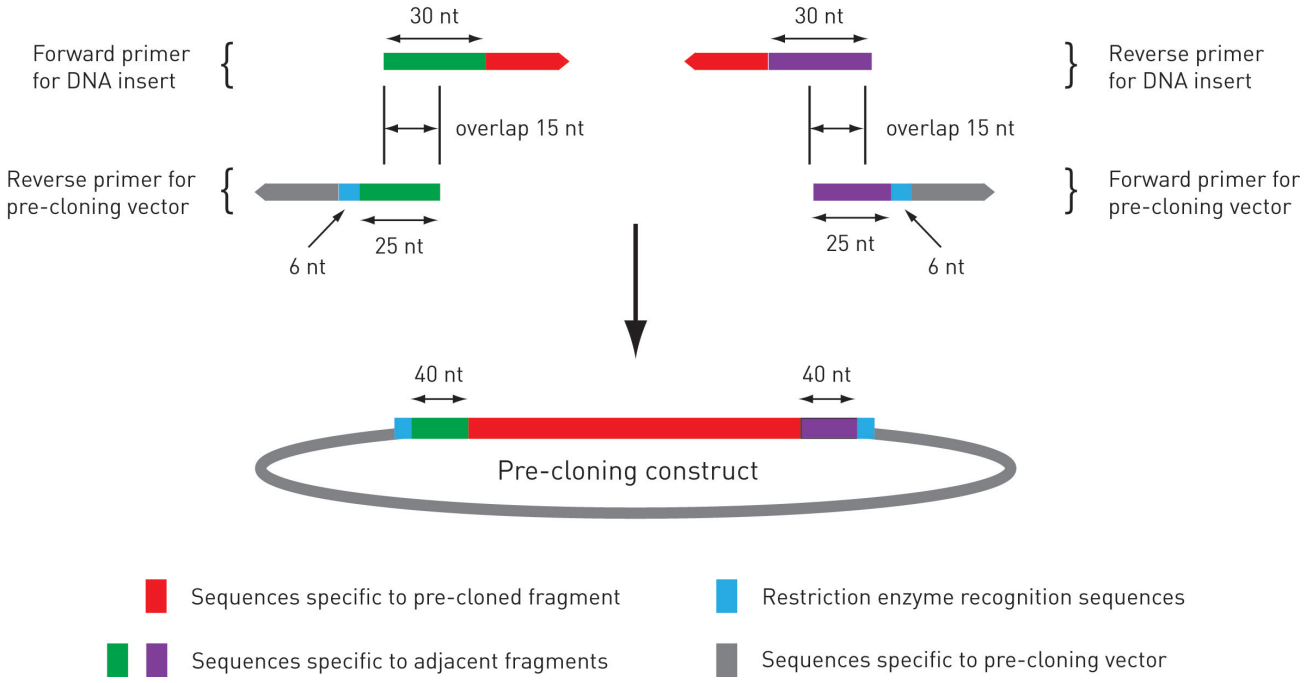
The 5′ end of each primer used for amplifying the pre-cloning vector contains a 25-nt homologous sequence (shown in green and purple), of which a 15-nt portion overlaps the homologous sequence provided by the primers used for the DNA fragment amplification. The rest of the primer consists of restriction enzyme recognition sites (6 nt; shown in blue) and sequences specific to the pre-cloning vector (shown in grey).

The two 15-bp sequences shared by the DNA fragment and the pre-cloning vector allow joining of the fragment and the vector through homologous recombination. In the final pre-cloning construct, the pre-cloned DNA fragment is flanked by a 40-bp homologous region (formed by the combination of 30- and 25-bp homologous sequences sharing a 15-bp overlap) and a restriction enzyme site on each side. These 40-bp regions provide the required end-terminal homology with the adjacent fragments in the final seamless cloning and assembly reaction (the final assembled construct is not shown below; refer to page 16 for a graphical representation of the final assembled construct).

$$\text{Length of tails} = \frac{\text{Homology requirement + RE sequence + Overlap between primers}}{2} = \frac{40 + 6 + 15}{2} = 30^*$$

* (40 + 6 + 15)/2 = 30.5, rounded down to 30.

Note that the tails of the pre-cloning vector primers include the sequences for the restriction enzyme recognition.



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 (see page 33).
- Generated your linear cloning vector according to the guidelines on page 10.
  Note: You can also use the linear pYES7L or pUC19L vectors, included in the GeneArt® Seamless PLUS Cloning and Assembly Kit, for your *in vitro* cloning and assembly reactions. 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 inserts according to the guidelines on pages 11–19.

When assembling large constructs (>25 kb) using the linear pYES7L cloning vector, we do **not** recommend PCR amplification of the vector to add the necessary end-terminal homology with the outermost DNA inserts. PCR amplifying the pYES7L cloning vector for large assemblies significantly reduces the colony output; however, this does not affect the cloning efficiency (i.e., most recovered colonies will contain the correctly assembled construct).

On the other hand, using a non-PCR amplified pYES7L vector for such assemblies requires the use of very long PCR primers to add the required end-homology to the outermost DNA inserts. In such cases, we recommend the "stacked primers approach" for splitting the homology requirements between two primer pairs (see page 14).

If you nevertheless decide to use the PCR-amplified pYES7L cloning vector for large assemblies, we recommend plating a larger volume (100–200 µL) from each transformation to ensure a sufficient number of colonies to screen for the presence of the insert(s) by colony PCR.

Materials Needed

- DNA inserts to assemble (up to 4 DNA inserts of 200 ng each for a final construct of 40 kb in length, including the cloning vector)
  Note: You can use the GeneArt® Seamless PLUS Cloning and Assembly Kit to assemble a larger number of inserts or to create constructs that exceed 40 kb in length; however, the cloning efficiency will be lower. We have had success with assembling up to 7 DNA inserts and a vector. Note that the GeneArt® Primer and Construct Design Tool only supports the seamless assembly of up to 4 inserts.
- Linear pYES7L, linear pUC19L, or your own linearized *E. coli* cloning vector (50 ng)
- Control insert (for assessing the efficiency of the assembly reaction)
- GeneArt® 2X Enzyme Mix
- Deionized, sterile water

Continued on next page

Seamless Cloning and Assembly Reaction, continued

- The GeneArt® 2X Enzyme Mix might appear slightly turbid; this is normal and does not affect the enzyme activity. Do **not** centrifuge the enzyme mix to produce a clarified supernatant.
- It is crucial to add the GeneArt® 2X Enzyme Mix to the seamless assembly and cloning reaction **separately at the end** after you have mixed all the other reaction components as described below.
- After adding the GeneArt® 2X Enzyme Mix to the reaction mixture, promptly return it to –80°C.

  Note: Although we have observed no signs of underperformance after subjecting the GeneArt® 2X Enzyme Mix to up to 10 freeze-and-thaw cycles or leaving it at room temperature for up to 1 hour, we recommend promptly returning it to –80°C storage to ensure best performance for subsequent uses.

Seamless Cloning and Assembly Reaction

1. In a microcentrifuge tube, set up the seamless cloning and assembly reaction. **It is crucial that you add the GeneArt® 2X Enzyme Mix as the last component** (see Step 2).

Insert(s) (200 ng each)*	x µL
Linear cloning vector (50 ng)**	1 µL
Deionized water	to 5 µL

   * For <13kb constructs, use a vector:insert molar ratio of 1:2; for >13kb constructs, use 200 ng of each insert. See page 36 for instructions on how to calculate molar ratios.

   **You can use 50 ng of pYES7L, linear pUC19L, or your own linearized cloning vector. The volume of your own linearized cloning vector depends on its concentration.

2. Quickly thaw the GeneArt® 2X Enzyme Mix on ice and pipette up and down to mix thoroughly. Add 5 µL of the thawed GeneArt® 2X Enzyme Mix to the reaction mix, and immediately return it back to –80°C.
3. Mix the reaction components completely by pipetting them up and down 3 times and then gently tapping the sides of the tube 3–5 times.
4. Briefly centrifuge (<500 rpm for <5 seconds) to collect the reaction components to the bottom of the microcentrifuge tube.
5. For small assemblies (<13 kb) containing inserts with 15-bp end-terminal homology, incubate the reaction mix at room temperature for **15–30 minutes**. Do **not** incubate more than 30 minutes.

   For large assemblies (>13 kb) containing inserts with larger end-terminal homology, incubate the reaction mix at room temperature for **60 minutes**. Do **not** incubate more than 60 minutes.
6. After incubation is complete, place the reaction mix on ice for 2–5 minutes before proceeding to the transformation step (next page).

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

Transforming One Shot® DH10B™ T1^R SA Competent Cells

Materials Needed

- Seamless cloning and assembly reaction mix (from step 6, previous page)
- One Shot® DH10B™ T1^R SA Chemically Competent Cells

  Note: Do **not** use electrocompetent *E. coli* for transformation. Only chemically competent cells result in successful seamless cloning and assembly of DNA inserts.
- S.O.C. medium
- 37°C water bath
- LB plates containing the appropriate selection antibiotic.

  If you have used the linear pYES7L vector for cloning, use LB plates containing 100 µg/mL spectinomycin (for the recipe, see **Support Protocols**, page 36).

  If you have used the linear pUC19L vector for cloning, use LB plates containing 50–100 µg/mL ampicillin and X-Gal (for the recipe, see **Support Protocols**, page 36).

  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 to verify the transformation efficiency

Preparation

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

1. Equilibrate a water bath to 37°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® DH10B™ T1^R SA Cells for each transformation.

Continued on next page

Transforming One Shot® DH10B™ T1R SA Competent Cells, continued

The One Shot® DH10B™ T1R SA Chemically Competent *E. coli* cells and the protocol below have been optimized for transformation of small, medium, and large DNA constructs assembled using the GeneArt® Seamless PLUS Cloning and Assembly Kit. They are not optimized for transformation of supercoiled or covalently ligated molecules.

One Shot® DH10B™ T1R SA Chemical Transformation

1. Add 3 µL of the seamless cloning and assembly reaction from step 6, page 21, into a vial of One Shot® DH10B™ T1R SA Chemically Competent *E. coli* and mix gently by tapping the tube several times. **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® DH10B™ T1R SA Cells and follow the transformation procedure.
2. Incubate the transformation mix on ice for 30 minutes.
3. Transfer the transformation mix to a **37°C** water bath and incubate for exactly 10 minutes.
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, spread 5–150 µL from each transformation on a pre-warmed selective plate. 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. See **Analyzing Transformants**, page 25, for more information.

Control Experiments

Control Reactions

When using the GeneArt® Seamless PLUS 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 GeneArt® Seamless PLUS Cloning and Assembly Kit includes the following components for use in the control reactions:

- Control *lacZ* Insert, a 1.6 kb DNA insert containing the *lacZ* gene and sharing a 15-bp homology with the linear pYES7L vector at both ends. Use the Control *lacZ* Insert with the linear pYES7L vector.
- Control Insert, a 1.3 kb DNA insert sharing a 15-bp homology with the linear pUC19L vector at both ends. Use the Control Insert with the linear pUC19L vector.

Note: The GeneArt® Seamless PLUS Cloning and Assembly Kit contains sufficient reagents (GeneArt® 2X Enzyme Mix and One Shot® DH10B™ T1^R SA Cells) for 20 cloning and assembly reactions. 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. It is crucial that you **add the GeneArt® 2X Enzyme Mix last**.

Component	Positive control	Negative control
Deionized water	5 µL	7 µL
Linear pYES7L or pUC19L vector (50 ng/µL)	1 µL	1 µL
Control *lacZ* Insert or Control Insert (50 ng/µL)	2 µL	2 µL
GeneArt® 2X Enzyme Mix	2 µL	—
Total volume	10 µL	10 µL

2. Mix the reaction components completely by pipetting them up and down 3 times and then gently tapping the sides of the tube 3–5 times.
3. Briefly centrifuge (<500 rpm for <5 seconds) to collect the reaction components to the bottom of the microcentrifuge tube, and incubate the control reaction mixes at room temperature for **15 minutes**.
4. After 15 minutes of incubation, place the reaction mix on ice for 2–5 minutes before proceeding to the transformation step (page 23).

   Note: Do not let the samples stay on ice for more than 5 minutes before transformation.
5. Plate the control transformations on LB plates containing the appropriate antibiotic (100 µg/mL spectinomycin for pYES7L or 50–100 µg/mL ampicillin for pUC19L) for selection and 30–100 µg/mL X-Gal for blue-white screening.

Continued on next page

Control Experiments, continued

Expected Results

- If you have used pYES7L vector and the Control *lacZ* Insert, you can expect >85% blue colonies on your positive control and mostly white colonies on your negative control plates.
- If you have used pUC19L vector and the Control Insert, you can expect >85% white colonies on your positive control and mostly blue colonies on your negative control plates.
- You may still get positive colonies (i.e., blue with pYES7L vector or white with pUC19L vector) on your negative control plates at a very low frequency.
- The transformation efficiency of One Shot® DH10B™ $T1^R$ SA Cells should be $\geq 1 \times 10^9$ transformants per µg of control plasmid.

Analyzing 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.

Analyzing 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 spectinomycin if you have used the linear pYES7L vector or 100 µg/mL ampicillin if you have used the linear pUC19L vector for cloning).
   Note: Pick 10 colonies for an assembly reaction involving 4 inserts plus the vector. For assembly reactions involving less than 4 inserts, 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 the PureLink® HQ Mini Plasmid Purification or the PureLink® HiPure Plasmid Miniprep kits (see page 40 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 inserts that were PCR amplified to rule out any errors made by the DNA polymerase during amplification.

Analyzing 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 40 for ordering information)
- Appropriate forwards and reverse PCR primers (20 µM each)
- LB plates containing the appropriate selection antibiotic

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Analyzing Transformants by Colony PCR, continued

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.

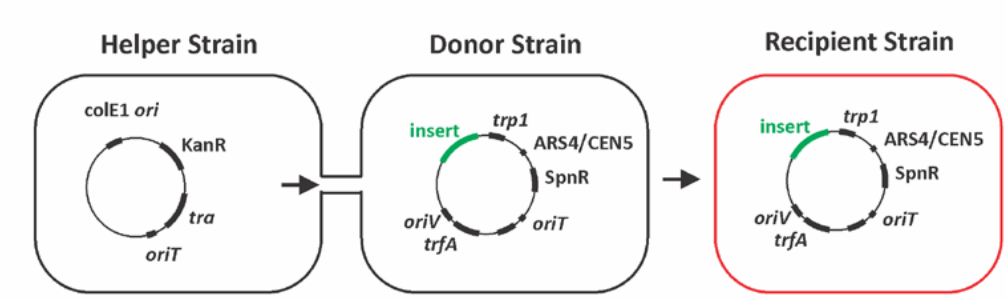
   Note: If you have used the linear pYES7L vector for cloning, use LB plates containing 100 µg/mL spectinomycin. If you have used the linear pUC19L vector for cloning, use LB plates containing 50–100 µg/mL ampicillin
2. Isolate a single colony and inoculate with it 1–2 mL of LB containing the appropriate 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.

Horizontal DNA Transfer (Conjugation)

Introduction

If you have used pYES7L as the cloning vector for the seamless assembly of your inserts, you can perform tri-parental mating using the Stbl3™ cells containing the helper plasmid pRK2013 to horizontally transfer your recombinant construct from the DH10B™ T1R SA host into variety of recipient strains such as *Agrobacterium tumefaciens*, *Rhizobium meliloti*, and *Saccharomyces cerevisiae*.

A diagram showing the principle of tri-parental mating is shown below. The helper plasmid pRK2013 (Figurski and Helinski, 1979) can be self-mobilized due to the presence of the *oriT* and *tra* genes and be transferred to the donor strain, where it provides the *tra* functions in *trans* to mobilize the donor plasmid (i.e., pYES7L containing your assembled construct) to the recipient strain (the donor plasmid contains an *oriT*). The donor plasmid is able to replicate in a variety of hosts thanks to the presence of the RK2 broad range host replication features (*oriV* and *trfA* gene) and yeast replication elements (ARS4/CEN5). Note that the capacity of pYES7L varies depending on the recipient species used (see **Guidelines for Conjugation**, below). The helper plasmid can also be transferred to the recipient strain but it will likely fail to replicate as it lacks broad range host replication functions (unless the recipient is also an *E. coli* strain).



Guidelines for Conjugation

- The conjugation procedure below works for most Gram-negative bacteria and yeast used as recipients.
- The capacity of the pYES7L (i.e., the size of the assembly it can carry) varies depending on the recipient species used. For example, while *A. tumefaciens* and *S. cerevisiae* can stably maintain a 55-kb pYES7L construct (vector + 47-kb insert), *R. meliloti* cannot maintain a construct >30 kb (vector + 23-kb insert).
- Other bacteria and cell types may also be used as recipient strains, provided that the appropriate replication origin and selective marker are incorporated into the constructs.

Continued on next page

Horizontal DNA Transfer (Conjugation), continued

Materials Needed

- DH10B™ T1R SA cells harboring the assembled inserts on pYES7L (i.e., donor strain)
- Stbl3™/pRK2013 (i.e., helper strain)
- Recipient strain bearing a selective marker (e.g., *Agrobacterium tumefaciens*-Rif^R or *Rhizobium meliloti*-Rif^R)

  Note: For most Gram-negative species, spontaneous rifampicin (Rif) resistant cells can be obtained by plating high density cultures onto solid media containing 50–100 µg/mL rifampicin.
- LB medium without antibiotics
- LB media and plates containing the appropriate selection antibiotics (see **Recipes**, page 36):

  LB + 100 µg/mL spectinomycin (LB + Spec)

  LB + 100 µg/mL spectinomycin + 100 µg/mL rifampicin (LB + Spec + Rif)
- 37°C shaking and non-shaking incubator
- 30°C shaking and non-shaking incubator (depending on the recipient strain's growth conditions)

Conjugation Protocol

Day 1

1. Innoculate the recipient strain (e.g., *A. tumefaciens*-Rif^R or *R. meliloti*-Rif^R) into 5 mL of appropriate selective medium (e.g., LB + Rif) and incubate at the appropriate temperature (e.g., 30°C) on a rotary shaker until the cells reach the stationary phase. (e.g., up to 3 days for *A. tumefaciens* or *R. meliloti*).

Day 4

2. Dilute the cultures above 1:2 to 1:4 in 5 mL of the appropriate selective medium (e.g., LB + Rif), and continue the incubation overnight.
3. Innoculate the donor strain (i.e., DH10B™ T1R SA cells harboring the assembled inserts on pYES7L) into 5 mL of LB + 100 µg/mL Spec, and incubate at 37°C on a rotary shaker overnight.
4. Inoculate the helper strain (i.e., Stbl3™/pRK2013) into 5 mL of LB + 25 µg/mL Kan, and incubate at 37°C on a rotary shaker overnight.

Day 5

5. Transfer 1.5 mL of each culture into a microcentrifuge tube and centrifuge at 4,000 rpm for 5 minutes.
6. Wash the pellet with 1.5 mL of fresh LB medium and centrifuge at 4,000 rpm for 5 minutes.
7. Resuspend each pellet in 150 µL of LB.

Continued on next page

Conjugation Protocol, continued

8. Mix Donor (D), Helper (H), and Recipient (R) strains in microcentrifuge tubes using the following combinations:

   R: 45 µL of R (recipient negative control)

   D+H: 15 µL of D + 15 µL of H (donor plus helper negative control)

   D+H+R: 15 µL of D + 15 µL of H + 45 µL of R

9. Mix the cultures well, spot on the center of pre-warmed LB plates, and incubate the cultures at 30°C overnight.

Day 6

10. Using inoculation loops, scrape the cells from each plate (from Step 9) and resuspend in 500 µL of recipient medium (e.g., LB for *A. tumefaciens* or *R. meliloti*).
11. For **R**, plate 100 µL of undiluted cells onto LB + Spec +Rif agar plate. Incubate the cells at 30°C for 3–4 days as a negative control.

    Note: If your recipient strain has a different selection marker, replace Rif with the appropriate selection marker.
12. For **D+H**, prepare 10^{-2}, 10^{-4}, and 10^{-6} dilutions, and plate 100µL of each dilution onto LB + Spec agar plates. Incubate the cells at 37°C overnight to determine the conjugation efficiency.
13. For **D+H**, plate an additional 100 µL undiluted onto an LB +Spec + Rif agar plate. Incubate the cells at 30°C for 3–4 days as a negative control.

    For **D+H+R**, prepare 10^{-2} and 10^{-4} dilutions, and plate 100 µL of each dilution and an undiluted sample onto LB + Spec + Rif agar plates. Incubate the cells at 30°C for 3–4 days until colonies appear on the selective plates.

    Note: If your recipient strain has a different selection marker, replace Rif with the appropriate selection marker.

Determining Conjugation Efficiency

Use the following equations to determine the colony output and conjugation efficiency:

$$\text{Colony output} = 5 \times (\text{number of colonies on the plate}) \times (\text{dilution factor})$$

$$\text{Conjugation efficiency} = \frac{\text{Colony output with D+H+R (on LB + Spec + Rif)}}{\text{Colony output with D+H (on LB + Spec)}}$$

Expected Results

- Few or no colonies should be observed for D + H plated onto LB + Spec + Rif. Any colonies that are observed are spontaneous Rif^R donors.
- No colonies should be observed for R plated onto LB + Spec + Rif.
- The expected conjugation efficiency is ~10^{-6} for *R. meliloti*, ~10^{-2} for *A. tumefaciens*, and ~10^{-8} for yeast.

Troubleshooting

The table below lists some potential problems and solutions that help you troubleshoot your experiments using the GeneArt® Seamless PLUS 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 22.
	One Shot® DH10B™ T1^R SA cells 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® DH10B™ T1^R SA competent cells. Competent *E. coli* can only be thawed once without dramatic loss in competency. • Store One Shot® DH10B™ T1^R SA cells 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. For the linear pYES7L cloning vector, use 100 µg/mL spectinomycin. For the linear pUC19L cloning vector, use 50–100 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 inserts do not share the required end-terminal homology	Make sure that your DNA inserts and the linearized-cloning vector share the required end-terminal homology. Refer to page 11 for the requirements on PCR primer design.
	Ends of the DNA inserts generated by PCR were damaged	Employ extra caution to minimize any potential damage to the ends of your DNA inserts 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 inserts and/or vector were used	• Make sure that you use the correct amounts of DNA inserts, and/or vector for cloning. • For maximum cloning efficiency, use a vector-to-insert molar ratio of 1:2 for <13kb constructs; for >13kb constructs, use 200 ng of each insert.

Continued on next page

Troubleshooting, continued

Symptom	Cause	Solution
No colonies after transformation, but the control transformation is successful	GeneArt® 2X Enzyme Mix handled incorrectly	• Quickly thaw the GeneArt® 2X Enzyme Mix on ice, and immediately return to –80°C after use. The enzyme mix may be subjected to 10 freeze/thaw cycles without a loss in activity. • Do **not** leave the GeneArt® 2X Enzyme Mix at room temperature or on ice 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. • If you observe turbidity in the 2X GeneArt® Enzyme Mix, do not centrifuge or vortex. Resuspend the enzyme mix by pipetting up and down.
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. The best results are obtained with vectors that are first digested with restriction enzymes and then PCR amplified. • Alternatively, use the linear pYES7L or the 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.
	GeneArt® 2X Enzyme Mix is **not** added last	It is crucial that you add the GeneArt® 2X 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 the appropriate time (15–30 minutes for small assemblies of <13 kb total construct size, 60 minutes for large assemblies of >13 kb total construct size). Do **not** overincubate. 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 A: Tools for Construct Design

GeneArt® Primer and Construct Design Tool

Introduction

Use the web-based GeneArt® Primer and Construct Design Tool to guide you when you are designing your cloning construct and DNA inserts. Based on your input, the tool designs the PCR primers used for amplifying your DNA inserts and creating the end-terminal homology required for seamless assembly, identifies potential pitfalls linked to your specific sequences, performs *in silico* cloning using your sequences, and allows one-click online ordering for custom primers (for countries with enabled online ordering). The GeneArt® Primer and Construct Design Tool is available at **http://bioinfo.invitrogen.com/oligoDesigner**.

Guidelines for Using the GeneArt® Primer and Construct Design Tool

- Select **GeneArt® Seamless PLUS Cloning and Assembly Kit** as the product type.
- Click **Browse** to upload your cloning vector sequence **or** choose from our suggested vectors. You can also copy and paste your sequence into the text box under **Enter sequence (copy/paste)**. Accepted input formats are FASTA and plain text.
- Click **Browse** to upload your fragment (i.e., insert) sequences one by one, **in the order you want to assemble them**. You can also copy and paste your sequence into the text box under **Enter sequence (copy/paste)**.
- If your adjacent sequences do not contain the required end-terminal homology, check the **PCR** box next to one or both sequences. This allows the tool to design PCR primers that will add the required homology to your inserts when they are generated using PCR.
- Click **Assemble and design oligos** to perform *in silico* cloning using your uploaded sequences. The GeneArt® Primer and Construct Design Tool will verify the uploaded sequences and determine the next steps in the design process (pre-cloning, primer design, etc.)
- If pre-cloning is required, click **Browse** to upload your pre-cloning vector sequence **or** choose from our suggested vectors. Select a **Restriction Enzyme** for releasing your pre-clone fragment from the drop-down menu and click **Finish**.
- The GeneArt® Primer and Construct Design Tool performs *in silico* cloning using your uploaded sequences and presents you with a graphical representation of your assembled construct, including the priming sites for your PCR primers (see next page for an example). The tool also provides you with the sizes and sequences of PCR primers as well as their melting temperature (T_m).
- Click **Add to Cart** to order your PCR primers directly from Life Technologies.
- Click the **Download GenBank File** to save the output from the web tool as a GenBank file.

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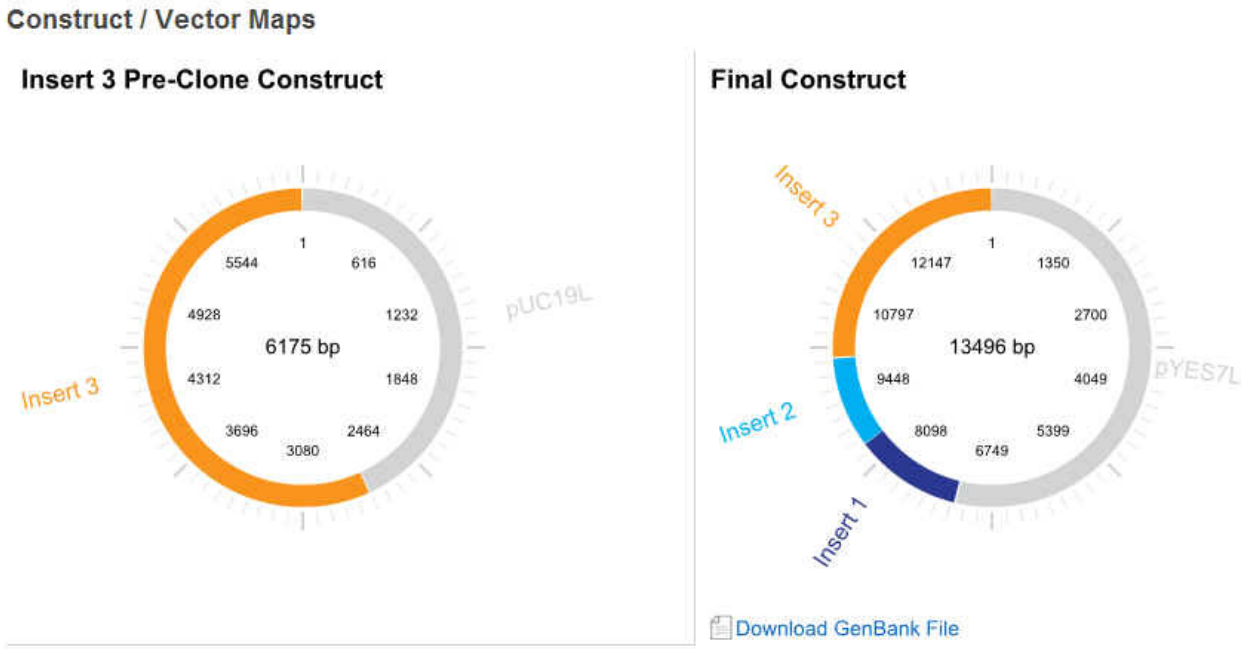
GeneArt® Primer and Construct Design Tool, continued

Output example from GeneArt® Primer and Construct Design Tool

The example below shows the output from GeneArt® Primer and Construct Design Tool for a 3-insert assembly into the pYES7L vector. In this example, Inserts 1, 2, and 3 were PCR amplified to introduce the required end-terminal homology for recombination (i.e., PCR box checked when uploading its sequence to the GeneArt® Primer and Construct Design Tool). Because Insert 3 is >2.5 kb in length and the total size of the final assembled construct is >13 kb, Insert 3 was pre-cloned into the suggested pUC19L vector. For **GeneArt® Primer and Construct Design Rules**, see page 35.



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GeneArt® Primer and Construct Design Tool, continued

GeneArt® Primer and Construct Design Rules

The GeneArt® Primer and Construct Design Tool uses the following rules when designing PCR primers used for amplifying your DNA inserts and creating the end-terminal homology required for seamless assembly.

- After a total construct design is submitted, the tool validates internal homology within each fragment itself using a sliding scale of 10 bp to 400 bp.
- For a final construct of <13 kb, if two adjacent fragments have at least 15 bp of homology between them and neither fragment is selected for PCR, the tool assembles them according to the existing overlap.
- For a final construct of between 13 kb and 25 kb, if two adjacent fragments have at least 40 bp of homology between them and neither fragment is selected for PCR, the tool assembles them according to the existing overlap without adding extra homology.
- For a final construct of > 25 kb, if two adjacent fragments have at least 80 bp of homology between them and neither fragment is selected for PCR, the tool assembles them according to the existing overlap without adding extra homology.
- If the primer created exceeds 40 nt in length and no pre-cloning was required, the tool splits the primer into two smaller primers with an overlap of 15 bp between them.
- If the total size of the construct (inserts + vector) is >25 kb, then pre-cloning is required of all the fragments regardless of their size.
- If the total size of the construct (inserts + vector) is between 13 kb and 25 kb, then pre-cloning is required for the fragments that are between 2.5 kb and 10 kb in size.
- If the total size of the construct (inserts + vector) is <13 kb, then pre-cloning is not required of any of the fragments, regardless of their size.
- If the total size of the construct (inserts + vector) is between 13 kb and 25 kb, and the fragments are <2.5 kb, then pre-cloning is not required.
- If the total size of the construct (inserts + vector) is between 13 kb and 25 kb, and the fragments are <2.5 kb, then the tool displays a warning message recommending the screening additional colonies.
- If the total size of the construct (inserts + vector) is between 13 kb and 60 kb, and only 1 fragment (which is less than 10 kb) is entered, then pre-cloning is not required.
- For pre-cloning, the tool verifies that the total size of the repository vector (i.e., the "pre-cloning vector") and DNA fragment is <15.5 kb.
- For pre-cloning, the tool displays a list of the restriction enzymes whose sequences do **not** match the sequences of the large fragment or the repository vector.

Appendix B: Support Protocols

Recipes and Calculating Molar Ratios

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.

   Note: Use spectinomycin and rifampicin at a final concentration of 100 µg/mL. Use ampicillin at a final concentration of 50–100 µg/mL.
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.

Calculating Molar Ratios

For maximum cloning efficiency, use a vector:insert molar ratio of 1:2 when assembling constructs that are <13 kb in length.

1. Determine the concentration of your DNA insert solutions in µg/mL by OD_{260} or fluorescence.
2. Use the following formula to calculate the amount of insert needed to give a molar ratio of 1:2 between the linearized pUC19L and insert. Note that the amount of pUC19L is 50 ng.

$$\text{x ng insert} = \frac{(2)\ (\text{bp insert})\ (50\text{ ng linearized pUC19L})}{(2{,}659\text{ bp pUC19L})}$$

   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.

Appendix C: Vectors

pYES7L

Map of pYES7L

The figure below summarizes the features of the pYES7L cloning vector (7,266 bp). The vector is supplied linearized. Note that the replicating active form of this vector is circular in bacteria and in yeast.

The complete sequence and the restriction map for pYES7L is available online at www.lifetechnologies.com or by contacting Technical Support (see page 41).

Comments for pYES7L:
7,266 nucleotides

TRP1 gene: bases 172–846 (c)
ARS4/CEN5: bases 1,382–1,902
OriT: bases 2,196–2,307
rrnB T2: bases 2,450–2,477 (c)
rrnB T1: bases 2,609–2,652 (c)
trfA gene: bases 3,408–4,553 (c)
Spectinomycin resistance gene: bases 5,085–6,094 (c)
Spn (Spectinomycin promoter): bases 6,096–6,229 (c)
OriV: bases 6,243–6,859

(c) = complementary strand

Features of pYES7L

The linear pYES7L (7,266 bp) vector contains the following elements. All features have been functionally tested.

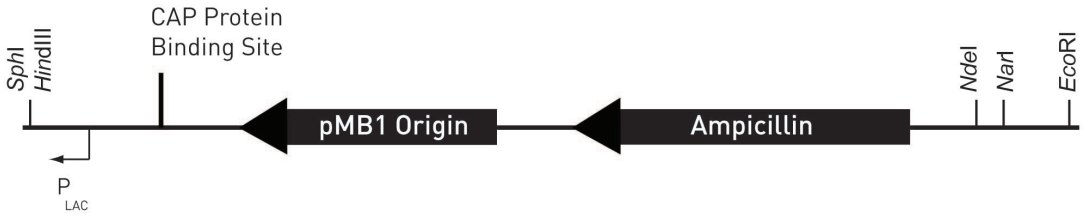
Feature	Benefit
TRP1 gene	Permits the selection of yeast transformants in tryptophan-free medium
ARS4/CEN5 origin	Allows the stable maintenance of the plasmid in yeast
OriT	Origin of transfer; participates in conjugation
rrnB T1 and rrnB T2	Transcription terminators
trfA gene	Allows broad host range replication
Spectinomycin resistance gene	Allows selection in *E. coli*
OriV	RK2 replication origin; allows the stable maintenance of the plasmid in Gram-negative bacteria
*Not*I, *Pac*I, I-*Sce*I, I-*Ceu*I	Restriction enzyme and homing endonuclease recognition sites that could be used for restriction mapping of the final assembled construct

pUC19L

Map of pUC19L

The figure below summarizes the features of the pUC19L cloning vector (2,659 bp). The vector is supplied linearized. Note that the replicating active form of this vector is circular in bacteria and in yeast.

The complete sequence and the restriction map for pUC19L is available online at www.lifetechnologies.com or by contacting Technical Support (see page 41).



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	Restriction enzyme recognition sites that could be used for restriction mapping of the final assembled construct

Appendix D: Ordering Information

GeneArt® Products

GeneArt® Seamless PLUS Cloning and Assembly Products

Some of the components of the GeneArt® Seamless PLUS Cloning and Assembly Kit are also available separately from Life Technologies. These products are listed below. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 41).

Product	Amount	Cat. no.
GeneArt® Seamless PLUS Cloning and Assembly Kit	1 kit	A14603
GeneArt® Seamless Cloning and Assembly Enzyme Mix	20 reactions	A14606
GeneArt® Linear pUC19L Vector for Seamless Cloning	20 reactions	A13289

Other GeneArt® Products

Life Technologies also offers other GeneArt® products that can be used for seamless *in vivo* assembly of up to 10 DNA inserts and vector and for site directed mutagenesis. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 41).

Product	Amount	Cat. no.
GeneArt® Seamless Cloning and Assembly Kit	1 kit	A13288
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 with Sapphire™ Technology	10 reactions	A13287
GeneArt® Vector Conversion Cassette with Sapphire™ Technology	10 reactions	A13291
CSM Media for MaV203 Yeast Cells	1 kit	A13292
GeneArt® Site-Directed Mutagenesis System	1 kit	A13282
GeneArt® Site-Directed Mutagenesis PLUS Kit	1 kit	A14551

Additional Products

Accessory Products

The products listed below may be used with the GeneArt® Seamless PLUS Cloning and Assembly Kit. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 41).

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

Obtaining Support

Technical Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/sds**.

Limited Product Warranty

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

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Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit lifetechnologies.com/support or email techsupport@lifetech.com

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17 May 2013